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Original Research

Is there a role for homeopathy in breast cancer surgery? A first randomized clinical trial on treatment with *Arnica montana* to reduce post-operative seroma and bleeding in patients undergoing total mastectomy

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ABSTRACT

Aim: This study aimed to evaluate the benefits of *Arnica montana* on post-operative blood loss and seroma production in women undergoing unilateral total mastectomy by administering *Arnica Montana 1000 Korsakovian dilution (1000 K)*. **Materials and Methods:** From 2012 to 2014, 53 women were randomly assigned to *A. montana* or placebo and were followed up for 5 days. The main end point was the reduction in blood and serum volumes collected in drainages. Secondary end points were duration of drainage, a self-evaluation of pain, and the presence of bruising or hematomas. **Results:** The per-protocol analysis revealed a lower mean volume of blood and serum collected in drainages with *A. montana* (−94.40 ml; 95% confidence interval [CI]: 22.48-211.28; $P = 0.11$). A regression model including treatment, volume collected in the drainage on the day of surgery, and patient weight showed a statistically significant difference in favor of *A. montana* (−106.28 ml; 95% CI: 9.45-203.11; $P = 0.03$). Volumes collected on the day of surgery and the following days were significantly lower with *A. montana* at days 2 ($P = 0.033$) and 3 ($P = 0.0223$). Secondary end points have not revealed significant differences. **Conclusions:** *A. montana* 1000 K could reduce post-operative blood and seroma collection in women undergoing unilateral total mastectomy. Larger studies are needed with different dilutions of *A. montana* to further validate these data.

KEY WORDS: *Arnica montana*, breast cancer, homeopathy, mastectomy, seroma

INTRODUCTION

Surgery is not free from significant complications, despite advancement in surgical techniques and in perioperative care. One of the major complications, particularly for breast and soft tissues surgery, is post-operative bleeding which represents a critical and, in some cases, lethal risk factor [1,2]. Moreover, post-operative bleeding after breast surgery causes a severe discomfort to the patient, entailing the need for surgical re-intervention and sometimes blood transfusions. Another frequent complication of breast surgery is seroma, which often requires numerous accesses for outpatient drainage, finally resulting in a delay of adjuvant therapy administration and a significant psychological burden [3].

A recent study conducted in the United States has extracted from the 2011 Nationwide Inpatient Sample the hospital discharge rates for primary diagnosis of breast cancer and has selectively identified discharges with post-operative bleeding [3]. Results showed that even if only 2.5% of the discharges had bleeding complications and eventually needed re-operation, globally, bleeding complications extended the length of stay by 1.3 days while increasing hospital costs by $5495 per admission. Moreover, even higher health costs and patients' discomfort are associated with the treatment of chronic or refractory seroma [3].

The risk of blood loss or seroma could be limited by identifying and then correcting potential triggers, but although this topic has

been extensively studied, both hemorrhage and seroma are still largely reported [4,5]. Homeopathy could provide remedies based on compounds with anti-hemorrhagic and anti-inflammatory properties, but their use in diluted homeopathic solutions has been poorly investigated [6-9]. Indeed, some homeopathic remedies have been already evaluated in various surgical settings, however showing controversial effects [10-17]. *Arnica montana* is a plant belonging to the Compositae family and it grows in East and Central Europe [18]. In particular, its anti-inflammatory action is linked to the lactone helenalin that seems to be involved in the inhibition of the pro-inflammatory transcription factor nuclear factor kappa B (NF-κβ) [19-27]. A. *montana* has been recently evaluated in several surgical settings such as esthetic and orthopedic surgeries, but currently, no single study is available in literature on the effects of this homeopathic remedy in breast cancer surgery. In the present study, a homeopathic preparation of A. *montana* 1000 Korsakovian dilution (1000 K) was administered preoperatively and postoperatively in a placebo-controlled, double-blind clinical trial to patients undergoing unilateral total mastectomy, with or without reconstruction, to evaluate any favorable or adverse effect on post-operative blood and seroma collection from surgical drains.

MATERIALS AND METHODS

Study Design

This randomized, double-blind, placebo-controlled study was carried out between 2012 and 2014 in a single university hospital department (Breast Unit, "Luigi Sacco" University Hospital, Milan, Italy). Fifty-three women affected by breast cancer who were candidates for unilateral total mastectomy, with or without reconstruction, were enrolled in the study. The study was authorized by the Ethical Committee of the same university hospital (Trial Register number: 93/2011, approved by Ethical Committee on February 15, 2012) and was conducted in accordance with the International Conference on Harmonization of Good Clinical Practice guidelines. Only patients who had given written informed consent were considered for inclusion. Women were included in the study if they were: (a) aged between 20 and 75 years; (b) hospitalized for unilateral total mastectomy, with or without reconstruction; (c) and subjected to standard treatment, as described in the "concomitant therapies" section. Other variables, such as weight and breast size, were recorded and considered.

Women were excluded from the study if they were: (a) receiving complex surgery such as breast reconstruction by transverse rectus abdominis or latissimus dorsi myocutaneous flaps, or in case of needed axillary dissection; (b) admitted at the emergency department; (c) receiving anticoagulants and antiplatelet treatments; (d) affected by bleeding disorders; (e) patients with liver pathologies or with concomitant severe general diseases; (f) patients receiving drug therapy with aromatase inhibitors or anticancer therapies; (g) patients with a history of stroke or of acute myocardial infarction, <24 months before; (h) patients diagnosed with other concurrent tumors; (i) patients who had participated in other clinical trials in the 3 months prior to the study; (j) and patients with values of prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen outside the normal range of the clinical site (i.e., 0.85-1.18 s for PT, 0.85-1.20 s for PTT, and 170-400 mg/dL for fibrinogen). The day before surgery (day 0), vital signs (blood pressure, heart rate, and blood oxygen saturation) were evaluated, along with any concomitant medication and any adverse event that could have occurred since the screening visit. Moreover, based on the results of blood tests, the eligibility of the patient was re-verified. The enrolled patient was then assigned to a treatment group according to the randomization list.

Randomization was created by the Contract Research Organization (CRO) Opera S.r.l. and generated using the random function (proc plan) of SAS (version 9.4) software. At screening, each patient was assigned to a code composed of the identification number of the clinical site in two digits (e.g., 01) and the identification number of the patient to three digits (e.g., 001-002-003). Time and date of each administration were recorded. The CRO Opera S.r.l held the key to the randomization list in a sealed envelope, which was not opened until the end of the study. The key was used only after freezing of the database and finalization of the statistical analyses.

The patients, according to the randomization list, received sublingually a dose of five drops of A. *montana* 1000 K 3 times a day, or placebo with identical times of administration, from 1st day before the surgery until the 4th day after the surgery (in total 6 days). On the day of surgery (day 1), the previously assigned treatment was continued. All the patients were operated by the same surgical team. During the operation, surgical drains with a caliber of 15 French (equal to 5 mm in diameter) were positioned. Surgeons did not know the allocation of patients in the treatment groups. From the end of surgery and for every 12 h, a control was carried out, the amount of blood and serum volumes collected through drainages was recorded, and for every 24 h, a blood test for the evaluation of the complete blood count (CBC) and the performance of coagulation factors was carried out. In the days following the surgery (days 2-4), the patients continued the previously assigned treatment. Vital signs, any concomitant medication, adverse event, and the presence of bruises or hematomas were monitored. Finally, patients were asked to provide an opinion on the perception of pain after surgery, using a visual analog scale (VAS). Day 5 concluded the study. On this day, the treatment previously assigned was continued, and the liquid collected by drains (if still positioned) was measured. If it was still not possible to remove the drainage, the amount of fluid drained was registered for the last time and, based on other parameters, the date of the possible discharge of the patient was indicatively anticipated. Conversely, if all parameters resulted in the normal range and drains were removed, the patient had the possibility to be discharged before the 5th day; in this case, the patient was not prematurely withdrawn, but she was considered having correctly completed the study.

Endpoints

The principal endpoint of the study was the efficacy of A. *montana* versus placebo in reducing the post-operative blood loss and seroma production in patients undergoing

unilateral total mastectomy, with or without reconstruction. The secondary end points included the following:

- Duration of drainage, which was calculated from day 1 to the day of drainage removal
- Time to reach a collected volume below 10 ml
- Self-evaluation of pain, measured by VAS after surgery
- The average time of hospitalization after surgery, which was measured in days following surgery
- The presence of bruising and hematomas or breast swelling, measured both in terms of presence versus absence and of description or size
- The differences between the volume collected on day 1 and the volume collected in each of the following days
- Possible adverse reactions to treatment.

Sample Size

The appropriate accrual hypothesized for the study was set to 60 patients, with a 1:1 ratio per treatment group (A. *montana* 1000 K vs. placebo). Considering a possible 10% dropout rate, this sample size could allow us to have a final population of 53 women, presenting a mean blood and serum collection of 280 ml in the placebo group and of 200 ml in A. *montana* group (SD 100 ml), considering adequate a statistical power equal to 80%. Finally, 53 women with breast cancer and candidate for unilateral total mastectomy, with or without reconstruction, were enrolled in the study.

Study Treatment

A. *montana* 1000 K was in the form of drops in 30% hydro alcoholic solution, with sublingual absorption. The 1000 K dilution is a very high homeopathic dilution produced according to the French Pharmacopoeia, starting from a mother tincture (complete plant extract) which undergoes 1000 steps of successive dilution and shaking in a 30% water–alcohol solution. The mother tincture of the product used in this study contained a minimum content of 0.04% of sesquiterpenes (expressed as dihydrohelenanin tiglate). According to the Korsakovian dilution method, the same flask was used for the entire preparation: At each step, the flask was emptied of most of the liquid but not dried, and then immediately filled again with the solvent. Because the residual volume after emptying the flask certainly cannot be >10% of the total used in each dilution, the 1000th K dilution is sufficiently high that it certainly does not contain toxic amounts of the plant. The placebo drops were identical in appearance to the active drops, but included only 30% hydro alcoholic solution without any homeopathic dilution. The studied homeopathic treatment and the placebo were manufactured and supplied in strictly identical packaging by Laboratoires Boiron S.r.l. (Segrate, Milan, Italy).

Concomitant Therapies

A standard treatment used in the management of surgical procedures was administered to all the patients enrolled in the study. In particular, it was provided as follows:

- Antibiotic prophylaxis with cefazolin 2 g intravenous (IV) once;
- Antithrombotic prophylaxis using low molecular weight heparin at a dose of 4000 IU 12 h before the surgery;
- Analgesic therapy with paracetamol 1000 mg IV for every 6 h for the first 24 h and tramadol 100 mg IV, if needed.

For patients undergoing reconstruction, morphine 20 mg in continuous infusion with antiemetic medication was administered for the first 48 h.

Statistical Analysis

Interval data were described as the number, mean, and standard deviation (SD). Ordinal data were described as the absolute and relative frequencies with 95% confidence intervals. Comparisons of means were carried out by analysis of variance. The differences in the blood and serum volumes collected by the drainage between A. *montana* and placebo were evaluated with three linear regression models:

- Model 1: Includes the treatment;
- Model 2: Includes the treatment and the volume collected on the day of the intervention;
- Model 3: Includes the treatment, the volume collected on the day of surgery, and patient weight.

Methods of survival analysis (Kaplan–Meier) were used to evaluate the duration of drainage need and time to reach a collected volume below 10 ml. Survival curves were compared using the log-rank test. Quantitative variables were compared using Student's *t*-test for normal distributions. The level of significance was set at 5% ($P = 0.05$). All statistical analyses were carried out using SAS (version 9.4) software.

RESULTS

The 53 women enrolled in the study (26 in the A. *montana* group and 27 in the placebo group) were all included in the intention-to-treat (ITT) population, defined as all patients who took at least one dose of A. *montana* 1000 K or placebo. The per-protocol (PP) population included all women of the ITT dataset, except those women violating the protocol. In particular, in the A. *montana* group, three patients were excluded (two had PT, PTT, and fibrinogen altered values, and one patient received axillary dissection). In the placebo group, seven patients were excluded (five had PT, PTT, and fibrinogen altered values, and two were on aromatase inhibitor or anticoagulant therapy introduced after enrollment). The baseline characteristics of the included patients are described in Table 1. There were no significant differences between treatment groups.

Blood and serum collected by drainages dramatically increased between day 1 and day 2 and then decreased in both treatment groups [Figure 1]. In the PP dataset, the collected volumes were higher in placebo group, but in the ITT dataset, the difference was less noticeable [Figure 1a]. In the PP dataset, the differences between the volume collected on day 1 and the volume collected in each of the following days revealed a difference steadily lower in the A. *montana* group compared to placebo [Figure 1b]. These differences were statistically significantly different between the

two treatment groups on day 2, both in the univariate model ($P = 0.034$) and in the model including treatment and collected volume ($P = 0.033$), and on day 3, only in the latter model ($P = 0.0223$) adjusted for weight and volume to the day of surgery. The difference between the two treatments was not statistically significant on days 4 and 5. Globally, a lower mean volume of blood and serum collected by drainage was observed in A. *montana* group compared to placebo (in PP analysis: 334.35 vs. 428.75 ml, $P = 0.11$) [Tables 2 and 3]. A statistically significant difference was shown in regression model 3 applied on PP dataset, when the estimates were also adjusted for the volume collected from the drainage on day 1 and the weight of the patient [Table 4].

During the 5 days of the study, the drainage was removed in 36 patients, equally distributed in the two groups. The analysis of time of drain removal revealed no difference between the two groups of patients both in the ITT dataset (log-rank test $P = 0.7287$) and in the PP dataset (log-rank test $P = 0.8653$), although in A. *montana* group, drainage was apparently removed earlier [Figure 2a]. The analysis of the time up to collected volume below 10 ml neither revealed difference between the two treatments in the ITT analysis (log-rank test $P = 0.8653$) nor in the PP analysis (log-rank test $P = 0.6138$), but showing a better performance of the patients treated with A. *montana* in the latter [Figure 2b].

The presence of bruising and hematomas or breast swelling did not differ significantly by treatment group, $P = 0.67$ and $P = 0.57$, respectively. Even the perception of pain, measured by VAS, showed no statistically significant differences between the two treatment groups ($P > 0.05$). All the recorded adverse events were not severe (e.g., rush, headache, nausea), not correlated with the A. *montana* treatment, and completely recovered at the end of the study. Moreover, they were equally distributed between the two groups: Five patients experienced at least an adverse event in the A. *montana* group versus four in the placebo group.

Table 1: Baseline characteristics of the ITT population

Characteristics	Statistics	Placebo (*N*=27)	*Arnica* (*N*=26)
Age	Mean±SD	56.59±12.65	55.77±11.41
	Median	52.28	53.60
	Min-Max	33.91-75.42	38.15-73.22
Race	Asian (%)	-	1 (3.8)
	Caucasian (%)	27 (100)	25 (96.2)
Height	Mean±SD	160.96 (4.74)	162.46 (7.27)
	Median	162	160.50
	Min-Max	150-70	150-178
Weight	Mean±SD	64.19±13.05	66.95±13.17
	Median	60.40	62.90
	Min-Max	48.00-104.50	50.10-96.30
BMI	Mean±SD	24.74±4.71	25.33±4.59
	Median	24.34	22.81
	Min-Max	18.25-40.82	20.04-38.58
Systolic blood pressure (mmHg)	Mean±SD	119.74±17.90	120.00±15.30
	Median	120.00	120.00
	Min-Max	85.00-150.00	100.00-160.00
Diastolic blood pressure (mmHg)	Mean±SD	73.52±11.99	75.19±7.41
	Median	70.00	80.00
	Min-Max	50.00-100.00	60.00-90.00
Heart rate (beats/min)	Mean±SD	73.78±8.45	74.88±11.67
	Median	74.00	75.00
	Min-Max	60.00-88.00	50.00-102.00

SD: Standard deviation, ITT: Intention-to-treat

DISCUSSION

In this study, which to our knowledge is the first to test the effect of A. *montana* in breast cancer surgery, we have measured

Table 2: Mean daily volume and total volume in the two treatment groups for the ITT dataset

Treatment	ITT dataset				95% CI
	Day	Patients (*N*)	Volume Mean (ml)	SD	
A. montana 1000 K *N*=26	1	26	39.42	40.68	23.79-55.06
	2	26	124.42	49.94	105.23-143.62
	3	26	98.08	57.08	76.14-120.02
	4	25	70.20	65.54	44.51-95.89
	5	20	55.00	97.73	12.17-97.83
	Total	26	371.73	239.97	279.49-463.97
Placebo *N*=27	1	27	37.78	30.21	26.38-49.17
	2	27	133.15	82.38	102.07-164.22
	3	27	107.41	70.39	80.86-133.96
	4	26	71.15	59.02	48.47-93.84
	5	21	49.52	60.09	23.82-75.23
	Total	27	385.37	235.36	296.59-474.15

SD: Standard deviation, ITT: Intention-to-treat, *A. montana*: *Arnica montana*, CI: Confidence interval

Table 3: Mean daily volume and total volume in the two treatment groups for the PP dataset

Treatment	PP dataset				95% CI
	Day	Patients (n)	Volume mean (ml)	SD	
A. montana 1000 K *N*=23	1	23	42.17	40.42	24.70-59.65
	2	23	119.78	45.11	100.27-139.29
	3	23	91.09	50.99	69.04-113.14
	4	22	57.73	35.81	41.85-73.61
	5	18	33.33	37.10	14.88-51.78
	Total	23	334.35	164.64	263.15-405.55
Placebo *N*=20	1	20	43.50	28.43	30.20-56.80
	2	20	154.50	77.15	118.39-190.61
	3	20	127.00	66.34	95.95-158.05
	4	20	67.50	45.67	46.13-88.87
	5	16	45.31	52.87	17.14-73.48
	Total	20	428.75	214.32	328.44-529.06

SD: Standard deviation, *A. montana*: *Arnica montana*, CI: Confidence interval, PP: Per-protocol

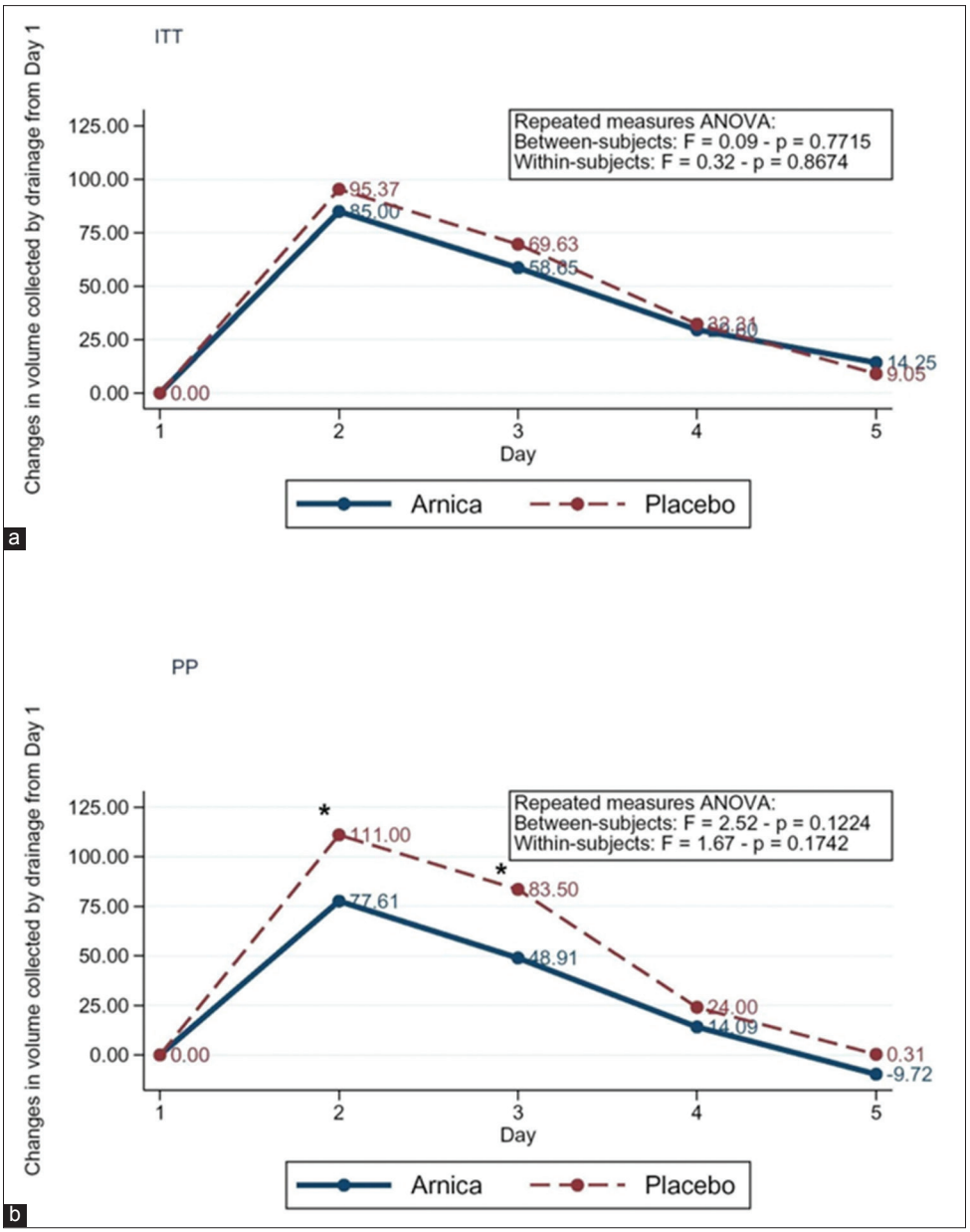

Figure 1: Changes in volume collected by drainage from day 1 to each following day per treatment: (a) Intention-to-treat population and (b) per-protocol population; *P < 0.05

the efficacy of the homeopathic treatment versus placebo in reducing the post-operative bleeding and seroma production in patients subjected to total mastectomy. The difference in the volume of blood and serum collected by drainages between the group of women treated with A. *montana* and those treated with placebo was statistically significant in the PP dataset, but only in the model which included, in addition to the type of treatment, the volume collected on the day of surgery, and the weight of the patient. Thus, we determine that these two additional variables have a considerable effect on the efficacy of the treatment, and further studies are needed to investigate their specific contributions. In general, the other data obtained, such as a slightly earlier removal of the drainage and a faster reduction in the volume of blood and serum collected in the drainage, were not statistically significant and only suggested a slight advantage in the post-operative recovery process of patients treated with A. *montana*. The variability is similar in the two groups and it appears not due to different responses to the drug tested. The high Korsakovian dilution used in this study compared to other studies could explain the failure to reach the threshold of significance despite the fact that the differences are quantitatively small but noticeable [6,11]. This high Korsakovian dilution was chosen to surely avoid any toxicity from A. *montana* extract, to administer a harmless experimental treatment.

Overall, the efficacy of homeopathic treatments in surgical settings is still controversial. In obstetrics, evaluating the effect of A. *montana* and *Bellis perennis* on blood loss, Oberbaum *et al.* found that the mean hemoglobin levels remained the same

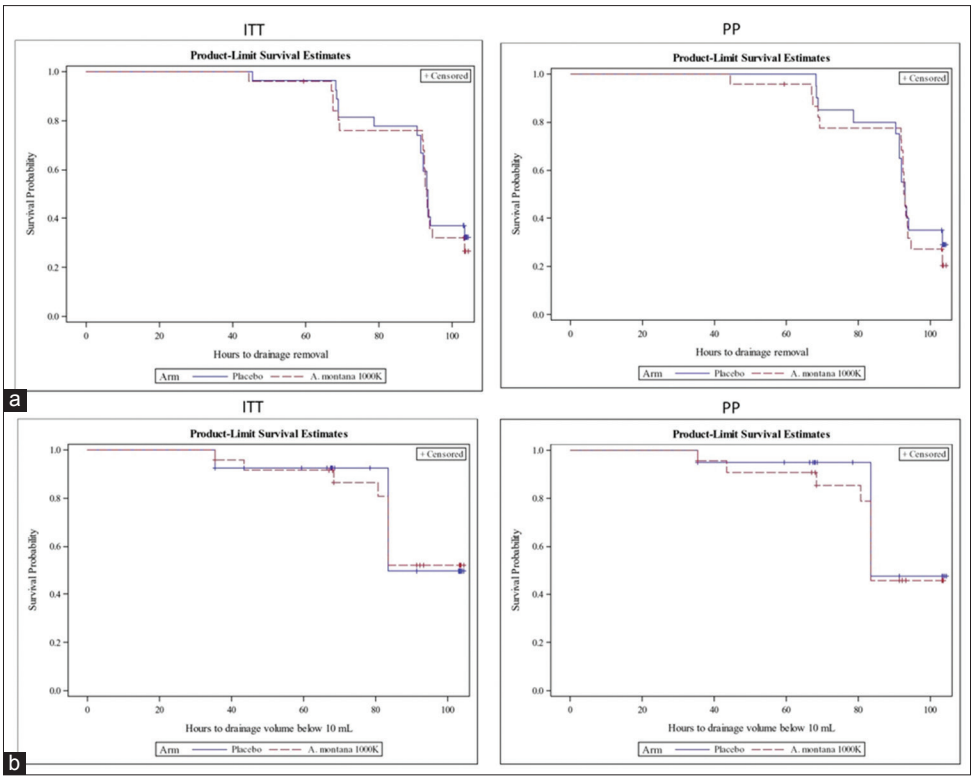

Figure 2: Duration of drainage (a) and time to drainage volume below 10 mL (b) per treatment (intention-to-treat and per-protocol datasets)

Table 4: Estimates of the mean difference in total volume collected by drainage during the study between the two treatment groups (*Arnica*–Placebo) by dataset

Dataset	Model[1]	Variables	Parameter estimate[2]	95% CI	*t*-value	*P* value
ITT	1	Treatment	−13.64	−138.51-111.23	−0.22	0.83
	2	Treatment	−18.85	−129.87-92.17	−0.34	0.73
	3	Treatment	−28.69	−138.27-80.89	−0.53	0.60
PP	1	Treatment	−94.40	−211.28-22.48	−1.63	0.11
	2	Treatment	−90.28	−186.71-6.14	−1.89	0.07
	3	Treatment	−106.28	−203.11-−9.45	−2.22	0.03*

[1]Regression models are as follows: (1) Model 1: Treatment only, (2) Model 2: Treatment and volume collected on the day of the intervention, (3) Model 3: Treatment, volume collected the day of surgery, and patient's weight, [2]Mean difference in volume (mL) collected by drainage during the study between *Arnica* and placebo groups (*Arnica*–Placebo). *$P<0.05$. CI: Confidence interval, PP: Per-protocol, ITT: Intention-to-treat

after the administration of the homeopathic treatment whereas the levels decreased significantly in the placebo group (12.7 versus 11.6; $P < 0.05$) [17]. A systematic review has analyzed A. *montana* in eight randomized controlled trials (RCTs) from 1966 to 1997 [10]. Two of the included trials, one on the effect of A. *montana* on the delayed onset of muscle soreness (DOMS) and the other on the prevention of post-operative complications, showed a statistically significant result in favor of the homeopathic treatment. The other six trials did not demonstrate statistically significant differences, but some of these trials were not considered qualitatively adequate for a proper meta-analysis [10]. Other RCTs had conflictual data. Only one of the three RCTs on DOMS and none of the two on surgical patients showed statistically significant between-group differences, in particular, on the recovery after total abdominal hysterectomy and on the stripping of varicose veins [13,28-32]. In a double-blind, placebo-controlled clinical study involving patients undergoing prolonged venous perfusion, A. *montana* reduced pain symptoms, hyperemia, edema, and formation of hematomas [33]. A. *montana* treatment also slightly increased a number of coagulation factors and platelet aggregation.

In vitro studies are more promising. In fact, different authors have shown that helenalin, the most active component of A. *montana*, not only blocks the transcriptional NF-κB in T-cells, B-cells, and epithelial cells, but it also inhibits human neutrophil migration and chemotaxis and the activities of 5-lipoxygenase and leukotriene C4 synthase [34]. All these mechanisms are known to be associated with the induction of pain and inflammation, as observed in animal models of inflammatory pain [34,35]. However, these studies were conducted with pure helenalin rather than with plant extract and with much higher doses than those used here. A recent work carried out on human macrophage cells differentiated with interleukin-4 demonstrated that homeopathic doses of A. *montana* stimulate the expression of genes involved in the repair of connective tissue and, in particular, fibronectin [36]. Given the importance of this protein to the adhesion, proliferation, and movement of cells (both epithelial and connective), this could be a further target at the cellular level of this plant's action.

Undoubtedly, one as yet unsolved problem of homeopathy concerns the mechanism of action of dilutions so high that

the presumed active substances are often not detectable in molecular terms. Many laboratory studies that use homeopathic dilutions report dose-response curves that are non-linear and sometimes pseudo-sinusoidal, with peaks occurring at different dilutions, alternating with other inactive doses [37-39]. In relatively recent times, evidence has been found, especially in physicochemical studies, pointing to the presence of nanoparticles even in extremely high dilutions, or the formation of coherence domains among the molecules of the water–alcohol solvent, as predicted by quantum electrodynamics [40,41]. Although these findings fall outside the scope of the present work, if consolidated by further experimental studies, they might furnish a basis of plausibility to this complementary pharmacological approach.

The main limitations of the study were the relatively small sample, the analysis of post-operative recovery, which was limited only to 5 days, and the use of a high Korsakovian dilution which could have negatively impacted on the statistical significance of our findings. Observational studies with different Korsakovian dilutions could be useful to highlight any significant effect of *A. montana* and further validate these findings.

CONCLUSIONS

The scientific community often claims that homeopathy effects are not supported by rigorous clinical trials. This study, although reporting statistical significance only for some specific data settings, suggests a reduction in post-operative blood loss and seroma production in a group of women who underwent breast cancer surgery and treated with A. *montana* 1000 K.

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Original Research

Effect of *Nigella sativa* (black seeds) against methotrexate-induced nephrotoxicity in mice

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ABSTRACT

Aim: To evaluate the protective effect of Nigella sativa (NS) against nephrotoxicity of methotrexate (MTX) in mice. **Materials and Methods:** Four groups of Swiss albino male mice, eight in each group were used. The study was carried on between October 2014 and April 2015. Group 1 (control) were administered 0.3 ml distilled water orally daily for 21 days and injected with normal saline (0.25 ml) IP weekly. Group 2 (MTX group) were treated with MTX, 10 mg/kg IP weekly, while Group 3 were treated with 0.125 ml of NS oil by mouth daily and injected with normal saline (0.25 ml) IP weekly. Group 4 received 0.125 ml of NS oil by mouth daily and injected with 10 mg/kg MTX IP weekly. Oral treatments were administered using a special curved smooth tip nontraumatic metal needle and IP injections were given for 3 weeks at days 7, 14 and 21. Animals were sacrificed at day 23. Malondialdehyde (MDA) and glutathione (GSH) measurements were performed on kidney homogenate. Histopathology of the kidneys were prepared and examined. **Results:** MTX has resulted in a small elevation in MDA and reduction in GSH levels in kidney homogenate which was returned back to control values when NS and MTX were administered in combination. Statistical significance was achieved with elevation of GSH by MTX and NS compared to MTX alone. MTX caused histopathological changes suggesting nephrotoxicity in 6 animals out of 8, while no changes were found in all animals treated with MTX and NS. **Conclusion:** NS is protective against MTX-induced nephrotoxicity.

KEY WORDS: Antioxidant, methotrexate, *Nigella sativa,* kidney toxicity

INTRODUCTION

Methotrexate (MTX) was introduced in 1948 and remains one of the most commonly used antimetabolite agents in cancer therapy. It was approved for the management of various types of malignancies such as leukemia, breast cancer, and lymphoma [1]. It has been used also for nonmalignant diseases such as rheumatoid arthritis [2], psoriasis [3] and for the treatment of ectopic pregnancy [4]. MTX has serious side effects including hepatotoxicity, myelosuppression, pulmonary disorder, gastrointestinal toxicity [5,6] and nephrotoxicity [7]. There are various ways to minimize MTX nephrotoxicity such as intravenous hydration, leucoverin rescue, alkalinization of urine and the use of glucarpidase [8]. There is an increasing evidence from animal studies supporting the protective effect of a medicinal plant Nigella sativa (NS, black cumin) against nephrotoxicity produced by gentamicin [9], paracetamol [10], cadmium [11], cyclosporine A [12], doxorubicin [13], and cisplatin [14]. The protective effect of NS is mainly attributed to its antioxidant potential which has been inferred from the ability of NS in reversing drug induced changes in parameters of oxidative stress, malondialdehyde (MDA) and glutathione (GSH) levels, toward placebo levels [15,16]. This study, therefore, was designed to investigate the effect of NS against MTX-induced nephrotoxicity in mice.

MATERIALS AND METHODS

Animal Handling

Thirty-two adult Swiss albino male mice were obtained from the animal house at Basrah College of Medicine. Their weights ranged between 20 and 30 g and their ages between 4 and 6 weeks. The study was conducted between October 2014 and April 2015. The animals were kept for acclimatization in separate cages in the animal house 2 weeks before the study, with a 12:12 h light/dark cycle and at a room temperature around 25°C. A standard food was prepared in the form of pellets containing carbohydrate, starch, moisture, and a crude protein not <20% and a crude fat not <6% of the total weight of the pellet. Food and ordinary drinking water were provided *ad-libitum*. The animals were carefully handled in accordance with the internationally accepted guidelines for handling laboratory animals (National Institutes for Health USA Publication, 1985), and measures were undertaken to minimize pain and discomfort

during experimentation. The study protocol was approved by a local Institutional Ethical Committee.

Preparation of NS

Seeds of NS were purchased from a local market in Basrah, and authenticated by an expert herbalist. A voucher specimen was kept in the Department of Pharmacology for future reference. Viability of the seeds was tested by cultivating 100 seeds in a small garden, implanted numbers of plants were counted and their growth was followed up for at least 4 weeks.

Extraction of the NS essential oil was performed in the Marine Science Center at Basrah University. The seeds were crushed by electric grinder into a fine powder then the essential oil was extracted with petroleum ether at 40-60°C via a soxholet apparatus (25% yield of essential oil) and the solvent was removed by rotatory evaporator under vacuum, then the oil was stored in dark sealed containers.

Study Design

The animals were divided into 4 groups (8 mice in each group). The first group (control) received (0.3 ml) of distilled water orally every day for 21 days and at the same time injected with (0.25 ml) normal saline IP weekly at days 7, 14 and 21 of the experiment. The second group (MTX group) were given distilled water (0.3 ml) orally each day and injected with MTX IP weekly (MTX ampule, Ebewe-Pharma, Austria) at a dose of (10 mg/kg) at days 7, 14 and 21, while animals in the third group received 0.125 ml of NS oil by mouth daily and injected with normal saline (0.25 ml) IP at the same weekly schedule. Animals in the fourth group received the same treatment of Group 3 but instead of normal saline, the animals were injected with MTX at a dose 10 mg/kg IP. Oral treatment was administered using a special curved smooth tip nontraumatic metal needle. Animals in all groups were sacrificed at day 23 after an overnight fasting. The following parameters were obtained; MDA and GSH in tissue homogenate of the kidney, specimens of kidney for histopathological examination, body weight and kidneys weight of the animals were measured and a ratio kidneys weight to animal body weight was calculated.

Measurement of MDA in Kidney Homogenates

MDA levels in kidney homogenates were measured by thiobarbituric acid (TBA) test, based on the reaction of MDA with TBA which produces a pink pigment that can be measured by a spectrophotometer at 532 nm [17].

Measurement of GSH in Kidney Homogenates

Enzyme-linked immunosorbent assay (ELISA) kit specific for mice (Cusabio reagents, Cusabio Laboratories, Wuhan, China) was used for measurement of GSH levels in kidney homogenates. This kit employs a technique known as "double antibody sandwich technique." ELISA (HumaReader HS, Germany) is the equipment used for the measurement.

Histopathological Examination

Slides were prepared in duplicates from kidneys specimens, stained with hematoxylin and eosin. The slides were coded, blindly examined by a senior histopathologist using light microscopy (Olympus CX-series). Histopathological changes were graded as previously described by Morsy *et al.*, [18]: 0 (Normal), 1 (mild), 2 (moderate), and 3 (severe), for 0%, 25%, 50%, and 75% histopathological changes of fields examined, respectively.

Statistical Analysis

SPSS version 19 (IBM, NY, USA) was used for statistical analysis. A nonparametric Mann–Whitney U-test for independent samples was used for comparing variables. The data were presented as mean ± SD, $P < 0.05$ is considered significant.

RESULTS

Effect of NS, MTX and their Combination on MDA in Kidney Homogenate

There was an increase in MDA level in the homogenate of the kidney in the group treated with MTX compared to the control group, however, the change was small and did not achieve statistical significance (1852 ± 369 to 2008 ± 750 μmol/L). MTX with NS had resulted in a small and insignificant reduction in MDA levels compared to the MTX treated group (1705 ± 496 vs. 2008 ± 750 μmol/L). The effect of NS on MDA levels was discarded from the analysis because of unexplained variability in the data possibly due to technical errors in measurement [Table 1].

Effect of NS, MTX and their Combination on GSH in Kidney Homogenate

There was a small and insignificant reduction in GSH level in the group of mice treated with MTX compared to the control. Treatment with NS has also resulted in a small and insignificant reduction in GSH compared to the control. While treatment with the combination MTX and NS resulted in elevation in GSH level which is statistically higher than the value of GSH of the MTX treatment ($P = 0.048$) but still lower than the level of the control ($P = 0.065$) [Table 1].

Table 1: Effect of NS, MTX and their combination on MDA and GSH levels in tissue homogenate of the kidney

Parameters of oxidative stress (μmol/l)	Control	NS	MTX	MTX+NS
Kidney MDA	1852±369	¥	2008±750	1705±496
Kidney GSH	62±13.4	60±5.8	58±3.9	61.8±2.9*

*: Significantly different from the correspondence value of MTX, P=0.48. ¥: Data not available, NS: *Nigella sativa*, MTX: Methotrexate, MDA: Malondialdehyde, GSH: Glutathione

Effect of NS, MTX and their Combination on Animal Body Weight

Changes in animal body weight from day 1 to day 23 were recorded and a mean change from the corresponding pretreatment value was calculated for each treatment. The body weight of animals treated with normal saline (control group) for 23 days was increased compared to pretreatment value with a mean change of 1.02 ± 3.27 g, however, this increase did not achieve statistical significance, while a reduction in animal body weights were observed in the animals treated with MTX (−2.53 ± 2.41 g) or NS (−2.03 ± 2.57 g); these changes were significantly different from the control value ($P = 0.01$). Animal body weights increased again toward the control values in the group treated with the combination NS and MTX (1.01 ± 0.25 g) [Figure 1a].

Effect of NS, MTX and their Combination on Animal Kidney Weight Body Weight Ratio

A baseline kidney weight was not possible to obtain for comparison with kidney weight at day of sacrificing the animals, therefore, for this analysis it was assumed that kidney weight is proportionally related to animal's body weight, such proportion is approximately valid as described by Gad, 2008 [19].

The ratios of kidney to animal weight for NS and the combination NS + MTX were significantly lower than the value of the control, $P = 0.05$ [Figure 1b]. The ratio for MTX was lower than that for the control but it did not reach statistical significance.

Histopathological Examination

Control and NS treated groups

There were no histopathological changes found in the control group or in the group treated with NS (Score 0). Glomeruli and renal tubules maintain normal morphological features [Table 2, Figure 2a and b].

MTX treated group

Treatment with MTX produced no changes in two animals (Score 0), moderate changes in two (Score 2) and in four animals severe changes were seen (Score 3). The changes involved renal tubules and seemed not involving the glomeruli; these changes were focal degeneration of tubular epithelial cells, interstitial chronic inflammatory cell infiltration of lymphocytes and plasma cells and perinephric fat necrosis [Figure 3a].

NS and MTX groups

There were no histopathological changes (Score 0) detected in all animals ($n = 8$) treated with the combination NS + MTX [Figure 3b].

DISCUSSION

MTX is a drug basically used for its cytotoxic, anti-inflammatory, and antiproliferative effects [20], but organ toxicities are frequently reported, and may result in discontinuation of treatment. Thus attempts to minimize these toxicities are well appreciated. NS is a medicinal plant with a wide range of properties and effects on various body systems including hepatoprotective and nephroprotective properties [10,21].

This study was then designed to investigate the protective effect of NS against nephrotoxicity induced by MTX. Mice were selected as a convenient model for MTX nephrotoxicity.

Table 2: Scoring of histopathological changes of kidney damage induced by treatments with NS, MTX and their combination

Scores	Number of animals			
	Control	NS	MTX	MTX+NS
0	8	8	2	8
1	0	0	0	0
2	0	0	2	0
3	0	0	4	0

Scores 0, 1, 2 and 3 are for normal, mild, moderate, and severe changes, respectively. NS: *Nigella sativa*, MTX: Methotrexate

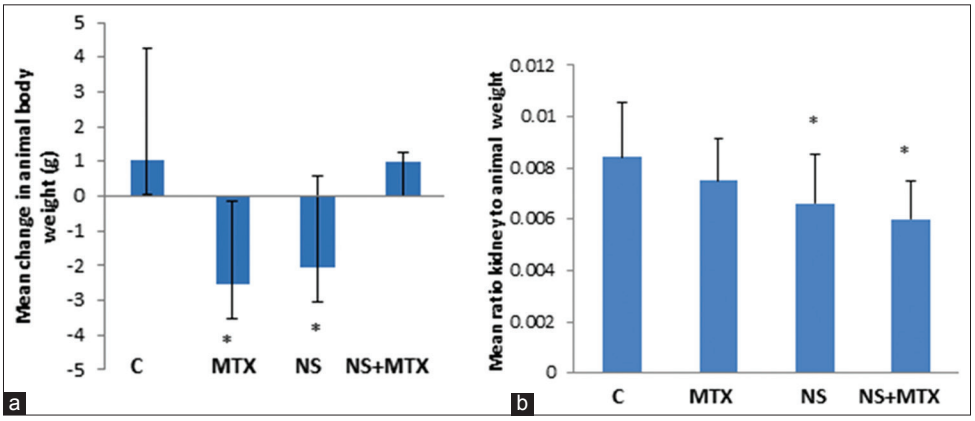

Figure 1: (a) Changes in animal body weights between day 1 (before treatment) and after 23 days of treatment with MTX, NS and the combination, (b) the ratio of kidney weights to animal weights. C: Control, MTX: Methotrexate, NS: *Nigella sativa*, NS+MTX: Combination of NS and MTX. *: Significantly different from the control (P=0.05)

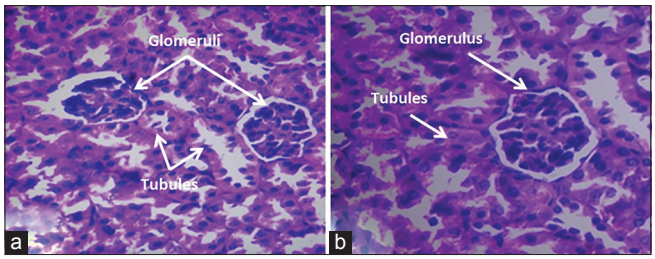

Figure 2: Renal tissue from a mouse treated with normal saline, (a) *Nigella sativa*, (b) histopathological features revealed normal glomeruli and tubules (×40 hematoxylin and eosin)

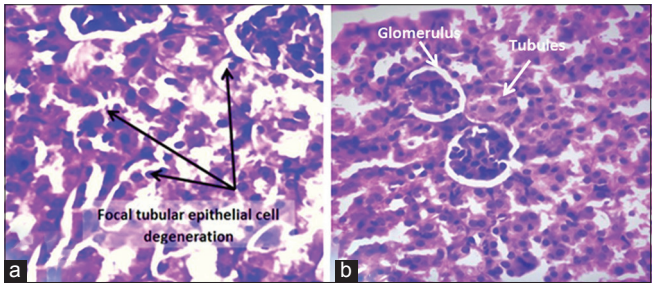

Figure 3: Renal tissue of a mouse treated with methotrexate (MTX), (a) showed severe focal degeneration of tubular epithelial cells (×40, hematoxylin and eosin [H&E]), (b) it is a renal tissue from a mouse treated with the combination *Nigella sativa* and MTX. Normal glomeruli and tubules are seen (×40, H&E)

Two parameters of oxidative stress (MDA and GSH) were assessed. Treatment with MTX has resulted in a slight increase in the level of MDA in kidney homogenate which is then slightly decreased in the group treated with NS and MTX combination; the changes were small and did not achieve statistical significance.

The rise in MDA and reduction in GSH may reflect oxidative effect of MTX, and the changes of these parameters toward placebo values following the combination NS with MTX may be attributed to the antioxidant effect of NS [22].

Histopathological examination of the kidneys was performed and used as a tool for confirmation of toxicity induced by MTX and to evaluate the nephroprotective effect of NS. There were no changes detected in the control and NS groups, while in the MTX treated group; tubular epithelial cell degeneration ranging in severity was observed in 6 of 8 treated animals.

This study revealed that MTX is toxic to the tubules and seems not affecting the glomeruli. This result, in part, is in agreement with the study of Morsy *et al.*, 2013 [18] who reported nephrotoxicity in rats following IP administration of MTX at a dose of 7 mg/kg/day for 3 consecutive days. These authors observed severe histopathological changes including degeneration of the renal tubules as a result of direct toxicity of MTX with disruption of in-between tubules basement membranes with cystic luminal dilatation and flattening of lining cells, but in contrast to the findings of this study, these authors had reported degeneration of glomeruli induced by MTX treatment. There were many possibilities for the lack of effect of MTX on glomeruli in this study; first, it could be attributed to spacing of MTX dosing since in this study a dose of 10 mg/kg was given once weekly for 21 days rather than daily for 3 consecutive days; second, species differences may also contribute to differences in MTX effect on the glomeruli; finally, changes in glomeruli may exist but may not be seen by ordinary microscope.

NS treatment had resulted in preventing histopathological changes in the kidneys in all animals treated with NS and MTX.

MTX causes renal toxicity by different mechanisms including direct tubular toxicity [7] possibly due to oxidative stress [23] or due to accumulation and precipitation of MTX or its metabolites in distal tubules causing intrarenal obstruction leading to tubular damages. On the other hand, this may further decreases renal elimination of MTX leading to increased plasma levels and more toxicity.

It is not known for NS to have an effect on accumulation of MTX in renal tubules; therefore, it is possible for NS to minimize tubular damage caused by direct toxicity of MTX through its antioxidant potential [22].

Animal body weights were measured at the beginning and at the end of the experiment. There was a reduction in body weight in the group treated with MTX and in the group treated with NS. Reduction in body weight by MTX was reported by de Araújo *et al.* [24] which may be attributed to intestinal mucositis a finding noticed in this study and resulting in reduction in food consumption in the MTX and NS treated animals, in addition, diarrhea was noticed in animals treated with MTX, both these findings may contribute to weight loss in the animals. Reduction in body weight by NS and the mechanism behind this effect is not clearly understood.

Changes in animal's body weight were paralleled with changes in kidneys weight. For the sake of comparison, changes in kidney weight were presented as a ratio of kidney to animal body weight. Small kidneys were observed in the group treated with NS or even smaller in the MTX treated group.

The significance of these changes and their effect on kidney function needs further investigations.

In medical practice absence of persistent kidney protective agents against drugs which produce kidney toxicity, herbs may offer an important alternative treatment in preventing kidney toxicity. It can be concluded that the medicinal plant NS (black cumin) is protective to the kidney against toxic effects of MTX. As NS demonstrates a good safety profile [25] it is worth trying NS orally along with MTX treatment to those patients who need this type of treatment.

Study Limitations

The mouse as a small animal has limited amount of blood sufficient to perform additional test such as renal function test

or parameters of oxidative stress in the serum, therefore, the study depended on parameters obtained from homogenates of kidney tissue with further confirmation by histopathological examination.

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Original Research

Antinociceptive and anti-inflammatory effects of aqueous extract of *Chenopodium opulifolium* schrad leaves

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ABSTRACT

Aim: *Chenopodium opulifolium* is a specie of the Chenopodiaceae commonly used as vegetables in local diet and for treating different ailment in Uganda. This study was conducted to evaluate the antioxidant, antinociceptive and anti-inflammatory effects of the aqueous extract of *C. opulifolium* leaves (AECO). **Materials and Methods:** The dried leaf of the plant was extracted by maceration in water. Qualitative and quantitative phytochemical analysis, antioxidants, and membrane stabilizing effects were determined in the extract. The extract was then investigated for acute toxicity, antinociceptive (writhing, hot plate and open field test), and anti-inflammatory (egg albumin-induced paw edema) effects in rodents. **Results:** Phytochemical analysis revealed the presence of alkaloids, tannins, phlobatannins, flavonoids, and saponins in AECO. Total caffeic acid derivatives and total flavonoids content were 91.7 mgCAE/g sample and 94.7 mgRE/g sample, respectively. AECO demonstrated antioxidant effects in both 1,1-diphenyl-2-picryl-hydrazyl and NO assays. Significant membrane stabilizing activity was observed in both the heat and hypotonic solution-induced lysis of erythrocytes. The acute toxicity test showed that AECO (5000 mg/kg) did not cause any significant change in behavior or death in rats. AECO (100-400 mg/kg) produced a significant antinociceptive effect in both the writhing and hot plate tests, but no significant reduction in the locomotory activity in mice. Furthermore, the extract significantly ($P < 0.05$) reduced egg albumin-induced rat paw edema by 44.2%, 44.5%, and 51.2%, respectively, after 120 min. **Conclusion:** The results showed that *C. opulifolium* extract possesses significant antioxidant, antinociceptive and anti-inflammatory effects, and these affirm the reasons for its folkloric use.

KEY WORDS: Anti-inflammatory, caffeic acid, *Chenopodium opulifolium*, erythrocytes, writhing

INTRODUCTION

Medicinal plants represent unique biodiversity resources on the African continent contributing immensely to the health and wellness of its population over the centuries. The contributions of the variety of medicinal plants to local economies, cultural integrity, and health care and ultimately the well-being are increasingly being discussed [1]. In Sub-Saharan Africa for example, households in rural areas rely heavily on plant resources for food, fodder, and herbal medicine. The World Health Organization decade old statistics estimated that about 80% of the African population depends on herbal medicines for some aspect of primary health care needs [2]. Inaccessible and unaffordable modern medicines are major factors that still promote the use of medicinal plants among rural Ugandans for their daily health care needs [3]. Medicinal plants being a dominant component of the traditional medicine practice in Uganda, is used for the prevention, diagnosis, and treatment

of social, mental and physical illness [4]. One of such plants used is *Chenopodium opulifolium* Schrad. ex Koch & Ziz, with a synonym *Chenopodium ugandae* (Aellen) Aellen, a member of the Chenopodiaceae family that grows in Uganda [4].

The Chenopodiaceae is a large family comprising about 102 genera and 1400 species. The genus chenopodium includes a variety of weedy herbs (more than 200 species) that are native to Europe, Africa, Asia, North and South America [5]. The importance of the Chenopodium species was due to their variety of medicinal properties, commercially used as spices or drugs because of the presence of useful secondary metabolites. The most characteristic constituents are flavonoids, essential oils, and terpenes [6]. The reported biological activities of the chenopods include the antiviral, antimicrobial, antifungal, anti-inflammatory, analgesic, and immunomodulatory effects [7].

C. opulifolium known locally as "Kavumbavumba" in central or "Omwentago" in Western Uganda has been reported to have several ethnobotanical uses [8,9]. The leaf is used as vegetable in local diets in East Africa [10]. The leaves or root decoction is drunk to induce menstruation and hasten childbirth and to relieve irregular painful menstruation [11]. The leaves can be used as eye ointment or macerated leaf used for female asthenia, and abdominal colic for newborns [12]; and for managing pediatrics cases of febrile illnesses [13]. *C. opulifolium* leaf is reported to be used in the management of Herpes simplex viral infection in HIV/AIDS [14]. The water decoction or infusion of its leaves has been reported to be used for the treatment of malaria and cough, either alone or in combination with *Azadirachta indica*, *Momordica foetida*, *Ocimum gratissimum*, *or Vernonia amygdalina* [15].

The ethnobotanical information on this plant did not match the pharmacological or toxicological information. *C. opulifolium* medicinal properties have not been extensively studied in biological systems, and no published data are available in scientific literatures. This study was, therefore, carried out in an attempt to screen for the antioxidant, antinociceptive, and anti-inflammatory effects of the aqueous extract of *C. opulifolium* (AECO) leaves.

MATERIALS AND METHODS

Materials

Chemicals and drugs

Glacial acetic acid, indomethacin, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium nitroprusside, and quercetin were all product of Sigma-Aldrich, USA.

Animals

Adult Wister rats (120-180 g) or Swiss mice (18-25 g) of either sex were used throughout this study. The animals were kept under environmentally controlled laboratory room conditions at 21-27°C. The animals were housed in cages lined with wood shavings and were fed with standard pelletized feed (Nuvita® Animal Feeds Ltd., Jinja Uganda) and water *ad-libitum*. The animal experiments were conducted according to the National Institute of Health Guide for the care and use of laboratory animals [16] and ethical guidelines for investigation of experimental pain in conscious animals [17].

Plant Material

The leaves of *C. opulifolium* locally known as "omwentago" were collected in Ishaka, Bushenyi District, in Western Uganda. The plant was authenticated by Assoc Prof Dominic Byarugaba, a botanist at Kampala International University; a voucher specimen was prepared as herbarium sample and deposited at the school of pharmacy herbarium (KIU-WC/10/001). Fresh leaves of the plant were dried by placing them under shade, and then, reduced to fine powder by grinding using a metallic mortar and pestle in Kampala International University pharmacy laboratory.

Extraction

Extraction of *C. opulifolium* powdered leaf was done by maceration method. Briefly, powdered leaf (100 g) was soaked in 1 L of distilled water and shaken for 48 h on a laboratory rotator (Nuve SL 350 Quality System, Digisystem Laboratory Inc., Taiwan). The extract was filtered and evaporated over water bath at 50°C and was finally dried in the oven set at 40°C. The extract obtained was darkish brown in color denoted as AECO and was stored in the refrigerator at 4°C.

Qualitative Phytochemical Screening

The aqueous extract used for pharmacological screening was subjected to preliminary qualitative phytochemical analysis using standard protocols [18,19]. The presence of the compounds to be tested was rated as positive (+) or negative (−). These compounds included tannins, phlobotannins, saponins, terpenoids, flavonoids, alkaloids, and reducing sugars.

Determination of Total Caffeic Acid Derivatives Content (TCAD)

TCAD in the aqueous extract was determined using the spectrophotometric method with Arnow's reagent [20]. 0.2 mL of AECO (1 mg/mL), 1 mL HCl (0.5 N), and 1 mL Arnow's reagent and 1 mL NaOH (1 M) were mixed in test tubes and allowed to stand for 5 min. The volume was made up to 5 mL and after 30 min, the absorbance was read at 500 nm with a spectrophotometer (Spectronic 21D Milton Roy, USA). Caffeic acid content was determined from caffeic acid calibration curve with a linear regression curve ($R^2 = 0.999$).

Determination of Total Flavonoids Content in AECO Leaves

The total flavonoid content (TFC) in the extract was determined by the $AlCl_3$ spectrophotometric method as

described by Sultana *et al*. [21]. 1 mL of AECO (1 mg/mL) was dispensed into three separate test tubes. After that, 0.3 mL of 10% (w/v) $NaNO_2$ was added to the test tubes, and left to react for 5 min, 0.3 mL of 10% (w/v) $AlCl_3$ was added and left for 1 min to react. Thereafter, 2 mL of 1M NaOH was added and the mixtures shaken. Aliquots of the mixtures were transferred to a cuvette, and the absorbance values measured with a spectrophotometer (Spectronic 21D Milton Roy) at 510 nm. A mixture of 1 mL of 80% (v/v) methanol, 4 mL of deionized water, 0.3 mL of 10% (w/v) $NaNO_2$, 0.3 mL of 10% (w/v) $AlCl_3$, and 2 mL of 1 M NaOH was prepared and used as the blank. Rutin was used as a standard for the calibration curve ($R^2 = 0.985$).

Evaluation of Free Radical Scavenging Effects of AECO

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

The free radical scavenging properties of AECO were assessed using DPPH spectrophotometric assay by Aderogba *et al*. [22] with slight modifications. To 2 mL of AECO (12.5, 25, 50, 100, 200 and 400 μg/mL in methanol), quercetin (25 μg/mL) or methanol (2 mL) were added 3 mL of freshly prepared DPPH solution (0.1 mM) in methanol. The mixture was incubated in the dark cupboard for 30 min at room temperature, and the absorbance was measured at 514 nm on an ultraviolet (UV)/vis spectrophotometer (INESA). The percentage free radical scavenging activity was calculated accordingly:

% inhibition=[(absorbance of control–absorbance of test sample)/absorbance of control] ×100%

Nitric oxide free radical scavenging assay

Nitric oxide radical scavenging activity assay according to the method described by Kumaran and Karunakaran [23] was used. Briefly, sodium nitroprusside (10 mM in 0.1 M sodium phosphate buffer, pH 7.4) was mixed with AECO (12.5, 25, 50, 100, 200 and 400 μg/mL) or quercetin (25 μg/mL) and incubated at room temperature for 150 min. The control contained the same reaction mixture except the extract. After incubation period, 0.5 mL of Griess reagent (equal volume of 1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance was read at 546 nm in a UV/vis Spectrophotometer (INESA).

Evaluation of Membrane Stabilizing Properties

Preparation of 10% erythrocytes suspension

Blood was collected by cardiac puncture from healthy male Wistar rats lightly anesthetized with ether. The collected blood was mixed with equal volume of sterilized Alsever solution; packed red cells were obtained by repeated centrifugation (3000 rpm for 10 min), in isotonic phosphate buffer solution (154 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The packed cell was re-suspended to 10% (v/v) in the isotonic buffer.

Effect of AECO on heat-induced rat erythrocytes hemolysis

The effects of AECO on hemolysis of rat red blood cell (RBC) induced by heat was evaluated according to the modified method by Anosike *et al*. [24]. Briefly, AECO and indomethacin were dissolved in isotonic phosphate buffer solution (154 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The assay mixture consisted of 5 mL graded doses of extract (100, 200, 400, 800 and 1000 μg/mL); and 0.5 mL of 10% RBC suspension. The control was prepared as above without the extract, while RBC was omitted from the extract control tubes. Each sample was prepared and arranged in quadruplicate sets (4 sets per dose). A pair of the tubes was incubated at 54°C for 20 min in a regulated water bath. The another pair was maintained at −10°C in a freezer for 20 min. Afterward, the tubes were centrifuged at 4000 rpm for 3 min, and the hemoglobin contents of the supernatant were estimated at 540 nm using Spectronic 21 D (Milton Roy) Spectrophotometer.

Effect of AECO on hypotonic solution induced rat erythrocytes hemolysis

The effects of AECO on hemolysis of rat RBC induced by hypotonic solution was evaluated according to the method described by Shinde *et al*. [25] with some modifications. The extract was dissolved in hypotonic sodium phosphate buffer solution (50 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4). The hypotonicity-induced hemolysis assay mixture consisted of 5 mL graded concentration (100, 200, 400, 800 and 1000 μg/mL), in hypotonic solution, and 0.5 mL of 10% RBC suspension.

The control was prepared as above except that the extract was omitted, while the extract controls lacked erythrocyte suspension. Each sample was prepared and arranged in quadruplicate sets (4 sets per dose). The mixture was incubated for 1 h at room temperature (23 ± 2°C), and afterward, centrifuged for 3 min at 4000 rpm. The absorbance of the hemoglobin content of the supernatant was estimated at 540 nm using Spectronic 21D (Milton Roy) spectrophotometer.

Acute Toxicity Study

Acute toxicity refers to adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given within 24 h. The safety of the leaf extract was tested as described by Adzu and Haruna [26]. Rats were grouped into five groups of three rats each. The extract was administered orally to four groups in increasing doses of 250, 500, 1000 and 5000 mg/kg body weight, and the fifth group (control group) received distilled water at 10 mL/kg body weight. All the rats were kept in the same conditions. They were observed for pharmacotoxic signs continuously for 4 h and at the 24 h post administration to score for mortality. Thereafter, they were observed for up to 7 days post administration. Pharmacotoxic signs include salivation, diarrhea, piloerection, sedation, restlessness, sensitivity to sound and touch, gasping, cyanosis, blood in urine and edema.

Evaluation of Antinociceptive Properties of AECO

Acetic acid-induced writhing in mice

The method as described by Koster *et al*. [27] was used. Five groups of mice ($n = 5$) were used in this study comprising the vehicle (0.1 mL/10 g distilled water), indomethacin (10 mg/kg), or AECO (100, 200, 200 mg/kg). Each mice were placed singly inside the plexiglass observation chamber immediately after oral treatment for 1 h before injection of 0.6% acetic acid (0.1 mL/10 g body weight). The frequency of writhing occurring between 5 and 20 min was counted. Writhing movement was accepted as a contraction of the abdominal muscles accompanied by stretching of hind limbs.

Hot Plate Test

Thermal noxious stimulus was produced in mice by placing them on the hot plate (UgoBasile hot/cold plate 35100, Italy) according to a method described by Turner [28]. Five groups of mice ($n = 5$) were used, in this study, comprising the vehicle (0.1 mL/10 g distilled water), pentazocine (5 mg/kg), or AECO (100, 200, 200 mg/kg). The mice were placed singly on the hot plate which was maintained at 55 ± 1°C. Reaction time was recorded when the animals licked their fore and hind paws or jumped; at before (0) and 30, 60, 90 and 120 min after administration of test drugs. The mice which reacted within 20 s were selected for the study. The mean percentage maximum possible effect (% MPE) was calculated as:

$$\%\text{MPE} = \frac{\text{Post-drug latency} - \text{Pre-drug latency}}{\text{Cutt-off time} - \text{Post drug latency}} \times 100$$

Assessment of Effect of AECO on Locomotory Activity in Open-field

To assess the possible nonspecific muscle relaxants or the sedative effects of AECO, the motor performance of the mice was evaluated on the open-field apparatus (Archer, 1973). Groups of mice ($n = 5$) were treated with vehicle (0.1 mL/10g, p.o.), AECO (100, 200, 400 mg/kg, p.o.) 1 h before the performance of the test. The mice were placed in the center of the UgoBasile activity cage apparatus and allowed to have free ambulation for 5 min of observation of the locomotion frequency (horizontal activity and vertical activity).

Evaluation of *In Vivo* Anti-inflammatory Activity in Egg Albumin-induced Edema in Rat Paw

The phlogistic agent employed in this study to induce acute inflammation was fresh egg albumin according to the method described by Akah and Nwambie [29]. The rats were fasted overnight prior to the experiment and were divided into five groups of five rats each. Group 1 (negative control) received distilled water (10 mL/kg body weight), Groups 2-4 received AECO (100 -400 mg/kg body weight), while Group 5 (positive control) received indomethacin (10 mg/kg). All treatments were orally administered using oral canula.

Edema was induced 30 min later in all the rats by a single subplantar injection of 0.1 mL of raw egg albumin to the left hind paw. Edema formation was taken as increase in paw circumference measured by wrapping a white cotton thread around the injected paw and measuring the circumference on a meter rule [30]. Paw circumference was taken at 30, 60, 90 and 120 min after egg albumin injection. The percentage inhibition of edema was calculated using the formula:

$$\text{Percentage inhibition} = \frac{(C_t - C_o)_{\text{control}} - (C_t - C_o)_{\text{treated}}}{(C_t - C_o)_{\text{control}}} \times 100$$

Where C_t - paw (edema) circumference at time (t) after albumin injection and C_o - paw (edema) circumference before egg albumin injection.

Statistical Analysis of Data

Results were expressed as mean ± standard error of the mean and statistical comparison of means was performed using analysis of variance, all levels of significance were set as $P < 0.05$. Significant main effects were further analyzed by *post-hoc* test using Bonferroni's multiple comparison test. Graphs and statistical analysis were performed using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism® software version 5.01 (GraphPad Software, Inc. La Jolla, CA 92037 USA).

RESULTS

Result of Phytochemical Analysis of AECO

The screening for the secondary metabolites revealed the presence of alkaloids, tannins, phlobotannins, flavonoids, and saponins (data not shown). Steroids, terpenoids, and reducing sugars were not detected in AECO. The TCAD determined by the Arnow's reagent method showed that AECO contained an amount equivalent to 91.7 ± 0.01 mgCAE/g of sample. The amount of TFC in the extract estimated from the rutin calibration curve was 94.7 ± 0.03 mgRE/g of the sample (Figure 1).

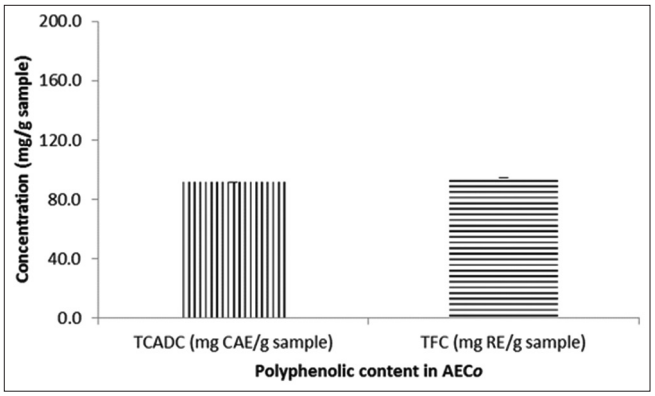

Figure 1: Total caffeic acid derivatives content and total flavonoid content in aqueous extract of *Chenopodium opulifolium*

In Vitro Antioxidant Effects of AECO

The results as shown in Figure 2 below revealed that AECO possesses *in vitro* antioxidant activity by mopping free radicals generated in both the DPPH and NO free radical scavenging assays. There was a corresponding increase in antiradical activity with an increase in concentration. The antiradical activity was higher in the NO than in the DPPH scavenging assay. Quercetin (25 μg/mL) activity was better than AECO (400 μg/mL) in the DPPH assay but lower in the NO assay.

Membrane Stabilizing Activity

AECO (100 -1000 μg/mL) demonstrated *in vitro* anti-inflammatory activity by its ability to inhibit heat-induced rat erythrocytes hemolysis (Table 1). The high percentage inhibition of hemolysis (36.5%, 44.0% and 49.6%) obtained for doses 400, 800 and 1000 μg/mL, respectively, was statistically significant ($P < 0.05$). Similarly, results, as shown in Table 2, revealed that AECO (100-1000 μg/mL) inhibited hypotonic solution-induced rat erythrocytes hemolysis in a concentration-dependent manner. The high percentage inhibition of hemolysis (37.5% and 40.3%) obtained for concentrations 800 and 1000 μg/mL, respectively, was statistically significant ($P < 0.05$).

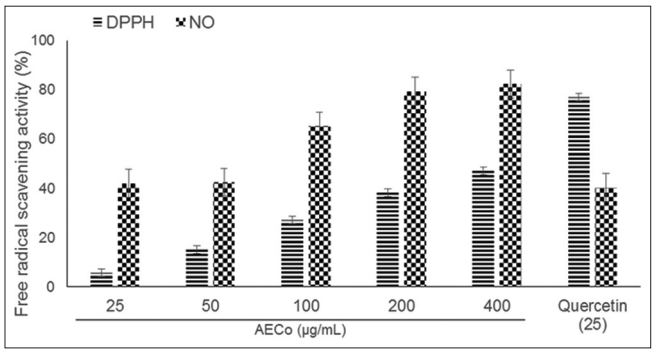

Figure 2: *In vitro* antioxidant activity of aqueous extract of *Chenopodium opulifolium*

Acute Toxicity

In the acute toxicity test of the AECO, there was no mortality or any signs of behavioral changes or pharmacotoxicity observed after oral administration of extract of the different doses even to the highest dose administered (5000 mg/kg body weight) in rats. No noticeable sign of toxicity was observed in all test doses until the end of the study period.

Anti-nociceptive Effect of AECO in Writhing Test

The pretreatment of mice with AECO 100, 200 and 400 mg/kg caused a significant ($P < 0.001$) and dose-dependent decrease in number of abdominal constriction in mice compared to control (Table 3). The percentage of inhibition of constriction was calculated as 49.1%, 52.2%, and 74.6% by AECO at 100, 200, and 400 mg/kg, respectively. Indomethacin (10 mg/kg) showed significant ($P < 0.001$) inhibition of abdominal constriction (75.9%).

Antinociceptive Effect of AECO in Hot Plate Test

In this model of thermally-induced nociception, pretreatment of mice with AECO (100-200 mg/kg) significantly prolong reaction time significantly ($P < 0.05$) compared to control as shown in the percent maximal possible effect curve (Figure 3). Dose-dependent maximal effect was observed at 60 min following administration. Pentazocine (5 mg/kg) showed a significant ($P < 0.001$) maximal possible effects when compared to control.

Table 1: Membrane stabilizing effect of AECO on heat-induced hemolysis of red blood cell

Treatment	Concentration (μg/mL)	Mean absorbance±SEM[a]		Percentage inhibition of hemolysis
		Heated solution	Unheated solution	
Control	-	1.034±0.062	0.036±0.011	-
AECO	100	0.795±0.013	0.038±0.001	24.1*
AECO	200	0.794±0.022	0.067±0.013	27.2*
AECO	400	0.759±0.003	0.124±0.024	36.5*
AECO	800	0.703±0.005	0.144±0.004	44.0*
AECO	1000	0.702±0.010	0.199±0.011	49.6*
Indomethacin	500	0.791±0.022	0.038±0.001	28.5*

[a]Values are triplicate measurements expressed as mean±SEM. *Denotes $P<0.05$ compared with control (Newmann Keuls *post-hoc* test).
AECO: Aqueous extract of *Chenopodium opulifolium*, SEM: Standard error of the mean

Table 2: Membrane stabilizing effect of AECO on hypotonicity - induced hemolysis of red blood cell

Treatment	Concentration (μg/mL)	Mean absorbance±SEM[a]	Percentage inhibition of hemolysis
Control	-	0.072±0.004	-
AECO	100	0.058±0.005	19.4
AECO	200	0.052±0.002	27.7
AECO	400	0.047±0.005	34.7
AECO	800	0.045±0.001	37.5*
AECO	1000	0.043±0.003	40.3*
Indomethacin	500	0.059±0.005	18.1

[a]Values are triplicate measurements expressed as mean±SEM. *Denotes $P<0.05$ compared with control (Newmann Keuls *post-hoc* test).
AECO: Aqueous extract of *Chenopodium opulifolium*, SEM: Standard error of the mean

Effects of AECO on Mice in the Open Field Test

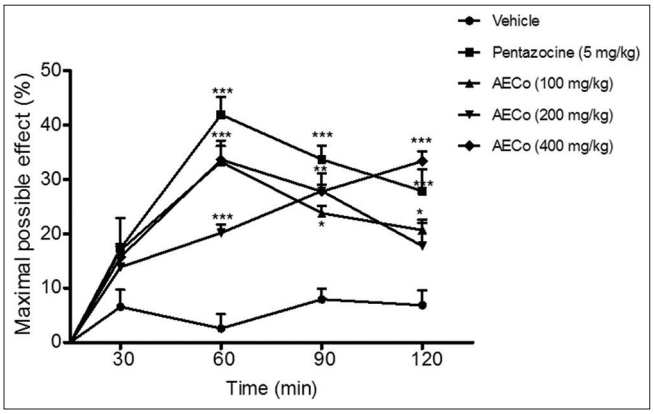

Figure 3: The effect of orally administered aqueous extract of *C. opulifolium* on the hot plate test. Result expressed as mean±standard error of the mean (*n*=5). The statistical significance was calculated by two-way analysis of variance followed by Bonferroni's test. **P*<0.05, ***P*<0.01, ****P*<0.001 when compared to vehicle

Table 3: Antinociceptive activity of AECO in acetic acid-induced abdominal constriction test in mice

Treatment	Dose (mg/kg)	No of writhing[a]	% inhibition
Vehicle	10 mL/kg	45.60±4.95	
AECO	100	23.20±1.53***	49.1
AECO	200	21.80±2.71***	52.2
AECO	400	11.60±1.63***	74.6
Indomethacin	10	11.00±1.14***	75.9

[a]Values are expressed as mean±SEM (*n*=5). Denotes *P*<0.001 compared with control (Newmann Keuls *post-hoc* test). AECO: Aqueous extract of *Chenopodium opulifolium*, SEM: Standard Error of Mean, ***: *P* < 0.001 compared with control (Newmann Keuls post hoc test),

Table 4: The effects of orally administered AECO in the open field test in mice

Treatment	Dose (mg/kg)	Parameters[a]	
		SMA[b]	Rearing
Vehicle	10 mL/kg	778.75±69.19	75.25±13.28
AECO	100	679.75±38.99	47.75±8.78
AECO	200	708.75±64.71	68.75±4.29
AECO	400	810.00±94.31	68.00±8.57

[a]Values are expressed as mean±SEM (*n*=5), [b]SMA. AECO: Aqueous extract of *Chenopodium opulifolium*, SEM: Standard error of the mean, SMA: spontaneous motor activity

Evaluation of the effect of oral administration of AECO on the spontaneous motor activity and rearing in mice did not show any significant alteration ($P > 0.05$) in those activities when compared with control (Table 4).

Anti-inflammatory Effect of AECO in Egg Albumin - Induced Edema in Rat Paw

The results obtained from this experiment are shown in Table 5. Egg albumin induced edema in all experimental animals at different time points, and maximum edema formation was observed at 30 min post albumin injection. Pre-treatment with AECO (100, 200 and 400 mg/kg) produced statistically significant ($P < 0.001$) and time-dependent inhibition of the edematous response during the 120 min duration measurement. The percentage inhibition of edema formation was 44.2%, 44.5%, and 51.2% by AECO 100, 200, and 400 mg/kg, respectively, while indomethacin administered at 10 mg/kg inhibited edema by 51.2%.

DISCUSSION

The findings of this study showed the antioxidants, antinociceptive and anti-inflammatory activities of AECO leaves. The phytochemical screening of AECO used in the pharmacological tests revealed the presence of alkaloids, tannins, phlobotannins, flavonoids, and saponins. Quantification of caffeic acid derivatives and TFC in the extract showed an appreciable amount of polyphenolics. The species of the family Chenopodiaceae are widely distributed weedy herbs often used commercially as spices or drugs because of the presence of useful secondary metabolites [31]. Phenolics, flavonoids, saponins, and triterpenoids were reported to be the major phytoconstituents of the *Chenopodium* genus, and about 300 and 79 compounds have been reportedly isolated [7]. AECO showed weak antiradical activity in the DPPH and potent activity in NO free radical scavenging assays. DPPH free radical scavenging assay is a primary assay for investigating the antioxidant properties of extracts. Antioxidant potentials usually correlate well with the phenolic composition of extract.

In a concentration-dependent manner, the extract shows membrane stability activity in both the heat and hypotonic solution-induced hemolysis of rat erythrocytes. The ability to protect the erythrocytes from stress-induced lytic effect of heat and hypotonic solution demonstrate the *in vitro* anti-

Table 5: Anti-inflammatory effects of AECO on egg albumin-induced rat paw oedema

Treatment groups	Dose (mg/kg)	Changes in paw sizes (cm)[a]			
		30	60	90	120
Vehicle	10 mL/kg	1.03±0.07	0.65±0.03	0.55±0.07	0.43±0.02
AECO	100	0.88±0.04 (14.6)	0.52±0.07 (25.0)	0.38±0.04 (30.9)	0.24±0.02 (44.2)*
AECO	200	0.64±0.04 (37.9)***	0.40±0.05 (38.5)**	0.32±0.05 (41.8)**	0.24±0.04 (44.5)*
AECO	400	0.54±0.09 (47.6)***	0.38±0.07 (41.5)***	0.32±0.05 (41.8)**	0.21±0.02 (51.2)*
Indomethacin	10	0.58±0.05 (43.7)***	0.38±0.04 (41.5)***	0.36±0.04 (34.5)*	0.21±0.02 (51.2)*

[a]Results expressed as mean±SEM (*n*=5). The statistical significance was calculated by two-way ANOVA followed by Bonferroni's test. **P*<0.05, ***P*<0.01, ****P*<0.001 when compared to vehicle. Percentage inhibitions of oedema formation are in parenthesis. AECO: Aqueous extract of *Chenopodium opulifolium*, SEM: Standard error of the mean, ANOVA: analysis of variance

inflammatory properties of the extract. During inflammation, there are lyses of lysosomes which release their component enzymes that produce a variety of disorders. The lysosomal enzymes are implicated in the pathogenesis of articular tissue degradation in several rheumatic diseases. Drugs by stabilizing the membrane can prevent the rupture of the lysosomes and inhibit the release of lysosomal enzymes [32]. The RBC membrane stabilization test serves as a model for lysosomal membrane stabilization since several agents capable of releasing hydrolytic enzymes from lysosomes also injure erythrocytes [24]. The presence of polyphenols such as flavonoids and tannins in AECO may contribute to the anti-inflammatory effects observed. Flavonoids are known to exert profound stabilizing effects on lysosomes membrane which is often attributed to their free radical scavenging properties [33].

Results of the acute toxicity test of the AECO showed that there was no mortality or any significant change in the behavior of the rats recorded up to the dose of 5000 mg/kg body weight. Acute toxicity test provides preliminary information on the toxic nature of a material for which no other toxicological information is available [34]. Based on the results of the preliminary toxicity testing on AECO, the highest dose was found to be non-lethal, hence we can safely conclude that median lethal dose (LD_{50}) is >5000 mg/kg body weight. The high safety margin of AECO may partly explain the historical use of *C. opulifolium* decoction or infusion in managing fever associated with malaria parasite infection in Uganda [13]. The information from acute toxicity studies is often used in dose setting for other studies, thus the set dose for anti-inflammatory screening of AECO was lower than 1/10th fraction of the highest tolerated dose [35,36].

C. opulifolium extract showed similar antinociceptive effect to Indomethacin in the acetic acid-induced abdominal writhing test. This model characterized by stereotypical behavior of abdominal constriction involves peripheral nociceptive mechanisms. Most importantly is the release of biogenic amines (e.g., histamine and serotonin), bradykinin, prostaglandin E_2 and $PGF_2\alpha$ which activates visceral nociceptors [37].

Thermally-induced nociception in the hot plate is considered to be selective for centrally acting analgesic compounds (morphine-like drugs). AECO showed positive activity in this test. In order to rule out the chances of false positive effect of AECO, we evaluated its effect on spontaneous locomotor function in the activity meter cage. AECO did not alter the ambulatory and rearing activities. The results revealed that the observed antinociceptive effect of AECO was not as a result of sedation or impairment of motor activity in mice. Taken together, effect in acetic acid-induced writhing and hot plate test revealed that AECO antinociceptive activity might involve peripheral, spinal and supraspinal inhibition of pain.

The AECO leaves exhibited an anti-inflammatory effect by inhibiting the edema induced by egg albumin in rat paw. This model is used as an *in vivo* model of acute inflammation that can be used to screen agents for anti-inflammatory effect [29,30]. The results showed that AECO suppressed inflammation at the early phase, an indication that it may be blocking the release of the early phase mediators. Histamine, bradykinin, serotonin, and prostaglandins are inflammatory factors released from damaged cells, and they are mediators of inflammation and pain. Prostaglandins are products of arachidonic acid metabolism via the cyclooxygenase pathway [29].

CONCLUSION

This study clearly revealed that the AECO leaf contains polyphenolics (flavonoids, caffeic acid, and tannins) that showed antioxidant, antinociceptive, and anti-inflammatory activities. This result provides a scientific credit for the use of *C. opulifolium* in traditional medicine as a remedy against inflammatory conditions and thus a candidate for further studies.

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Original Research

Ocimum basilicum extract exhibits antidiabetic effects via inhibition of hepatic glucose mobilization and carbohydrate metabolizing enzymes

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ABSTRACT

Aim: *Ocimum basilicum* L (Lamiaceae) is used as a traditional remedy for different ailments, including diabetes mellitus. This study investigated the antidiabetic effects of an extract of aerial parts of *O. basilicum*. **Methods:** Antihyperglycemic effect of the extract was determined by its effects on α-amylase and α-glucosidase *in vitro*, while antidiabetic properties were studied in alloxan induced diabetic rats treated for 28 days with extract and compared to those treated with oral metformin (150 mg/kg). The study and analysis was conducted between 2014 and 2015. **Results:** The treatment with 100 and 200 mg/kg extract significantly ($P < 0.05$) reduced fasting blood glucose concentration and slightly increased mean body weight in treated groups. Oral glucose tolerance was also significantly ($P < 0.05$, 0.001) improved in 100 and 400 mg/kg extract-treated groups. The extract caused a dose-dependent increase in liver glycogen content, while it decreased alanine transferase (18.9-30.56%) and aspartate transferase (6.48-34.3%) levels in a non-dose-dependent manner. A dose of 100 mg/kg also reduced serum cholesterol and triglycerides by 19.3 and 39.54%, compared to a 2.6% reduction of cholesterol seen in the metformin-treated group. The extract was observed to produce significant ($P < 0.001$) concentration-dependent inhibition of α-glucosidase (35.71-100%) and also α-amylase (23.55-81.52%), with estimated inhibitory concentration values of 1.62 and 3.86 mg/mL, respectively. **Conclusions:** The antidiabetic properties of the extract may be due to its ability to suppress endogenous glucose release, inhibit glycogenolysis and/or stimulate glycogenesis.

KEY WORDS: Antidiabetic, diabetes mellitus, hyperglycemia, *Ocimum basilicum*

INTRODUCTION

The term diabetes mellitus describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism resulting from defects in insulin secretion, insulin action, or both [1]. The incidence of diabetes has risen rapidly in low and middle-income countries, due to factors such as population growth, urbanization, and increasing the prevalence of obesity due to dietary changes and physical inactivity [2]. Type II diabetes mellitus (Type II DM) is the most common form of diabetes, caused by a combination of factors, including insulin resistance, a condition in which the body's muscle, fat, and liver cells do not use insulin effectively and develops when the body can no longer produce enough insulin to compensate for the impaired ability to use insulin. This type of diabetes comprises the majority of adult diabetic patients around the world and is still on the increase. Long-term effects of diabetes mellitus include progressive development of the specific complications of retinopathy with potential blindness, nephropathy that may lead to renal failure, and/or neuropathy. Management of Type II DM involves nonpharmacological measures such as dietary modification and exercise and pharmacological approaches with oral antidiabetics, aimed at controlling postprandial hyperglycemia and improving insulin action [3]. These oral hypoglycemic drugs possess a distinct mechanism of actions which enables them to be used independently or in combination. In many developing countries, however, access to these drugs and affordability may be contending issues in proper therapy and management. In these countries, traditional herbal medicines are popular and play an important role in DM management [4]. Recent reports have highlighted different plant extracts screened for antidiabetic activity, which were shown to improve tissue insulin sensitivity and inhibit carbohydrate metabolizing enzymes [5,6]. Extracts of *Ocimum basilicum* L (Lamiaceae) also commonly known as "Holy basil" have been reported to possess different pharmacological effects, including blood glucose lowering and hepatoprotective properties [7,8]. The plant is widely used in traditional medicine in different cultures and also known for its culinary uses. Reported phytochemical constituents found in *O. basilicum* extract include linalool,

methylchavikol, methyl cinnamate, linolen, rosmarinic acid, citral, eugenol, and geraniol [9-11]. A previous *in vitro* study showed that aqueous extract of the plant inhibited porcine α-amylase and rat intestinal sucrase and maltase [7]. In an earlier study, the blood glucose-lowering effects of oral administration of a dichloromethane:methanol (1:1) extract and solvent fractions of aerial parts of *O. basilicum* in diabetic rats were reported [12]. Presently, we investigated the effects of repeated oral administration of the extract on blood glucose control and hepatic glucose mobilization in alloxan-diabetic rats as well as carbohydrate metabolizing enzymes *in vitro* to propose some likely mechanisms of its antidiabetic activity.

MATERIALS AND METHODS

Materials

Drugs, reagents, and solvents

Metformin, dimethyl sulfoxide (DMSO), 3,5- dinitrosalicylic acid, sodium potassium tartrate, O- toluidine, D- maltose, trichloroacetic acid, oxalic acid, phenol, alloxan monohydrate (Sigma-Aldrich), D-glucose (Fluka Chemicals, USA), acetylene chloride, and methanol (BDH, England). Other reagents and solvents were of analytical grade.

Enzymes and substrates

Porcine pancreatic α-amylase (EC 232-565-6), yeast α-glucosidase, soluble potato starch, and p-nitrophenyl-α-D-glucopyranoside, (Sigma-Aldrich, Germany).

Animals

Adult Wistar rats (100-200 g) of both sexes obtained from the animal facility center of the Department of Pharmacology and Toxicology, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, were used. The rats were housed in steel cages and allowed access to potable water and standard pelleted feed. All animal experiments were in compliance with NIPRD's standard operating procedures and the National Institute of Health guide for Care and Use of Laboratory Animals [13].

Methods

The entire study and data analysis was conducted between 2014 and 2015.

Collection and Identification of the Plant Material

Aerial parts of *O. basilicum* were collected between November and February from Suleja, Niger State and identified at NIPRD where a voucher specimen (NIPRD/H/6594) is maintained. The aerial parts were air dried under shade for 2 weeks and the dry material pulverized to coarse powder using an electric hammer mill.

Extraction of Plant Material

A total of 1.2 kg of dry plant material was extracted by cold maceration in a mixture of dichloromethane:methanol (1:1) for 48 h with occasional agitation. After 48 h, the mixture was filtered, and the residue extracted again with fresh solvent and filtered. The combined filtrates were concentrated under vacuum and then dried in a hot water bath maintained at 50°C. The extract was transferred to an airtight container and stored in a refrigerator (4°C) until required.

Thin Layer Chromatography (TLC) and High-performance Liquid Chromatography (HPLC) Fingerprinting of Extract

Pre-coated thin layer chromatographic glass plates were used for TLC analysis. Extract solution was spotted on heat-activated plates and developed in a mobile phase system of ethyl acetate: Methanol (3:2). Detection was done with sulfuric acid/vanillin reagent spray and heat (110°C for 3 min). Conditions for HPLC were as follows: Mobile phase comprising solvent A: 0.2% v/v formic acid, solvent B: Acetonitrile, mode: Linear gradient, flow rate of 0.6 mL/min, injection volume: 20 μL of 500 μg/mL methanol solution of extract, UV detection at 254 nm, and column oven temperature 40°C. The HPLC operating conditions were programed to give the following; at 0 min, solvent B: 20%; 10 min, solvent B: 25%; 20 min, solvent B: 80%; 25 min, solvent B: 20% return to initial condition. The total run time was 25 min.

Pharmacological Studies

Induction of experimental diabetes

About 40 Swiss albino rats were subjected to an overnight fast with free access to drinking water. Diabetes mellitus was induced in on day 0 by a single intraperitoneal injection of freshly prepared alloxan monohydrate solution in distilled water (160 mg/mL). On day 3, the rats were fasted overnight and blood glucose levels measured on day 4 from tail vein blood using a blood glucometer and its corresponding strips (Accu-Chek, Mannheim, Germany). Rats with blood glucose ≥200 mg/dL were considered diabetic and were used for the study.

Experimental Design

The plant extract was reconstituted with distilled water and tween 80 (1% v/v). Diabetic rats were weighed, randomized, and divided into five groups ($n = 6$). The rats were treated orally for 30 days; Groups 1 and 2 served as controls and received distilled water (5 mL/kg) and metformin (150 mg/kg), respectively. Groups 3-5 received 100, 200, and 400 mg/kg extract. Individual body weight and fasting blood sugar levels of all the rats were measured at weekly interval during the experimental period.

Oral Glucose Tolerance Test

On the 25th day of treatment, the rats were fasted overnight but allowed free access to water. On the 26th day, the rats were

treated accordingly, then after 30 min, an oral glucose load of 1.5 g/kg was administered orally. Blood glucose levels were taken immediately and at 30, 60, 90, and 120 min after the glucose load.

Hepatic Glycogen and Serum Biochemistry Determination

On day 30, the rats were euthanized by chloroform inhalation and blood collected using plain sera tubes for biochemical analysis of protein (total protein and albumin), lipids (cholesterol and triglycerides), and hepatic enzymes. The liver of each rat was excised carefully and weighed. 1 g of each liver was cut for assay of hepatic glycogen according to a method described earlier [14]. Following glycogen hydrolysis, the concentration of glucose obtained was determined using a standard calibration plot of glucose obtained using O-toluidine. The absorbance measured at 355 nm and glycogen content was expressed as g/g liver tissue.

α-amylase Inhibition Assay

The assay was performed according to the chromogenic non-preincubation method described by Ali *et al.* [15] as modified by Okoli *et al.* [14]. Test incubations were prepared for 1.25-20 mg/mL of extract to study the concentration dependent enzyme inhibition and to calculate the concentration required to inhibit 50% enzyme activity inhibitory concentration (IC_{50}). For each concentration, blank incubations were prepared by replacing the enzyme solution with distilled water, while control incubations were prepared by replacing extract with 120 μL DMSO. All the tests were run in triplicate. Absorbance was measured at 540 nm (Shimadzu UV-160 spectrophotometer, Kyoto, Japan) and net absorbance (A) due to maltose generated was calculated as:

Net absorbance (A) due to maltose generated was calculated as:

$$A_{540nm}\ \text{extract} = A_{540nm}\ \text{test} - A_{540nm}\ \text{blank}$$

From the value obtained, maltose generated (%w/v) was calculated from the equation obtained from the maltose standard calibration curve (0.001-0.1% w/v).

The level of inhibition (%) was calculated thus:

Inhibition (%) = 100 - % reaction (at 3 min)

Where: % reaction= mean maltose in sample ×100/mean maltose in control

Alpha Glucosidase Inhibitory Activity Assay

A chromogenic method described previously was used [14]. A mixture of 0.32 mL of extract, 1.6 mL of buffer solution and 0.8 mL of enzyme solution was incubated for 5 min, followed by addition of 800 μL of substrate. Samples were further incubated for 15 min and the reaction stopped by addition of 320 μL of 200 mm sodium carbonate solution. The release of p-nitrophenol generated was measured at 400 nm. Enzyme and extract solutions were substituted with 800 μL buffer solution and 320 μL DMSO in blank and control incubations respectively. All tests were run in triplicate. The level of enzyme inhibition was calculated thus:

$$\text{Enzyme inhibition (\%)} = 100 - [((A_s - A_b)/A_c) \times 100]$$

Where A_c represents the absorbance of the control without test samples, A_s= sample absorbance and A_b denotes sample blank absorbance.

Statistical Analysis

Data were analyzed using one-way analysis of variance. The results expressed as mean ± standard error in mean and further subjected to LSD *post hoc* test for multiple comparisons. Differences between means accepted significant at $P < 0.05$.

RESULTS

Treatment of diabetic rats with 100 and 200 mg/kg doses of extract caused a decrease in fasting blood glucose levels in a non-dose-dependent manner, producing 59.21 and 38.67% decline in plasma glucose levels, respectively, by the end of the study period [$P < 0.001$ and $P < 0.05$; Table 1]. The hypoglycemic effect in extract-treated rats was higher than that in metformin-treated rats. Mean body weight of the diabetic rats treated with extract and the diabetic rats treated with metformin was observed to decrease slightly at the end of the study, but this change was statistically insignificant [$P > 0.05$, Table 2]. Administration of an oral glucose load increased blood glucose to peak levels in all the rats within 30 min and pretreatment with extract suppressed the rise in blood glucose levels. The extract (100 mg/kg) significantly ($P < 0.001$) decreased the blood glucose level at 120 min by 27.76%. The extract at 400 mg/kg also significantly ($P < 0.05$) suppressed the postprandial glucose level. The blood glucose lowering effect of extract was only limited to 90 min in the group treated with 200 mg/kg [Table 3]. Treatment with the extract also produced dose-dependent increase in glycogen content of the liver by 7.1 - 37.4% compared to the untreated control [Table 4]. The extract decreased the levels of aspartate aminotransferase and alanine aminotransferase in the diabetic treated groups, in a non-dose-dependent manner [Table 5]. The treatment with 100 mg/kg extract produced slight decrease in the alkaline phosphatase (ALP), total protein, albumin, and cholesterol levels but elicited marked reduction of serum triglycerides [39.54%, Table 5] compared to that caused by metformin.

The extract significantly ($P < 0.05$) inhibited α-glucosidase enzyme, in a concentration-dependent manner [Table 6]. Concentrations of 1.25 and 2.5 mg/mL produced 35.71 and 71.09% inhibition, while at concentrations of 5 and 20 mg/mL, enzyme inhibitory activity was observed to be 100%. The concentration required to inhibit the enzyme by 50% IC_{50} was estimated to be 1.62 mg/mL. The extract was also observed to inhibit α-amylase mediated generation of maltose in a concentration dependent manner at 2 min after the start of the

Table 1: Effect of extract on fasting blood glucose level in diabetic rats

Treatment	Dose (mg/kg)	Blood glucose concentration (mg/dL)				
		Day 0	Day 7	Day 14	Day 21	Day 28
Control	-	527.4±32.3	197.6±36.4***	250.2±61.1*	336.2±31.7*	376.6±40.1
Extract	100	567.0±14.9	103.2±9.0*** (−81.80)	166.3±34.4*** (−70.67)	159.2±15.97*** (−71.92)	231.3±23.95*** (−59.21)
	200	516.4±40.7	171.8±38.5*** (−66.73)	214.7±22.3* (−58.42)	166.3±54.1* (−67.79)	316.7±14.3* (−38.67)
	400	511.7±49.2	228.7±49.4** (−55.38)	175.0±61.3* (−65.80)	244.7±68.2 (−52.18)	425.7±87.6 (−16.81)
Metformin	150	545.4±35.3	112.4±5.1*** (−79.39)	271.0±21.9** (−50.31)	320.6±23.7*** (−41.21)	263.0±24.7** (−51.77)

*$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to Day 0 values (One-way ANOVA; LSD *post hoc*). Values in parenthesis represent reduction (%) calculated relative to Day 0 values

Table 2: Effect of extract on body weight of diabetic rats

Treatment	Dose (mg/kg)	Body weight (g)				
		Day 0	Day 7	Day 14	Day 21	Day 28
Control	-	110.5±4.9	108.3±3.6 (−2)	103±3.4 (−7.1)	97.8±3.7 (−11.5)	102±4.8 (−8.1)
Extract	100	119±11.8	114±10.6 (−4.5)	115±11.5 (−3.4)	115±11.1 (−3.4)	112.3±11.0 (−5.5)
	200	122.4±11.4	125.2±12.4 (+2.3)	116±5.0 (−5.4)	106.7±4.1 (−12.8)	106.0±6.7 (−13.4)
	400	126.0±22.0	127±21.0 (+0.4)	116.3±19.4 (−7.7)	101.0±7.4 (−20)	105.0±8.7 (−16.7)
Metformin	150	113.0±10.3	109.4±5.6 (−3.2)	108±4.8 (−4.8)	111.4±4.6 (−1.42)	107.2±4.2 (−5.1)

Values in parenthesis represent % reduction in body weight calculated relative to Day 0 values

Table 3: Effect of extract on oral glucose tolerance in diabetic rats

Treatment	Dose (mg/kg)	Blood glucose concentration (mg/dL)				
		0 min	30 min	60 min	90 min	120 min
Control	-	396.0±38.1	415.2±49.8	428.3±31.2	389.3±44.2 (−6.24)	399.8±42.9 (−3.71)
Extract	100	231.3±24.0	279.2±33.8	222.0±30.1** (−20.5)	222.3±31.7* (−20.4)	201.7±26.4*** (−27.8)
	200	316.8±14.3	419.3±32.0	367.0±28.2 (−12.5)	311.6±27.7 (−25.7)	339.0±32.2 (−19.2)
	400	425.7±87.6	541±29.7	469.0±39.3 (−13.3)	449.7±48.4 (−16.83)	398.0±35.0* (−26.4)
Metformin	150	263.0±24.6	280.8±16.4	204±21.7** (−27.5)	205.0±25.8* (−30)	167.2±21.3** (−40.5)

*$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to 30 min values (One-way ANOVA; LSD *post hoc*); Values in parenthesis represent % reduction calculated relative to 30 min values

Table 4: Effect of extract on hepatic glycogen content and liver weight of diabetic rats

Treatment	Dose (mg/kg)	Liver weight (g)		Liver glycogen content (g/g liver tissue)
		Absolute	Relative	
Control	-	4.11±0.35	4.02±0.17	0.0288±0.006
Extract	100	4.41±0.55 (+7.3)	3.94±0.28 (−2.0)	0.0310±0.008 (+7.1)
	200	3.97±0.21 (−3.4)	3.81±0.38 (−5.2)	0.0317±0.009 (+9.2)
	400	4.25±0.31 (+3.3)	4.09±0.35 (+1.7)	0.0460±0.011 (+37.4)
Metformin	150	3.81±0.09 (−7.3)	3.58±0.21 (−11.0)	0.0523±0.006* (+44.9)

Values in parenthesis represent % change in liver glycogen content and organ weight calculated relative to control. Relative organ weight was calculated using body weight of the respective rat on day 28 shown in Table 2, *: $P<0.05$

Table 5: Serum biochemistry of extract-treated diabetic rats

Group	AST	ALT	ALP	TP	ALB	CROL	LDL	TG
Diabetic control	479.8±85.4	215.0±22.5	1439±236.3	62.5±2.4	30.7±0.6	79.7±2.1	9.8±5.0	125.7±14.0
100 mg/kg	340.5±10.1 (−29)	149.3±13.2 (−30.56%)	1206±123.2 (−16.19%)	56.6±1.0 (−9.4)	26.5±1.9 (−13.6)	64.3±4.3 (−19.3)*	16.3±2.3	76.0±4.21 (−39.5)
200 mg/kg	315.0±48.7 (−34.3)	174.3±38.0 (−18.9)	1599±300.8	62.5±1.3	31.0±0.4	79.0±3.9	17.0±11.0	147.7±65.6
400 mg/kg	448.7±60.7 (−6.48)	171.3±17.2 (−20.33)	1645±285.4	61.7±4.4 (−1.12)	30.7±0.9	86.7±2.8	7.5±1.5 (−23.1)	158.7±65.6
Metformin 150 mg/kg	268.6±18.7 (−44.0)	144.0±10.1 (−33)	974±180.2 (−32.3)	67.2±1.9	32.2±0.4	89.4±6.0	22.4±6.7	122.4±19.1 (−2.6)

reaction [Figure 1]. At 3 min, a 20 mg/mL concentration of the extract produced the highest percentage inhibitory effect and was observed to suppress maltose generation by 81.52% while samples containing 1.25, 5, and 10 g/mL produced 23.55-58.33% inhibition [Figure 1]. The IC_{50} of the extract at 3 min was estimated to be 3.86 mg/mL.

TLC fingerprint of the extract showed 6 spots with retention factors ranging from 0.47 to 0.97. HPLC chromatogram revealed the characteristic peaks of the extracts with retention times ranging between 1.547 and 22.104 min [Figure 2].

DISCUSSION

The various mechanisms have been associated with the antihyperglycemic activities of medicinal plants which include peripheral utilization of glucose, increased synthesis of hepatic glycogen by enhancement of glycogen regulatory enzyme expression in the liver, inhibition of carbohydrate metabolizing enzymes, stimulation of pancreatic insulin release, and inhibition of hepatic glucose production [16-20]. The reduction of fasting blood glucose level by the extract implies that the extract may have exerted this effect through one or more of these mechanisms. The extract also enhanced glucose tolerance by suppressing postprandial rise in glucose level, likely through enhanced insulin sensitivity and/or increased glucose uptake by skeletal muscle and adipose tissue [21].

Table 6: α-glucosidase inhibitory activity of extract

Treatment	Concentration (mg/mL)	Absorbance ($\times 10^{-3}$)	Inhibition (%)
Control	-	293.7±9.4	-
Extract	1.25	188.7±30.8*	35.71
	2.5	85.3±15.2**	71.09
	5	0±28.6***	100
	20	0±0***	100

*$P<0.05$; **$P<0.01$; ***$P<0.001$ compared to the control (One-way ANOVA; LSD *post hoc* test); IC_{50}=1.62 mg/mL, ANOVA: Analysis of variance, IC_{50}: Inhibitory concentration

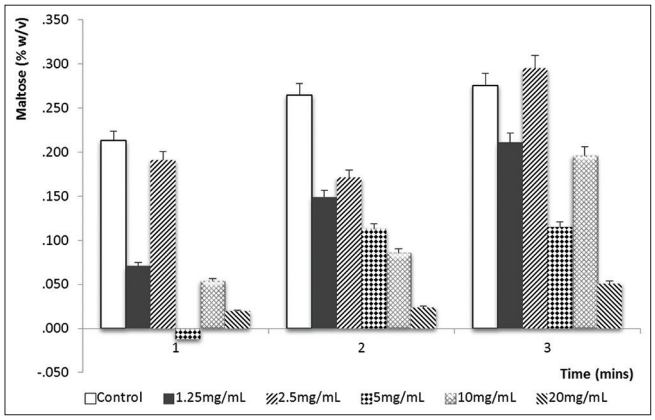

Figure 1: α-amylase inhibitory effect of the extract. Error bars represent standard errors of the mean of tests performed in triplicate

Effective suppression of the postprandial rise in blood glucose level reflects good tolerance of sudden glucose load, and this may occur as a result of increasing glucose uptake into tissue sites [22]. Postprandial glucose clearance by the liver translates to glycogen synthesis and storage which may be due to enhanced insulin release from β cells [23]. The extract inhibited α-amylase and α-glucosidase, and this indicates it may produce a postprandial antihyperglycemic effect by suppressing carbohydrate metabolism and the consequent glucose release from the lumen of small intestine following a meal [16,23]. This finding is consistent with a previous study which demonstrated the enzyme inhibitory actions of an extract of the plant [7]. Inhibition of both enzymes amounts to reduced glucose absorption and thus, suppressing postprandial hyperglycemia, which plays a central role in development and progression of diabetic complications [24]. Inhibition of the two enzymes *in vitro* by the extract can be correlated with enzyme inhibition *in vivo*, which is capable of decreasing glucose entering portal vein from the gut or glucose production from starch [25]. In addition to limiting the extent of glucose absorption, the extract enhanced glucose mobilization by stimulating hepatic glycogen synthesis shown by the increase in liver glycogen content as well as liver weight. This suggests that the extract may enhance glucose uptake by liver and skeletal muscles, an effect secondary to insulin stimulation and enhanced insulin sensitivity in target organs [26]. The reductions observed in hepatic marker enzymes in serum could have been caused by the hepatocellular

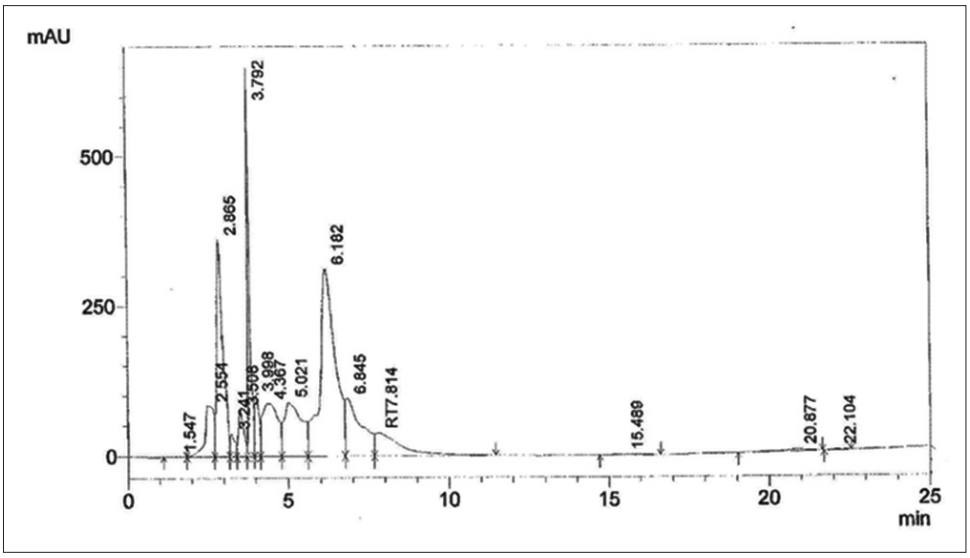

Figure 2: High-performance liquid chromatography fingerprint of extract

and cardiac protection offered by the extract and is consistent with an earlier report on an extract of *O. basilicum* [8]. Hepatic and cardiac tissues release aspartate and alanine transferases and therefore, the elevation of plasma concentrations of these enzymes are indicators of hepatic and cardiac damage as in the case of complications in diabetes mellitus [27]. The reduction in serum ALP activity recorded is suggestive of cellular membrane/hepatocellular membrane protective effects of the plant extract. ALP functions as a biochemical marker enzyme for maintaining membrane integrity. Increase in its plasma activity indicates disruption of cell membrane integrity, which occurs in diseases including diabetes mellitus [28]. These seeming hepatic protective actions of the extract suggest that once daily chronic application may not predispose to hepatic toxicity, albeit in the short term.

The burden of diabetes on individuals is due to its long-term microvascular and macrovascular complications. Hyperlipidemia is a major cause of macrovascular complication associated with diabetes. It is accompanied with premature atherosclerosis which is a major cause of cardiovascular disease [29]. Hyperlipidemia involves elevated total cholesterol and triglycerides. The extract in addition to good glycemic control also lowered the total cholesterol and triglyceride levels in diabetic rats consistent with earlier findings [19]. The extract reduced the total cholesterol, low-density lipoprotein and triglycerides and this may be attributed to a stimulatory effect insulin secretion which improves the action of lipoprotein lipase enzyme and/or by sensitization of target organs such as adipose tissue to insulin action.

The extract used on this study was finger-printed to establish an identity marker as a means of standardization. TLC and HPLC fingerprints of the extract can be used as tools for quality control and standardization of extracts of the plant obtained by similar method to that used for the extract in this study [30].

CONCLUSION

The extract of aerial parts of *O. basilicum* possesses antidiabetic effects, possibly mediated by limiting glucose absorption through inhibition of carbohydrate metabolizing enzymes and enhancement of hepatic glucose mobilization. Chronic oral administration may not predispose to the risk of hepatotoxicity in the short term. Further studies are warranted to evaluate effects of chronic administration of the extract in diabetes.

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Original Research

Geraniol attenuates hydrogen peroxide-induced liver fatty acid alterations in male rats

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ABSTRACT

Background: Hydrogen peroxide (H_2O_2) is an oxidant agent and this molecule naturally occurs in the body as a product of aerobic metabolism. Geraniol is a plant-derived natural antioxidant. The aim of this study was to determine the role of geraniol on hepatic fatty acids alterations following H_2O_2-induced oxidative stress in male rats. **Methods:** After randomization, male Wistar rats were divided into four groups ($n = 7$ each group). Geraniol (50 mg/kg, dissolved in corn oil) and H_2O_2 (16 mg/kg, dissolved in distilled water) were administered by an intraperitoneal injection. Administrations were performed during 30 days with 1-day interval. **Results:** Administration of H_2O_2 resulted with a significant increase in malondialdehyde (MDA) and a significant decrease in glutathione (GSH) peroxidase glutathione level; geraniol restored its effects on liver. However, hepatic catalase (CAT) activities were significantly higher in H_2O_2, geraniol, and geraniol+H_2O_2 groups than control group. The ratio of hepatic total saturated fatty acids increased in H_2O_2-treated animals compared with control. In addition, hepatic total unsaturated fatty acids reduced in H_2O_2 group compared with control. The percentages of both hepatic total saturated and unsaturated fatty acids were not different between geraniol+H_2O_2 and control groups. **Conclusions:** H_2O_2-induced oxidative stress may affect fatty acid composition in liver and body. Geraniol can partly restore oxidative hepatic damage because it cannot completely reverse the H_2O_2-induced increase in hepatic CAT activities. Moreover, this natural compound can regulate hepatic total saturated and unsaturated fatty acids percentages against H_2O_2-induced alterations.

KEY WORDS: Fatty acids, geraniol, hydrogen peroxide, liver, oxidative stress

INTRODUCTION

Reactive oxygen species (ROS) in particular hydroxyl and superoxide radicals, and non-radical oxidants such as hydrogen peroxide (H_2O_2), hypochlorous acid, and peroxynitrite are generated in organism, mainly as an outcome of aerobic metabolism [1,2]. However, the excessive productions of ROS can impair essential cellular components such as nucleic acids, proteins, and polyunsaturated fatty acids [3]. Even some other molecules such as peroxynitrite ($ONOO^-$) and H_2O_2 are not free radicals; they are accepted to generate free radicals through many biochemical reactions in various cases [4]. In general, all organisms are well protected against free radical damage by endogenous oxidative enzymes such as catalase (CAT)

and glutathione (GSH) peroxidase (GSH-Px). Whenever the balance between ROS generation and antioxidant defense is lost, "oxidative stress" results through a series of stages dysregulates the cellular functions leading to several pathophysiological conditions [5,6]. Several nonenzymatic antioxidant compounds such as phenolics, ascorbic acid, tocopherol, and other dietary compounds play an essential role in defending the body against free radical damage by scavenging or neutralizing oxidizing molecules and maintaining redox balance [7].

Recent studies have reported that the plant kingdom offers a wide range of natural antioxidant molecules including phenolic acids, flavonoids, and other secondary metabolites, and they can be useful for the treatment of various disorders [8,9]. Geraniol, a natural acyclic monoterpene, is the primary component of oils of rose and palmarosa [10] and several essential oils such as lemon, ginger, and orange [11]. This natural molecule possesses diverse biological effects, being an antioxidant [12], antibacterial [13], anti-inflammatory [14], and antiangiogenic [15] agent.

The mitochondrial respiratory chain is responsible for the primary source of ROS production in cells because this mechanism consumes about 80-90% of oxygen that a person utilizes and produces most of the ROS generated in the body. Another essential formation of ROS especially occurs in the liver [16]. This study aims to assess a possible protective role of geraniol on liver fatty acids composition in H_2O_2-induced oxidative stress. For this purpose, in our study biochemical analyses such as liver tissue and serum MDA, hepatic GSH-Px, GSH, and CAT concentrations besides liver and serum fatty acids percentages were evaluated.

MATERIAL AND METHODS

Animals and Experimental Procedure

A total of 28 adult Wistar albino male rats (230 ± 10 g body weight) were obtained from Experimental Research Unit of Firat University (Elazig, Turkey). The animals were housed under standard light/darkness cycle (lights on from 0700 to 1900 h), at a regular temperature (21 ± 1°C) and humidity (55 ± 5%) with free access to fresh water and food. The experimental applications were confirmed by Ethical Committee of Firat University (Document No: 146/2011-11), and the rats were treated in strict compliance with the international laws on the use and care of experimental animals.

Groups of animals were randomly divided into four groups as control, naringenin, lead acetate, and naringenin+lead acetate (n = 7 each group). Control group animals received vehicle solutions only. Geraniol (50 mg/kg, dissolved in corn oil) and H_2O_2 (16 mg/kg, dissolved in distilled water) were administered by intraperitoneal injection. Administrations were performed during 30 days with 1-day interval. The animals were sacrificed at the end of 30 days. Blood and hepatic tissue were obtained from animals. Serum and liver samples were stored at −20°C until the assays were performed.

H_2O_2, geraniol and other chemicals were obtained from Sigma (Dorset, UK) unless otherwise indicated.

Determination of Liver and Serum Oxidative Stress-related Parameters

Protein concentration was analyzed using Lowry method [17]. MDA level was measured at 532 nm and expressed as nmol g protein^{-1} [18]. CAT was measured by determining the decomposition of H_2O_2 at 240 nm and was expressed as kg protein^{-1} [19]. GSH-Px was analyzed according to Lawrence and Burk method [20] and was expressed as IU g protein^{-1}. GSH concentrations were determined using the method of Sedlak and Lindsay [21]. All analyzes were performed using a UV-visible spectrophotometer (Shimadzu-2R, Tokyo, Japan).

Lipid Extraction and Measurement of Fatty Acid Percentages

The liver tissue samples (3 g) were homogenized for analyzes. Nonlipid contaminants in the lipid solution were purified through the addition of 0.88% KCl solution. The total lipids were extracted by a mixture of hexane/isopropanol (3:2 v/v) according to the previous method [22]. Fatty acids were converted into methyl esters via adding of 2% sulfuric acid (v/v) in methanol [23]. These methyl esters were separated by a gas chromatography and measured via flame/ionization detection system (Shimadzu GC-17 Ver3, Japan). The chromatography process was performed via capillary column (Machery-Nagel, Germany) using nitrogen as a vehicle gas (flow rate 800 μl/min).

Statistical Analysis

Results were expressed as a mean±standard error of mean. Data were analyzed using one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference (HSD) test (SPSS 12.0 for Windows as a software program). For all analyzes, $P < 0.05$ was considered statistically significant.

RESULTS

Effects of Geraniol on Hepatic MDA, GSH, GSH-Px, and CAT Activity against H_2O_2-Treatment

Figure 1 represents the hepatic MDA concentration (nmol g protein^{-1}) of the groups. Administration of H_2O_2 caused significant increases ($P < 0.001$) MDA concentrations (23.5 ± 1.3) compared with control (16.3 ± 0.7), whereas its levels were lower in the liver of the geraniol+H_2O_2 (17.8 ± 1.4) group animals compared with H_2O_2-treated group. In H_2O_2-treated animals, liver GSH (Figure 2, nmol g protein^{-1}) and GSH-Px (Figure 3, IU g protein^{-1}) levels (2.9 ± 0.2 and 50.8 ± 6.5, respectively) were significantly lower ($P < 0.001$ and $P < 0.01$, respectively) than control group (3.5 ± 0.1 and 63.4 ± 7.7, respectively). These values were significantly

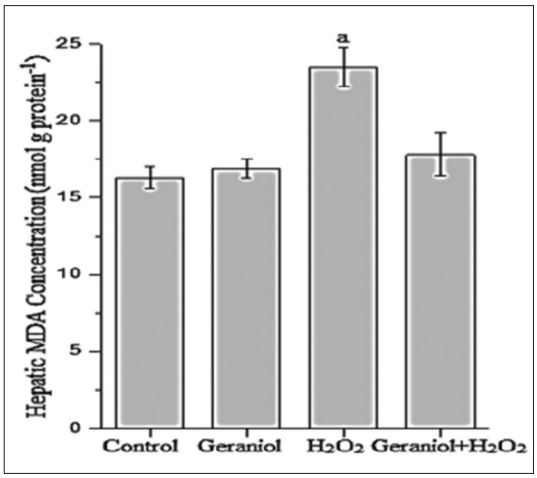

Figure 1: Hepatic malondialdehyde concentration of the groups. [a]*P*<0.001 vs. control group, (one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test), *n* = 7 for each group

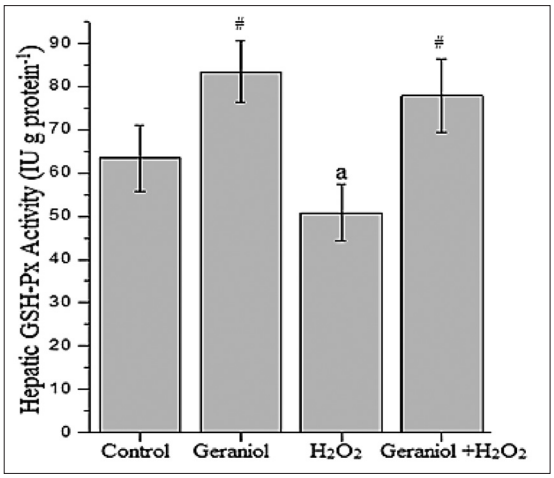

Figure 3: Hepatic glutathione-Px activity of the groups. [a]*P*<0.01 vs. control [#]*P*<0.001 vs. H_2O_2-treated group, (one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test), *n*=7 for each group

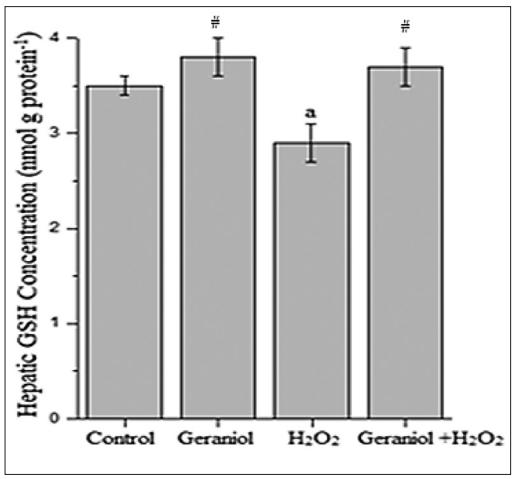

Figure 2: Hepatic glutathione concentration of the groups. [a]*P*<0.001 vs. control and [#]*P*<0.001 vs. H_2O_2-treated group, (one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test), *n* = 7 for each group

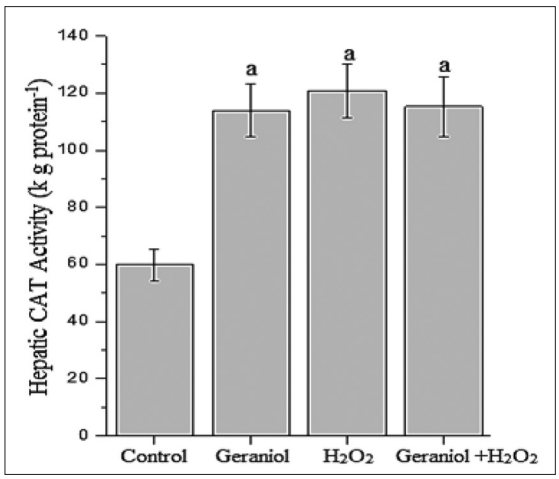

Figure 4: Hepatic catalase activity of the groups. [a]*P*<0.001 vs. control group, (one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test), *n*=7 for each group

increased following coadministration of geraniol with H_2O_2 in geraniol+H_2O_2 group (3.7 ± 0.2 and 77.9 ± 8.6, respectively) rats compared with H_2O_2 alone-treated animals ($P < 0.001$). Liver CAT activity (Figure 4, kg protein^{-1}) increased in the geraniol (113.9 ± 9.2), H_2O_2 (120.9 ± 9.4) and geraniol plus H_2O_2 (115.2 ± 10.4) groups compared with control (59.9 ± 5.5) group ($P < 0.001$). In geraniol+H_2O_2 group animals, CAT levels were generally lower than the H_2O_2 but this effect did not differ between groups.

Effect of Geraniol on Serum MDA Concentration against H_2O_2-Treatment

We examined that effect of geraniol on the liver as well as body oxidant status in rats' serum after H_2O_2 treatment (Figure 5). There was no statistical difference between geraniol plus H_2O_2 group (8.5 ± 0.4) and control (7.2 ± 0.3) in MDA levels (nmol g protein^{-1}). We found that there was a significant increase in serum MDA level of H_2O_2-treated group (12.8 ± 0.5) when compared with control animals ($P < 0.001$). However, MDA concentrations were found to be lower in geraniol plus H_2O_2-treated animals compared with H_2O_2 group.

Effect of Geraniol on Hepatic Fatty Acid Percentages against H_2O_2-Treatment

The values regarding effects of geraniol and H_2O_2 on the total and individual fatty acids were summarized in Table 1. The differences in mean total saturated fatty acids (∑SFA) were detected between H_2O_2 and control groups ($P < 0.05$). There was no a significant alteration in ∑SFA percentage of control group compared to geraniol alone and geraniol plus H_2O_2 groups. The ratio of total unsaturated FA (∑USFA) was significantly lower in H_2O_2-treated animals compared to control group ($P < 0.05$). Although it was not statistically

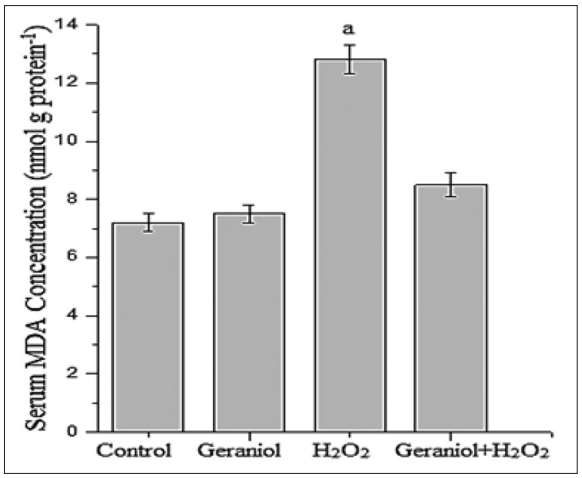

Figure 5: Serum malondialdehyde concentration of the groups. [a]*P*<0.001 vs. control group, (one-way analysis of variance followed by *post-hoc* Tukey's honestly significant difference test), *n*=7 for each group

Table 1: The percentages of fatty acids in liver

Fatty acids	Control	Geraniol	H_2O_2	Geraniol + H_2O_2
14:0	0.4±0.02	0.3±0.01	0.5±01	0.3±0.02
15:0	0.4±0.02	0.4±0.01	0.4±0.02	0.4±0.01
16:0	23.3±0.8	21.6±0.2	24.3±0.6	20.3±0.7[a]
16:1N7	2.6±0.7	1.5±0.1[a]	2.4±0.4	1.6±0.2[a]
17:0	0.7±0.1	0.9±0.04	0.7±0.1	0.9±0.03
18:0	15.1±0.1	16.9±0.2	15.7±1.03	17.9±1
18:1N9	7.1±0.6	4.8±0.2[b]	6.2±0.6	5±0.4[a]
18:1N7	3.5±0.2	3.6±0.1	3.6±0.1	3.8±0.2
18:2N6	16.9±0.7	16.3±0.4	17.8±0.6	16.5±0.2
18:3N6	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.02
18:3N3	0.2±0.01	0.1±0.01	0.2±0.05	0.1±0.01
20:1N9	0.4±0.02	0.2±0.01[a]	0.4±0.02	0.3±0.1
20:2N6	0.4±0.01	0.4±0.01	0.3±0.01	0.4±0.01[a]
20:3N6	1.4±0.1	1.4±0.03	1.1±0.1	1.4±0.1
20:3N9	0.2±0.01	0.3±0.01	0.2±0.02	0.3±0.02[a]
20:4N6	15.9±0.5	20.2±0.5[a]	15.3±1.5	19.8±1.1[a]
20:5N3	1.4±0.2	1.3±0.1	1.2±0.1	1.6±0.1
22:4N6	0.3±0.2	0.2±0.02	0.1±0.01	0.1±0.02
22:5N3	1.6±0.1	1.3±0.1	1.2±0.1[a]	1.3±0.1
22:5N6	0.3±0.01	0.3±0.02	0.3±0.01	0.3±0.01
22:6N3	7.4±0.2	7.4±0.2	7.3±0.7	7±0.2
24:0	0.3±0.01	0.2±0.1[a]	0.3±0.01	0.3±0.03
∑SFA	40.2±0.2	40.3±0.3	41.8±0.3[a]	40±0.3
∑USFA	59.7±0.4	59.7±0.4	58.1±0.3[a]	60±0.3
MUFA	13.5±1	9.3±0.2[c]	12.6±0.3	10.7±0.6[b]
PUFA	46.2±1.01	49.4±0.4[a]	45.5±2	49.2±1[a]
W3	10.6±0.4	10.2±0.2	10±0.8	10.1±0.3
W6	35.4±0.9	39±0.4[a]	35.3±1.2	38.8±1.1[a]

[a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001 versus control group, (Tukey's HSD test), *n*=7 for each group. MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids

significant, the monounsaturated fatty acids (MUFA) levels of H_2O_2 and control groups, the liver MUFA concentration was found to be lower in the geraniol and geraniol plus H_2O_2 groups compared to control ($P < 0.001$ and $P < 0.01$, respectively).

The significant difference in the mean polyunsaturated fatty acids (PUFA) was detected in geraniol and geraniol+H_2O_2 groups compared with control group ($P < 0.05$). Although not statistically significant, the PUFA levels of H_2O_2 and control groups, its liver ratio was found to be higher in the geraniol and geraniol plus H_2O_2 groups compared with control group animals ($P < 0.05$). No overt differences in mean W3 percentages were observed among groups. There was no statistical difference between H_2O_2 and control group in the W6 levels. However, this FA percentage was found to be higher in geraniol and geraniol+H_2O_2 groups compared to control ($P < 0.05$).

Effect of Geraniol on Serum Fatty Acid Percentages against H_2O_2-Treatment

The differences in mean 18:0 percentages were detected in alone H_2O_2-treated and geraniol plus H_2O_2 groups compared to control rats ($P < 0.001$). No overt differences in this FA percentage were observed between control and geraniol-treated groups. There was a significant difference in H_2O_2 and geraniol+H_2O_2 groups compared to control animals in the mean 18:2n6 ratio ($P < 0.01$). The differences of these FA levels were not detected between geraniol and control groups.

In the rats administered geraniol, ∑SFA level was similar to control rats. However, the ∑SFA concentration was significantly affected by H_2O_2 and geraniol plus H_2O_2 treatment compared to control animals ($P < 0.01$). Serum ∑USFA level was significantly higher in the geraniol-treated animals compared to control rats ($P < 0.05$). ∑USFA concentration was found to be lower in the H_2O_2 and geraniol+H_2O_2 groups compared to control animals ($P < 0.01$). Although not statistically significant, MUFA level was determined to be higher in the geraniol-treated animals compared to control animals. However, the concentrations of MUFA were significantly lower in H_2O_2 and geraniol+H_2O_2 groups compared to control animals ($P < 0.05$). Serum PUFA level was significantly lower in H_2O_2 and geraniol+H_2O_2-treated groups compared with control rats ($P < 0.05$). Although not statistically significant, PUFA percentage was determined to be higher in the geraniol-treated animals compared to control group. Administration of H_2O_2 decreased serum w6 level compared to control rats ($P < 0.05$). However, the W6 percentages of the geraniol+H_2O_2 group were similar to the control group. The values regarding effects of geraniol and H_2O_2 on the total and individual fatty acids in the serum were summarized in Table 2.

DISCUSSION

The study provides an argument for the protective role of geraniol on changes of liver and serum oxidative stress related molecules, enzymes, and fatty acids in rats with H_2O_2-induced hepatic damage. Intraperitoneal administrations of both geraniol and H_2O_2 affected antioxidant status in the liver and serum of rats. While H_2O_2 increased MDA levels, geraniol restored its effects on the liver and serum lipid peroxidation in male rats. Treatment of geraniol to animals inhibited H_2O_2-induced decrease of GSH and GSH-Px concentrations in the liver. However, geraniol did not reverse the H_2O_2-induced increase in CAT activity completely.

Table 2: The percentages of fatty acids in serum

Fatty acids	Control	Geraniol	H_2O_2	Geraniol+H_2O_2
16:0	28.7±1	26.3±0.3[a]	28.1±0.2	27.6±0.1
18:0	17.8±1	18.9±0.6	20.8±0.3[c]	20.6±0.4[c]
18:1N9	8.2±1	9.2±0.5	7.04±0.2	6.9±0.2
18:1N7	3.7±0.1	3.5±0.1	3.4±0.2	3.9±0.2
18:2N6	24.6±1	24.1±0.6	22.1±0.4[b]	22.2±0.1[b]
20:4N6	10.6±0.4	12.1±0.2[a]	11.6±0.1	13.4±0.4[c]
22:6N3	6.3±1	5.8±0.2	6.9±0.4	5.3±0.2
∑SFA	46.5±0.3	45.2±0.3	48.9±0.4[b]	48.2±0.4[b]
∑USFA	53.5±0.3	54.8±0.3[a]	51.1±0.4[b]	51.7±0.4[b]
MUFA	12±1.1	12.8±0.2	10.4±0.1[a]	11±0.2
PUFA	41.5±0.2	42±0.2	40.7±0.2[a]	40.9±0.2[a]
W3	6.3±1	5.8±0.2	7±0.4	5.31±0.2
W6	35.2±0.2	36.2±0.2	33.7±0.4[a]	35.6±0.2

[a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ versus control group, (Tukey's HSD test), $n=7$ for each group. MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids

Lipid peroxidation is an important toxic pathway because it involves the removal of hydrogen from fatty acid chains mediated by ROS [24,25] this way can lead to cell death in the body. The endogenous antioxidant enzyme includes GSH-Px that catalyzes the reduction of H_2O_2 to water through the oxidation of reduced GSH. CAT also participated in this conversion [26]. It was reported that oral administration of geraniol to 7,12-dimethylbenz(a)anthracene (DMBA)-treated mice significantly increased the activities of enzymatic antioxidants (CAT and GSH-Px) and non-enzymatic antioxidant (GSH) level in red blood cells, and skin tissues. Moreover, these parameters did not differ between geraniol alone treated and control animals [27]. Ibrahim *et al*. noticed [28] that 30 days administration of geraniol restores effects of the fructose-induced metabolic syndrome on hepatic and serum lipid peroxides related parameters in rats.

Hepatic nitric oxide (NO) content elevates in the fructose-induced model, possibly because of the increased synthesis of inducible NO-synthase activated by NF-κB [29,30]. Moreover, this mechanism is related with the increased lipid peroxidation and the decreased non-protein thiols (NPSH). Geraniol can suppress liver NO and lipid peroxides and enriches NPSH [28] via the activation of both GSH-Px and other reductase enzymes [31]. These reports that are related to hepatic lipid peroxidation and GSH-Px are consistent with our data. However, in this study, we observed that geraniol was not completely able to restore H_2O_2-induced increase in the hepatic CAT activity. Andrade *et al*. reported [32] that the inhalation of geraniol was increased serum alanine aminotransferase activity and hepatic lipid hydroperoxide in rats. Moreover, rats exposed to geraniol had higher CAT, SOD, and GSH-Px activities. The authors suggested that the lipoperoxide generation could be a result of ineffective antioxidant enzyme activities because these reactions were not sufficient to inhibit the ROS action and the lipoperoxide production in the liver of these animals. Koek *et al*. reported [33] that the activity of antioxidants enzymes increases early stage in the nonalcoholic steatohepatitis, but these alterations tend to decrease with progression of hepatic pathogenesis. The high concentration of H_2O_2 can be related with high activity of GSH-Px and CAT because these enzymes play an essential role in the elimination of H_2O_2. CAT is the most efficient enzyme in this interaction; also, it is so efficient that it cannot be saturated with H_2O_2 at any concentration [34]. Therefore, we can suggest that geraniol has an antioxidant role, but this effect is not full capacity on the H_2O_2-induced oxidative pathway in the liver of male rats.

Geraniol has anticancer efficacy that may be related to the inhibition of HMG-CoA reductase [35]. The latter role also confirms geraniol antiatherogenic effect, recently Jayachandran *et al*. suggested [14] that this natural monoterpene can ameliorate fructose-induced obesity and dyslipidemia in hamsters [28]. In 2011, it was reported [36] that geraniol activates *in vitro* several peroxisome proliferator-activated receptor (PPAR) subspecies. The interaction with geraniol and PPAR nuclear receptors that regulate the expression of target genes involved in lipid metabolism [37] is an important result because this data may provide a new treatment option for metabolic disorders such as hyperlipidemia, obesity, and diabetes. During the recent years, it was suggested that the antiatherogenic effect of geraniol is related to the activation of lipoprotein lipase to inhibit serum triglycerides, as well as lecithin cholesterol acyl transferase to elevate high-density lipoprotein cholesterol [14,28]. In this study, we first observed that the use of geraniol decreased high liver ∑SFA level caused by H_2O_2-treatment. Moreover, this monoterpene can protect the reduction of hepatic ∑USFA level induced by H_2O_2. Similar results were partly obtained for serum ∑SFA and ∑USFA levels.

As regards the individual fatty acids, the hepatic levels of palmitoleic acid, oleic acid, and eicosenoic acid, which are the member of MUFA, were affected by geraniol treatment. The percentages of hepatic oleic acid, palmitoleic acid, and eicosenoic acid decreased in the geraniol group and partly geraniol plus H_2O_2 groups. Therefore, the percentage of total MUFA decreased these groups. Interestingly, serum MUFA percentage did not differ between geraniol and control, while it decreased in H_2O_2 and geraniol plus H_2O_2 groups. Although not statistically significant, the percentage of hepatic arachidonic acid (20:4n6), which is a member of PUFA and is a partly essential fatty acid, was determined to be lower in the H_2O_2-treated rats, its level significantly increased in H_2O_2 and geraniol+H_2O_2 groups. In humans, levels of MUFA, such as oleic acid, are increased to the age of 18 years [38]. Several other PUFA particularly arachidonic acids are also decreased with age in the older persons [38]. Therefore, it can be said that these fatty acids change with metabolic and environmental factors over the years. Alterations in fatty acid composition of cells and its membrane are known to influence the activity of G proteins and protein kinase C (PKC) [39]. PKC and G proteins play a modulatory role in the regulating blood pressure [40]. Recently, it was reported that this natural compound can ameliorate the elevated systolic blood pressure against the fructose-induced metabolic syndrome [28]. Oleic acid is converted into nitrated-oleic acid in the presence of NO. The molecular mechanisms associated with physiologic roles of nitrated oleic acid remain unknown. It was suggested [41] that nitrated oleic exists in the blood and several organs, where it promotes vessel relaxation

and modulation of immune system cells. However, we did not determine whether geraniol and H_2O_2 affected blood pressure or NO-related metabolic parameters. Interestingly, although it was not statistically significant, oleic acid serum percentage was found to be higher in the geraniol-injected rats and lower in H_2O_2 and geraniol plus H_2O_2 groups. Otherwise, this fatty acid decreased in the liver of geraniol-treated group. Therefore, it can be said that the oleic acid and related fatty acids may have tissue dependent specific roles.

In this study, we observed that H_2O_2 causes oxidative stress in the liver and body; this effect can be restored partly through geraniol administration because it cannot reverse the H_2O_2-induced increase in CAT enzyme completely. In addition, this natural compound can be regulated in the liver several fatty acids percentage such as ∑SFA and ∑USFA levels against H_2O_2-induced alteration. However, geraniol has a distinct effect on other fatty acids like individual and total levels; this situation can be related to its other metabolic effects and interactions.

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Original Research

A prospective comparative field study to evaluate the efficacy of a traditional plant-based malaria prophylaxis

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ABSTRACT

Background: An unceasing threat of resistance of malarial parasites to available antimalarial drugs makes the development of new drugs imperative. Natural plant-based products are an alternative source for discovering new antimalarial drugs. **Aim:** To determine the prophylactic efficacy of a traditionally used plant-based drug on prevention of malaria in endemic villages of Odisha, India. **Methods:** A total of 267 healthy human volunteers of both sexes, aged 18-60 years were enrolled in Odisha, India, to receive either minimum 20 doses of aqueous extract of Traditional Plant-based Malaria Prophylactic drug 74, twice a week (experimental group), or no drug (control group) for 14 weeks. The primary criterion was the occurrence of malaria positive cases confirmed through expert microscopy during the study period. Analyses were by per-protocol (PP) and modified intention-to-treat (mITT). **Results:** A significant ($P < 0.01$) reduction (64%) of malaria incidence was observed in the experimental group compared to control group, 12.3% and 26.6%, respectively, as PP analysis. However, the reduction was nonsignificant as per mITT analysis ($P = 0.22$). The experimental group showed a relative risk of 0.36 compared to control group. **Conclusion:** This preliminary study constitutes a potential "proof of concept" for the development of malaria prophylactic drug and provide a scientific basis for the use of traditional remedy as a malaria preventive by tribal populations in India.

KEY WORDS: Herbal prophylaxis, India, malaria, malaria-endemic population, traditional medicine

INTRODUCTION

Malaria, a disease caused by the parasite of the genus *Plasmodium*, remains major public health concerns in tropical and subtropical countries. 214 million cases of malaria and over 438,000 deaths occurred globally in 2015 [1]. 58% of the malaria deaths occurred in the poorest 20% of the world's population, and these patients receive only poor quality care and have catastrophic economic consequences from their illness [2].

In India, malaria is one of the most common infectious diseases among tribal population with 22.6% of clinical episodes due to *Plasmodium falciparum* and 42% of episodes due to *Plasmodium vivax* globally [3,4]. In Odisha, eastern state of India, malaria is the leading cause of mortality and morbidity in the tribal population [5]. In many other states of India, malaria transmission intensity varies with season with high transmission after the monsoon rains in autumn and winter. In such conditions, a suitable control strategy implemented during these seasons may have a good impact on the reduction of malaria burden.

Malaria infection can be prevented using (anti-liver stage) chemoprophylaxis. However, most of the available drugs are confronted with the evolution of drug resistance [6-8]. On the other hand, the safety is a major concern in chemoprophylaxis.

For instance, primaquine, atovaquone, and doxycycline are contraindicated in pregnant women and children. Moreover, many of the drugs are not affordable and inaccessible to the needy populations.

As in almost all tropical endemic countries, malaria in India affects particularly people living in rural, remote areas, where most often affordable modern drugs are not available and where poor health-care infrastructure cannot assure prompt and appropriate treatment. The use of herbal medicinal plants especially those used in traditional medicine for the prevention of malaria is common in population of malaria endemic areas, among these, the use of Traditional Plant-based Malaria Prophylactic 74 (TPMP74) [9].

TPMP74 is a coded polyherbal traditional remedy, widely used in the form of aqueous extract (decoction), and all the plants are well recognized for its medicinal properties for a long time in Ayurveda. Each plant and aqueous extract of TPMP74 have been standardized through organoleptic, physicochemical, phytochemical, microbial, heavy metal, pesticide residue, and high-performance thin layer chromatography analysis [10].

Furthermore, it has also been demonstrated that TPMP74 possesses an inhibitory effect on the *Plasmodium yoelii* hepatic stages *in vitro* (50% inhibitory concentration = 0.74 mg/mL) with a therapeutic index of 9.54 and in mice treated with 2000 mg/kg/day, peak parasitemia reduction by 81% in the experimental 2.35% ± 0.14% as compared to controls 12.62% ± 0.52% ($P < 0.001$) [11].

Thus, TPMP74 which is considered as an important malaria prophylactic remedy needs to be further studied for its efficacy in human volunteers. The aim of this prospective comparative field study was to evaluate the efficacy of TPMP74 aqueous extract on lowering the occurrence of malaria in study population of malaria-endemic villages of Odisha, India.

MATERIALS AND METHODS

Ethical Issues

The protocol was approved by the Institutional Ethics Committee of the Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru (currently ITD-HST) (Protocol number: 05 TPPM-K). Written informed consent was taken from all the study participants. They were free to withdraw from the study at any time. Volunteers were followed closely and were given conventional treatment if necessary. The study was registered in Clinical Trial Registry - India (CTRI) and trial registration number is CTRI/2014/05/004610.

Settings

A prospective comparative field study was conducted in two rural villages (Tunpar and Kellar) of Koraput district (17° 50'N and 20° 30'N, 81° 27' and 84° 10' E) of Odisha state, India, during the period of high malaria transmission (June to December 2009). The two villages were situated within a radius of 3 km having similar climate, rainfall, and socioeconomic status [12] and had higher malaria incidences in the year 2007 and 2008 (unpublished data - Laxmipur PHC, Koraput district). The annual parasite incidence (API; cases/1000 persons/year) of Koraput district is 20.3 with 97% of *P. falciparum* cases [13,14]. The entomological and epidemiological parameters in these two villages were similar in intensity for malaria transmission (unpublished data-malaria baseline report, FRLHT, 2009).

Design

The study was designed as a prospective comparative study of volunteers recruited from Tunpar village who served as the experimental group to receive traditional prophylactic herbal remedy with those similarly recruited from Kellar village to serve as the control group given no specific malaria preventive medication. Owing to the very different nature of the intervention, it was not possible to blind the volunteers to the intervention being taken or to randomize the volunteers in the same village into two groups.

Participants

Before the start of the study, meetings were held in both the study villages to explain the purpose, methods of the study, and to answer their questions. People who were interested to participate in the study as volunteer were screened by medical examination and blood smears. Inclusion criteria for the study were (1) age 18-60 years, (2) having no chronic illness or symptomatic malaria, (3) having no parasites in the blood smear, and (4) healthy, without any co-morbid conditions. Noninclusion criteria were pregnancy, lactating mothers, those taking medication for any other complaints, and those unable or unwilling to provide written informed consent.

Intervention

TPMP74 aqueous extract (decoction) was freshly prepared on the day of administration by harvesting plant parts from the preidentified sources in the study area. The trained village health workers (VHWs) prepared the decoction in a central place (Government primary school, Tunpar) of the village. 45 mL of lukewarm decoction was administered twice a week (Tuesday and Friday) during the early evening (on an empty stomach) for 14 weeks to the participants of the experimental group and compliance on consumption of decoction was observed by the VHWs. The whole procedure was monitored by the study investigator. Samples of freshly prepared decoctions were analyzed to ensure the total dissolved solids of 16.32 ± 0.03% w/v, which confirmed the quality of decoction administered.

A data documentation system for the administration of decoction was devised and used by trained VHWs. This consisted of health card held by each volunteer and an attendance register held by the VHWs to record compliance and adherence to the study protocol. This health card was labeled

with the volunteers' number, name, and a table to record clinical findings on the day of administration of decoction.

Follow-up

Volunteers of both groups were actively and passively followed during the study period. The active follow-up was done biweekly and consisted of questioning the volunteers for the occurrence of symptoms consistent with malaria including fever, headache, body ache, and malaise. The passive follow-up was done through continuous availability of VHWs and auxiliary nurse midwife in the village, to evaluate any medical complaint at any time during the study period. Any volunteer, who complained of symptom potentially related to malaria, was given a complete medical check-up and laboratory assessment through blood smear examination. The positively diagnosed malaria cases were treated as per the standard guidelines of National Institute of Malaria Research, Government of India [15]. Apart from this clinical follow-up, all the volunteers were screened for malaria infection by blood smear examination at the end of the study. As per the study protocol, experimental group participants, who have received a minimum of 20 doses of TPMP74 out of the total of 28 doses in the study, were considered for statistical analysis. Volunteers were also monitored for intake of any antimalarial drugs as well as other drugs during the study period.

Laboratory Methods

Blood films were stained with Giemsa for 30 min, and parasite counts were done using standard WHO criteria [16].

Outcome Measures

The outcome of the study was based on the incidence of malaria as measured by laboratory diagnosis (blood smear) in both the experimental and control groups.

Statistical Analysis

Research pro forma was developed to enter baseline details, laboratory findings and follow-up details including regular consumption of the decoction. Every detail was entered by the VHWs and checked by investigators. All data were entered into an Excel sheet using MS Excel. Descriptive statistics, proportions, rates, and protective efficacies along with the required standard deviation and confidence intervals, Chi-square tests, per-protocol (PP) analysis and modified intention-to-treat (mITT) analysis were done using SPSS 18.0 (SPSS Inc., Chicago, USA). Since these were prospective cohort groups, relative risk was computed taking the incidence rate and the 95% corresponding confidence interval of malaria in experimental and control groups.

RESULTS

Participants

A total of 578 volunteers were enrolled in this study, 292 (male 142, female 150) in the experimental group and 286 (male 143, female 143) in the control group. Of the 292 volunteers enrolled in the experimental group, 149 (51%) did not complete the minimum 20 doses and 30 volunteers (10.2%) did not complete follow-up. Of the 286 volunteers enrolled in the control group, 132 (46.1%) did not complete follow-up. The main reason for loss to follow-up during the study period was migration of volunteers to another location as these are tribal people depend on daily wages. Thus, there were a total of 113 volunteers in the experimental group and 154 volunteers in the control group who completed the follow-up [Figure 1].

Baseline Characteristics

Baseline demographic and characteristics of participants in experimental and control groups are shown in Table 1. There was no statistically significant difference in age and use of malaria preventive measures between the two groups except for the gender. This shows that the volunteers of study and control groups have similar characteristics.

Impact of the Study on Malaria Incidence

The incidence of microscopically confirmed malaria in the two groups is presented in Table 2. PP analysis demonstrated that overall the incidence of malaria during the entire malaria endemic season was 14 among the 113 of the experimental group (incidence rate = 12.3%) and 41 among the 154 of the control group (incidence rate = 26.6%). The difference is statistically significant ($P = 0.005$). The experimental group showed a relative risk of 0.36, compared to the control group meaning that the risk to get malaria in the control group was 2.8 times higher than in the experimental one. However, mITT analysis was not significant ($P = 0.22$), where specific minimum standard

Table 1: Baseline demographic and characteristics of participants in experimental and control groups

Variables	Frequency (%)		*P*
	Experimental group (113)	Control group (154)	
Age	36.16±11.45	37.73±11.95	0.279
Male	65 (57.5)	57 (37.0)	0.0009*
Female	48 (42.5)	97 (63.0)	
Use of mosquito net	61 (54.0)	100 (64.9)	0.118
Use of mosquito coil	13 (11.5)	10 (6.5)	0.250
Use of mosquito mat	4 (3.5)	5 (3.2)	0.686
Use of cream	1 (0.9)	1 (0.6)	0.676
Herbal fumigation	12 (10.6)	8 (5.2)	0.177
Do not use	44 (38.9)	52 (33.7)	0.443

*Statistically significant. Percentage do not add up to 100 because of multiple responses

Table 2: Incidence rate of malaria in experimental and control group (in PP analysis)

Participants	Experimental group % (95% CI)	Control group % (95% CI)	*Z* score	*P*
All	12.3 (14/113) (6.1; 18.5)	26.6 (41/154) (19.5; 33.7)	2.83	0.005

CI: Confidence interval, PP: Perprotocol

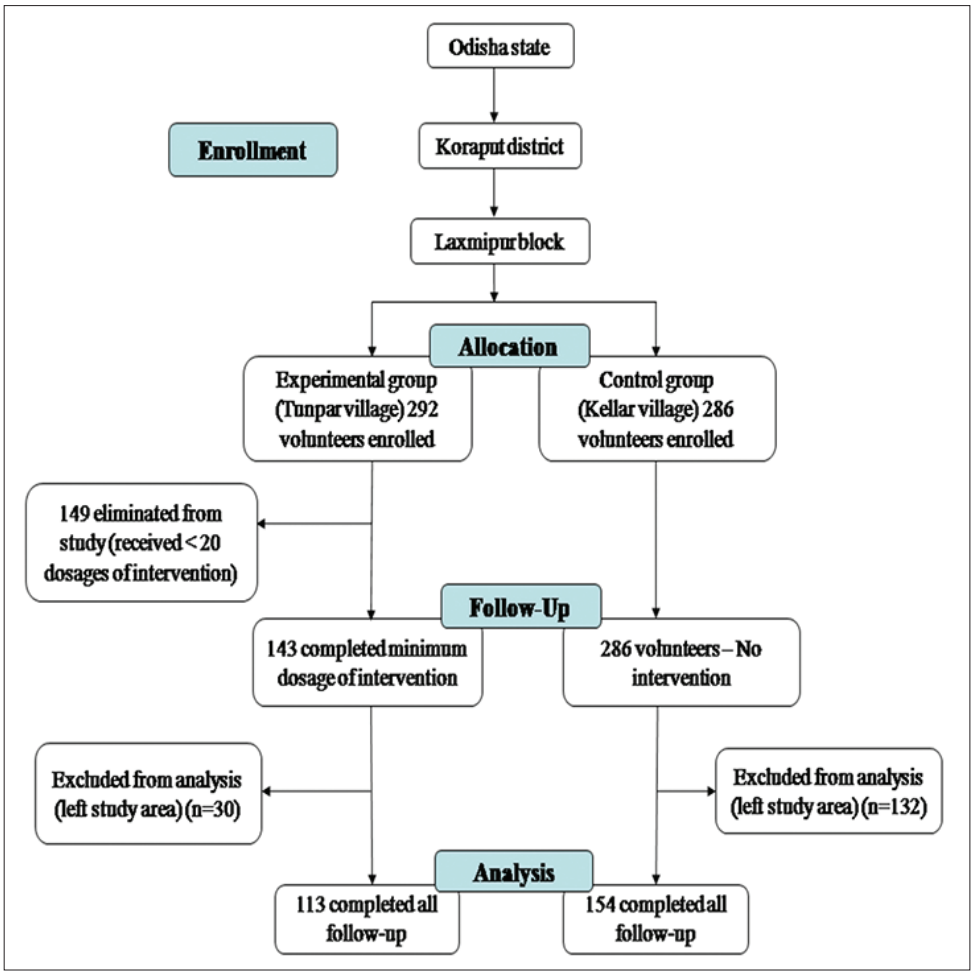

Figure 1: Village trial flow chart

criteria were minimum of 20 dosages during the 14 weeks of administration of TPMP74. However, due to the limited sample sizes, the power of the test was too low to be conclusive (28.9%). Hence, a higher number of volunteers could demonstrate a significant difference with a mITT analysis. One volunteer in the experimental group and two volunteers in the control group had *P. vivax* infection, whereas the remainder had *P. falciparum* malaria. However, there was no statistically significant difference in age, gender and use of malaria preventive measures among the volunteers who were positively diagnosed for malaria.

DISCUSSION

In this study, we wished to ascertain whether a plant-based preparation used by traditional healers in Odisha, India, as malaria preventive remedy has a demonstrable prophylactic potential. The selection of one over others traditional medicines was based on its perceived value to prevent, rather than to treat the malaria infection because drugs capable of providing prophylactic cover by inhibiting the liver stages have some advantages over other chemotherapeutic drugs [6].

We opted first to attempt to gather field evidence showing that the remedy as traditionally administered does indeed have an effect on the acquisition of malaria. A similar approach had been adopted in other studies [17,18]. In this field study, healthy volunteers were selected from two villages. For practical reasons (i.e.: No placebo remedy), it was difficult to recruit volunteers in the same village to serve as an experimental and a control group. Thus, volunteers from one village were the experimental group to whom the remedy was administered and those from the other served as the control group. The two villages were carefully selected to have similar malaria epidemiology. We are aware that such a study has limitations, principally a high attrition rate due to the migration of volunteers out of the village for economical reasons, and because of difficulties of some to adhere to the 28 (minimum 20) doses that needed to be administered during the study period. Nonetheless, we obtained clear indication that the remedy reduced the number of recorded microscopically confirmed malaria episodes in residents of malaria-endemic villages with constrained resources (a risk reduction of 64% in the PP analysis).

Intermittent preventive treatment (IPT) is a common approach followed in malaria transmission season to protect against malaria in endemic areas. Dicko *et al*. in Mali found a reduction of 42.5% in annual incidence of clinical malaria when IPT with sulfadoxine-pyrimethamine is given at 8-week intervals

in children targeting the transmission season [19]. Several studies have shown that IPT given to pregnant women, children and infants has reduced the incidence of clinical episodes of malaria by 20-60% depending on the transmission duration and seasonality [20-22].

Unlike the use of other malaria control strategies such as use of insecticide-impregnated material and IPT that requires daily implementation and external agency, whereas this traditional herbal usage strategy requires only training on the use of locally available herbs with standard preparation procedures. This simple strategy can be delivered through VHWs, local nongovernmental and governmental organizations. These herbal remedies may be affordable and accessible to the endemic population and can contribute to reducing the malaria burden in the communities, particularly in rural settings where health services are insufficient and inaccessible. It is also essential to achieve the cultural and social acceptance by the communities for a successful implementation of any malaria control strategy. As TPMP74 is a local health traditional practice by healers of their own community, it is quite easy for their acceptance.

Usually, the search for lead compounds from ethnopharmacological preparation is initiated on individual fractions or compounds isolated from each plant component from which it is prepared. However, traditional healers often use polyherbal preparations in the belief that not only each herb has a specific beneficiary role but also that the combination of these effects is important. The underlying principle is that plants combination may be synergistic or may potentiate the action of the individual plants [23]. To capture this, we opted to assess the first whether the traditional remedy, as administered to subjects, has an impact on malarial infections. Indications of a positive effect from such a study would then justify testing the same preparation under *in vivo* and *in vitro* experimental settings.

Earlier clinical observational studies on TPMP74 have shown that the 45 mL of lukewarm decoction is safe and tolerable in healthy human participants [24]. The dose, dosage (twice a week), form of medicine, and duration of intervention were followed as per the recommendations of traditional healers [9]. In this study, the participants of experimental group were closely monitored for safety and any adverse events. None of the participants showed any serious adverse event except 10 volunteers who developed on the 3rd dosage mild but self-limiting gastric irritation and nausea.

The prophylactic activity observed in villagers was mirrored in that observed when the same formulation of the remedy was assayed *in vivo* in mice challenged by *P. yoelii yoelii* sporozoites inoculation. In addition to a 3-fold reduction in peak parasitemia in treated as compared to control mice, a 1-day delay was observed for the 1st day of patency in the treated mice (6 days vs. 5 days in the controls-after sporozoites inoculation). Because of the exponential increase of the asexual parasites every 24 h, such a delay could reflect a dramatic reduction and/or delay in merozoites emerging after liver stage development indicating potential prophylactic activity [11].

CONCLUSION

The simple strategy of using plant-based remedy is likely to be one of the effective strategies in reducing malaria burden in areas like Koraput district where local people still depend on the local plants to combat malaria. This approach of malaria control needs further research in other areas where malaria transmission remains high and where traditional herbal remedies have a major impact. For the benefit of the large population who adheres to the traditional medicine practices, there is a need for further pre-clinical and multicentric trials to evaluate efficacy and safety on long-term ingestion of commonly used traditional herbal malaria prophylaxis.

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Original Research

Kolaviron, a biflavonoid of *Garcinia kola* seed mitigates ischemic/reperfusion injury by modulation of pro-survival and apoptotic signaling pathways

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ABSTRACT

Objective: The study was designed to investigate the ameliorative effect of Kolaviron (KV) on ischemic/reperfusion injury in experimental animal models. **Materials and Methods:** Male Wistar rats were randomly divided into two groups: Group 1 received corn oil as a vehicle and rats in Group 2 were administered KV at 200 mg/kg for 4 weeks. The rats were fed with rat standard chow pellet and water administered *ad libitum*. After 4 weeks of KV administration, hearts were excised and mounted on the working heart perfusion system. Western blot analysis for protein expression was carried out on frozen heart samples. **Results:** There was significant ($P < 0.05$) reduction in the activity of catalase, superoxide dismutase, and glutathione peroxidase with concomitant reduction in oxygen radical absorbance capacity in ischemic rat heart of control compared to group pre-treated with KV, respectively. Similarly, intracellular reactive oxygen species and malondialdehyde were significantly elevated in control compared to KV pre-treated rats. KV significantly increased total Akt/protein kinase B (PKB), phosphorylated Akt/PKB at serine 473 and also caused a significant reduction in p38 mitogen-activated protein kinase, Caspase 3, and cleaved poly adenosine diphosphate ribose polymerase. **Conclusion:** Taken together, KV offered significant cardioprotection via free radical scavenging activity and upregulation of pro-survival pathway.

KEY WORDS: Antioxidant, chemoprevention, Kolaviron, ischemic/reperfusion injury, oxidative stress

INTRODUCTION

Mitochondrial generation of reactive oxygen species (ROS) has been reported as one of the sources of important mechanisms of disease and redox signaling in the cardiovascular system [1]. Ischemic heart disease, myocardial infarction, and other pathologies associated with heart diseases continue to be leading causes of cardiovascular morbidity and mortality. ROS are known to induce the oxidation of membrane lipids with concomitant production of malondialdehyde (MDA), a specific biomarker of lipoperoxidation [2]. Furthermore, the restoration of coronary flow (CF) (reperfusion) after a prolonged period of ischemia therefore precipitates and aggravates oxidative stress, which is a major cause of myocardial injury. In the myocardium, nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase have been described as the two major enzymatic sources of ROS [3,4].

The survivor activating factor enhancement (SAFE) pathway has been shown to require the activation of the signal transducer

and activator of transcription 3 and it can successfully lessen cardiomyocyte death at the time of reperfusion [5]. Tamareille *et al.* and Lecour defined SAFE pathway as all kinases which are specifically activated at reperfusion and that can improve cardiomyocyte survival [5,6]. Previously, SAFE pathway was originally described solely in terms of the extracellular-signal regulated kinase (ERK1/2), mitogen-activated protein kinase (MAPK) and Akt/PI3K kinase pathways, but Tamareille *et al.* described the JAK-STAT signaling pathway as a critical "third arm" of the RISK pathway (sometimes referred to as the SAFE pathway) [6]. Recently, antioxidant therapy has become a promising pharmacological approach for the prevention of myocardial ischemia/reperfusion (MI/R) injury [7-9].

Our laboratory has reported the beneficial effects of *Garcinia kola* (GK) and Kolaviron (KV) ranging from hepatoprotective, nephroprotective, and chemopreventive [10-18]. Hence, this study was designed to elucidate the possible mechanism of action of KV a bioflavonoid of GK seed extract on ischemic reperfusion injury in isolated rat hearts.

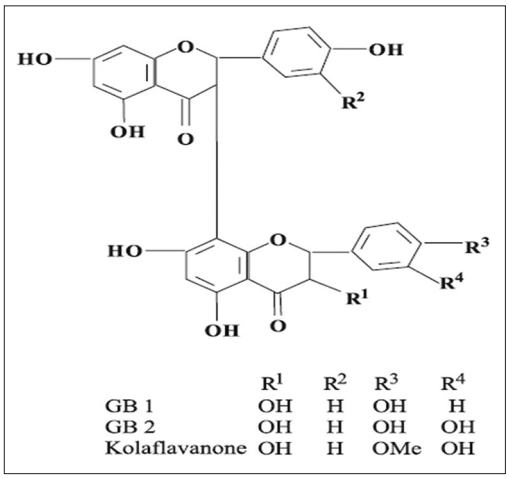
MATERIALS AND METHODS

Extraction of GK and Isolation of KV

KV was extracted from the seeds of GK according to the method of Iwu *et al.* with slight modification [19]. The seeds were sliced, air-dried, and powdered. The powdered seeds were defatted by extraction using n-hexane in a Soxhlet extractor apparatus for 24 h. The defatted dried marc was repacked and extracted with methanol. KV was fractionated from concentrated methanolic extract using chloroform to give a golden yellow solid which consists of Garcinia biflavanones – GB1, GB2, and kolaflavanone [Figure 1] [19].

Experimental Animals

A total of 40 rats were purchased from the primate colony of Tygerberg Hospital, Cape Town, South Africa. The rats were divided into 20 rats per group of two. They were housed in standard rat cages with 12 h light and 12 h dark cycle with temperature of 14 ± 4°C. They were fed with standard rat chow and given free access to water. The rats were acclimatized for 2 weeks before the commencement of the study. The rats in Group 1 received corn oil and those in Group 2 were administered KV at 200 mg/kg body weight for 4 weeks, respectively. The pharmacologic dose of KV (200 mg/kg) was used based on the previously established studies in our laboratory.



	R^1	R^2	R^3	R^4
GB 1	OH	H	OH	H
GB 2	OH	H	OH	OH
Kolaflavanone	OH	H	OMe	OH

Figure 1: Structure of kolaviron

Induction of Anesthesia and Perfusion Protocol

The rats were intraperitoneally anesthetized with sodium pentobarbital (50 mg/kg). About 10 min after anesthesia, blood was collected from the abdominal aorta into heparinized sample bottles for antioxidant capacity, and the hearts were immediately removed and mounted on a Langendorff apparatus. Krebs-Henseleit buffer (KHB) composed of NaCl 118.5 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, and glucose 11 mM (pH 7.4, 37°C) and saturated with 95% O_2, and 5% CO_2 as previously described [20]. The left ventricular pressure, the heart rate (HR), aortic output (AO), CF, systolic pressure (SP), and diastolic pressure (DP) were monitored via a transducer connected to a water-filled latex balloon inset into the left ventricle through the atrium and coupled to a pressure transducer (PowerLab, Australia). All the data were recorded and stored with the LG Computers, South Africa.

Animal Ethics

All animals used in this study received humane care in accordance with the Principle of Laboratory Animal Care of the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health Publications No. 80-23, revised 1978). The rats had free access to water and food. They were housed in an animal house at a constant temperature of 27°C, and they were exposed to a 12 h artificial day-night cycle. The ethical clearance for this study was approved and granted by the Health and Applied Sciences Research Ethics Committee of the Cape Peninsula University of Technology (Ethical number CPUT/HW-REC 2012/A03).

Isolated Heart Perfusion

Perfusion was maintained at a constant pressure of 75 mmHg, and the isolated hearts were stabilized for 20 min. The isolated heart was subjected to 5 min perfusion on Langendorff and 10 min perfusion on working heart with KHB. This was followed with 15 min global ischemia. The isolated rat hearts were for 10 min on Langendorff and 15 min on working heart [Chart 1].
Buffer: KHB.
Drugs: KV, GK and dimethyl sulfoxide as the control.

Blood Collection and Post-mitochondrial Fraction Preparation

About 3 ml of blood was collected from the abdominal aorta of the animals into heparinized tubes. The blood was centrifuged at 4000 g for 15 min to obtain the plasma.

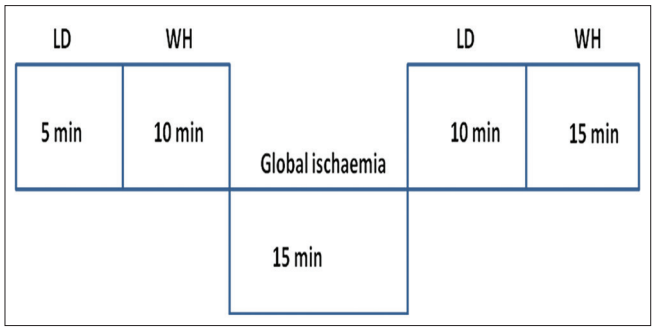


Chart 1: Heart perfusion protocol

The heart was harvested, rinsed and homogenized in aqueous potassium buffer (0.1 M, pH 7.4), and the resulting homogenate was centrifuged at 10,000 g (4°C) for 10 min to obtain the post-mitochondrial fraction.

Biochemical Assays

Protein determination was carried out bicinchoninic acid assay according to the method of Olsen and Markwell assay kit [21]. The oxygen radical absorbance capacity assay (ORAC) was determined as described by Cao and Prior [22]. The 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity of the hydrophilic extract was determined according to the method described by Seeram *et al.* [23]. The ferric reducing ability of the hydrophilic fraction was determined using the method described by Benzie and Strain [24]. The phenolic content of plasma was measured by the Folin-Ciocalteu method modified to remove protein interference [25]. Glutathione peroxidase (GPx) activity was measured according to the method of Flohé and Günzler [26]. The superoxide dismutase (SOD) activity was determined according to the method of Ellerby and Bredesen [27]. Catalase activity was determined by the method of Aebi [28]. The oxidized glutathione (GSSG) was determined as described by Griffith [29]. Total reduced glutathione (GSH) was determined according to the method of Tietze [30].

The intracellular ROS were determined described by Bartosz [31]. The fluorescence intensity is proportional to the ROS levels within the cell cytosol. The assay employs the cell-permeable fluorogenic probe 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA). In brief, DCFH-DA is diffused into cells and is deacetylated by cellular esterases to non-fluorescent DCFH, which is rapidly oxidized to highly fluorescent 2', 7'-dichlorodihydrofluorescein by ROS.

Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blot Analysis

Loading gel was prepared as follows 30% acrylamide, 0.5 mM Tris (pH 6.8), double distilled water (dd.H_2O), 10% SDS, ammonium persulfate, and tetramethylethylenediamine while the separating gel was also prepared in the same manner with 1.5 mM Tris at pH 8.8 (BIO-RAD; CA, USA). Gel electrophoresis was performed in a running buffer (×10, Tris/Glycine/SDS Buffer; BIO-RAD, CA, USA). Proteins (20 μg/lane) were separated by 10% SDS-PAGE and transferred in transfer buffer Tris 25 mM, glycine 192 mM, 0.01% SDS and 10% methanol and dd.H_2O (Millipore) onto polyvinylidene difluoride membranes. The separated proteins were pre-incubated with 5% nonfat in Tris buffered saline (Tris, sodium chloride, dd.H_2O and Tween-20) and incubated overnight at room temperature. Membranes were then probed with the following primary antibodies against protein kinase B (PKB)/Akt (1:1000), p-PKB/Akt at Ser473 (1:2000), p38 MAPK (1:1000), poly adenosine diphosphate ribose polymerase (PARP) (1:1000), cleaved PARP (1:1000), Caspase 3 (1:1000), and cleaved Caspase 3 (Asp 175) (1:1000). All primary antibody probes were purchased from Cell Signaling Technology, Danvers, MA, USA. And then, the membranes were incubated with horseradish peroxidase-conjugated secondary anti-rabbit immunoglobulin G (1:500; Santa Cruz Biotechnology, CA, USA).

Protein Quantification and Analysis

Protein bands were visualized using western blot detection reagent 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate (KPL, Gaithersburg, MD, USA) according to manufacturer's instruction. The membranes were then allowed to dry, scanned and the relative intensities of the membranes were thereafter quantified with UN SCAN-IT (Skill Scientific Incorporation, Orem, Utah, USA). UN SCAN-IT programme allows the protein bands on the membrane to be marked and the pixel value of each band determined. The higher the pixel value, the higher the protein concentration.

RESULTS

Effect of KV on Markers of Oxidative Stress, Enzymatic and Non-enzymatic Antioxidant System in Reperfusion Injury of Isolated Rat Hearts

In this study, there was significant ($P < 0.05$) increase in FRAP, Trolox equivalent antioxidant capacity (TEAC)/ABTS and ORAC levels in the plasma of rats pre-treated with KV at 200 mg/kg body weight compared to the control [Table 1]. As shown in Table 2, the antioxidant enzyme (SOD, catalase [CAT] and GPx) activities were significantly increased in KV pre-treated rats at the 20 min reperfusion time point compared to the control. The markers of oxidative stress (MDA and intracellular ROS) values were significantly ($P < 0.05$) reduced in the isolated rat hearts of the KV-treated group [Table 2]. Similarly, a significant ($P < 0.05$) increase in ORAC levels was found in the perfused ischemic cardiac tissues of rats pre-treated with KV for 4 weeks at 25 min reperfusion time points [Table 2].

Effect of KV on Functional Parameters in Reperfusion Injury of Isolated Rat Hearts

Pre-treatment with 200 mg/kg body weight of KV for 4 weeks was able to significantly improve AO compared to the control [Table 3]. Similarly, there was a significant increase in CF compared to the control as shown in Table 3. The improvement

Table 1: Effect of KV on antioxidant capacity in plasma in ischemic reperfused rat hearts

Groups	Total polyphenols (mg/GAE/L)	FRAP (μmol/AAE/L)	TEAC/ABTS (μmol TE/L)	ORAC (μmol TE/L)
KV (200 mg/kg)	321.51±15.49	680.44±34.69**	444.67±17.99***	817.93±34.99***
Corn oil (2 ml/kg)	304.60±40.04	542.44±21.61	354.80±30.06	731.25±42.83

The results are expressed as the mean±SEM (n=7). **and***Indicate significant difference at $P<0.01$ and $P<0.001$, respectively, in each column. FRAP: Ferric reducing antioxidant power, TEAC: Trolox equivalent antioxidant capacity, ORAC: Oxygen radical absorbance capacity, GAE: Gallic acid equivalent, TE: Trolox equivalent, AAE: Ascorbic acid equivalent, KV: Kolaviron (200 mg/kg bodyweight), corn oil (2 mL/kg), SEM: Standard error of mean

Table 2: Effect of KV on antioxidant enzymes and markers of oxidative stress in ischemic rat hearts (20 min reperfusion time point)

Groups	SOD[a]	CAT[b]	GPx[c]	MDA[d]	ORAC[e]	ROS[f]
KV (200 mg/kg)	2.34±0.97	0.29±0.063	0.057±0.0008	4.67±0.76	16.01±2.147	415.70±36.41
Corn oil (2 ml/kg)	0.97±0.26**	0.21±0.024**	0.032±0.0006*	8.81±1.84**	12.17±1.84*	548.40±15.57**

The results are expressed as the mean±SEM of seven rat hearts per group. *,**and***Indicate significant difference at $P<0.05$, $P<0.01$ and $P<0.001$, respectively, in each column. [a]Units/μmol/mg protein, [b]μmol/min/mg protein, [c]nmol/min/mg protein, [d]fluorescence detection unit, [e]nmol of MDA formed/g tissue, [f]μmol/TE/g tissue. SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase, MDA: Malondialdehyde, ORAC: Oxygen radical absorbance capacity, ROS: Intracellular reactive oxygen species, KV: Kolaviron (200 mg/kg bodyweight), corn oil (2 mL/kg), SEM: Standard error of mean

Table 3: The effect of KV on functional parameters for 25 min reperfusion

Functional parameters	Groups	Pre-ischemia	25 min reperfusion
AO (mL/min)	KV	38.76±0.92	24.01±1.81
	Control	35.62±0.96*	18.22±2.75
CF (mL/min)	KV	20.53±1.03	8.91±1.18
	Control	16.74±1.07*	15.52±0.73*
SP (mm Hg)	KV	163.21±3.05	145.80±2.55
	Control	158.01±3.16	141.36±4.08
DP (mm Hg)	KV	71.40±2.80	70.62±2.45
	Control	68.71±1.00	71.37±1.66
HR (bpm)	KV	288.45±4.89	286.26±6.18
	Control	309.13±8.94	298.74±7.74

The results are expressed as the mean±SEM of 10 rat hearts per group. *Indicate significant difference at $P<0.05$ in each column. HR: Heart rate, LVEDP: Left ventricular developed pressure, SP: Systolic pressure, CF: Coronary flow, AO: Aortic output, bpm: Beat per minute, KV: Kolaviron (200 mg/kg bodyweight), corn oil (2 mL/kg)

observed in the hemodynamic parameters (SP, DP and HR) in KV pre-treated rats were not significantly different from the control animals at the 20 min reperfusion time point [Table 3].

Our results showed an apparent increase in plasma reduced GSH in rats pre-treated with 200 mg/kg body weight of KV but with no significant difference when compared to the control [Figure 2]. Similarly, there was a significant ($P < 0.05$) reduction in plasma GSSG in rats pre-treated with KV (200 mg/kg) when compared with the control [Figure 2]. However, the increase in GSH/GSSG ratio obtained in rats administered with 200 mg/kg KV was not significantly ($P > 0.05$) different from the control [Figure 2].

Western Blots Results

Figure 3 showed that KV pre-treatment significantly reduced the expression of Akt and phosphorylated Akt (p-Akt), respectively, at 25 min reperfusion time point in isolated rat hearts. KV pre-treatment significantly reduced the expression of pro-apoptotic protein (p38 MAPK) compared to the control at 25 min reperfusion time point [Figure 4]. Furthermore, Caspase 3 and cleaved Caspase 3 expressions were significantly reduced in isolated hearts of animals pre-treated with KV for 4 weeks at 25 min reperfusion time points [Figure 5], respectively. Results obtained in this study showed that KV significantly increased and reduced PARP and cleaved PARP expressions, respectively, after 25 min reperfusion in hearts of animals pre-treated with KV compared to the control group [Figure 6].

DISCUSSION

This study provides evidence about the antioxidant property and cardioprotective effect of KV isolated from GK seed on isolated rat heart. The antioxidant capacity of KV was demonstrated with significant elevated plasma levels of FRAP, TEAC and ORAC in the rats pre-treated with KV compared to the untreated rats. The bioflavonoids present in KV might be responsible for this antioxidant capacity and the cardioprotective potentials. In addition, our data also show that KV pre-treated significantly increased AO and CF. The improvement in these hemodynamic parameters signifies the essential components of KV that offer cardioprotection via antioxidant property.

Moreover, we observed a significant increase in MDA levels, intracellular ROS and followed by a depletion of cardiac antioxidant enzymes; CAT, SOD, and GPx activities, respectively, after 20 min ischemic reperfusion injury in untreated rat hearts compared to the KV pre-treated rats. However, it has been documented that brief episodes of MI/R are associated with the generation of ROS [32,33].

The administration of antioxidants or free radicals scavengers is able to limit the evolution of myocardial damage reducing ROS-induced lipid peroxidation [34-36]. The medicinal use of KV as antioxidant, anti-inflammatory, and chemoprevention has been extensively documented elsewhere [14-16]. Moreover, the GB - GB1, GB2, and kolaflavanone present in KV could be used as a functional food to prevent or mitigate pathologies associated with oxidative stress and myocardial damage.

Western blot analysis revealed a significant increase in the expressions of Akt and p-Akt as pro-survival proteins in the

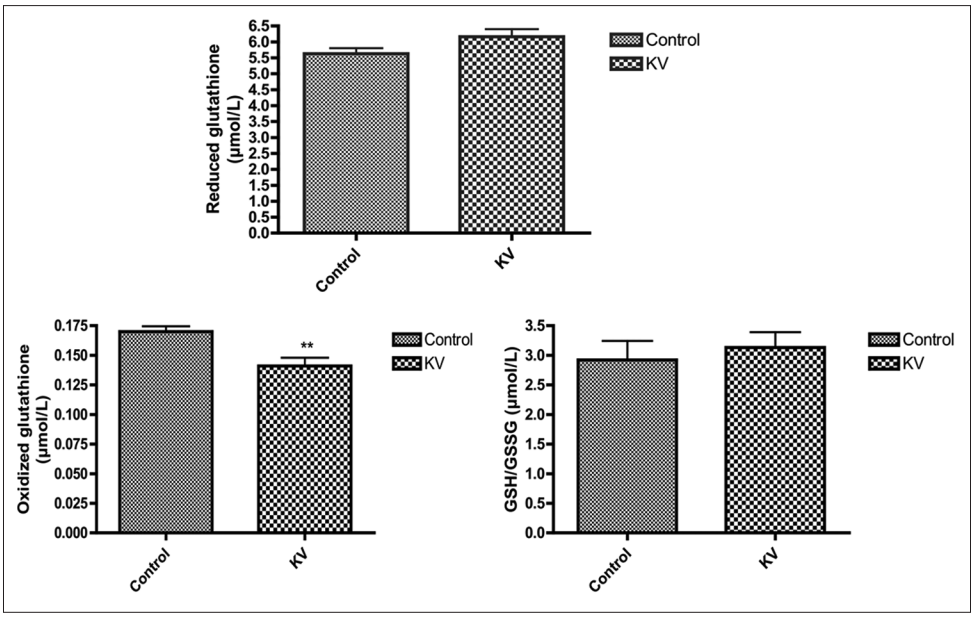


Figure 2: The effect Kolaviron (KV) on plasma reduced glutathione (GSH), oxidize GSH (GSSG) and GSH/GSSG ratio in 20 min reperfusion time point. Values are mean ± standard deviation, $n = 10$. Kolaviron; KV (200 mg/kg bodyweight), corn oil (2 mL/kg)

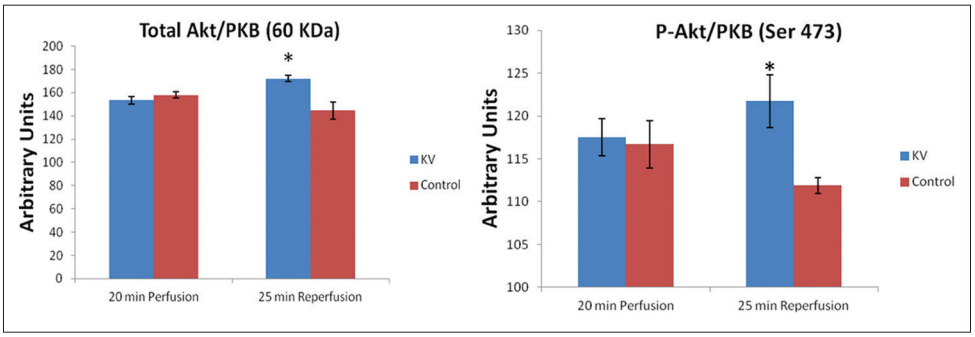


Figure 3: The effect of Kolaviron (KV) on total Akt/PKB and phosphorylated Akt/PKB at 20 min perfusion and 25 min reperfusion time point. Values are mean ± standard deviation, $n = 10$, $^*P < 0.05$ compared with control. KV (200 mg/kg bodyweight), corn oil (2 mL/kg)

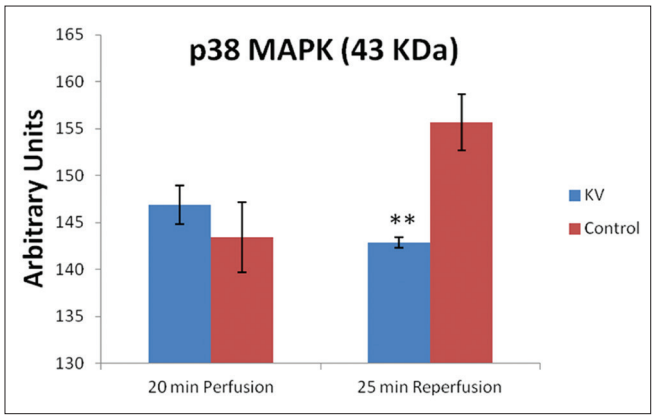


Figure 4: The effect of Kolaviron (KV) on p38 MAPK at 20 min perfusion and 25 min reperfusion time points. Values are mean ± standard deviation, $n = 10$, $^{**}P < 0.05$ compared with control. KV (200 mg/kg bodyweight), corn oil (2 mL/kg)

heart of rats pre-treated with KV compared to untreated group. This therefore also pointed to the fact that KV increased Akt phosphorylation in the pre-treated animals. On the other hand, pre-treatment with KV significantly suppressed Caspase 3, cleaved Caspase 3, p38 MAPK and cleaved PARP in the isolated rat hearts subjected to 20 reperfusion injury after global ischemia. The cytoprotecive and anti-apoptotic activity of KV might be associated with antioxidant and free radical scavenging activity of KV. This study, therefore, corroborates the previous study by Farombi *et al.* on the cytoprotecive of KV against DNA damage and oxidative stress [37]. Together, KV suppressed pro-apoptotic pathway and up-regulated pro-survival pathway in isolated rat heart of animals pre-treated with KV for 4 weeks. The antioxidant and free radical scavenging activity of KV might be responsible for the abrogation of death pathway and improvement in the survival pathway.

A great number of studies have suggested that MAPK signaling cascades were regarded as an important pathway in oxidative-stress-induced apoptotic cell death [38,39], and three MAPK subfamily members - ERK1/2 (beneficial), p38 MAPK and JNK (deleterious) - are activated following MI/R [40,41]. Furthermore, activation of ERK contributes to

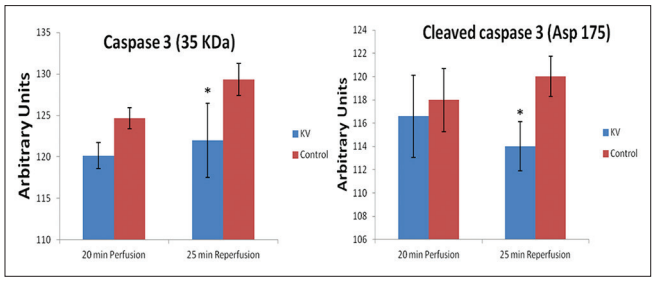


Figure 5: The effect Kolaviron on Caspase 3 and cleaved Caspase 3 at 20 min perfusion and 25 min reperfusion time points. Values are mean ± standard deviation, $n = 10$, $^*P < 0.05$ compared with control

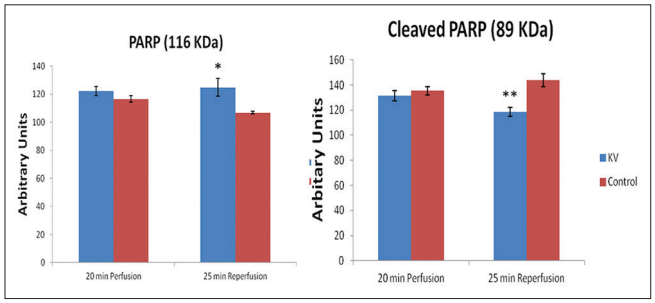


Figure 6: The effect of Kolaviron (KV) on apoptotic marker (cleaved Caspase 3) at 20 min perfusion and 25 min reperfusion time points. Values are mean ± standard deviation, $n = 10$, $^*P < 0.05$ compared with control. KV (200 mg/kg bodyweight), corn oil (2 mL/kg)

cell differentiation, proliferation, and survival; whereas JNK and p38 are activated by environmental stresses, promote apoptosis, and pro-inflammatory cytokines [42]. To clarify the possible singling pathway of KV on I/R-induced heart damage, we next tested the potential effects of KV on different pro-apoptotic cascade activation induced by I/R. The results indicated that activations of p38, Caspase 3, cleaved Caspase 3 and cleaved PARP protein expressions were observed in untreated rat hearts subjected to I/R.

In this study, we chose to study the impact of KV on p38 MAPK, another member of the MAPK family. However, pre-treatment with KV downregulated the expression of p38 MAPK in our model of MI/R. In the same vein, rats pre-treated with KV for 4 weeks significantly reduce Caspase 3, cleaved Caspase 3 and cleaved PARP protein expressions compared to the untreated group.

PI3K activates Akt by phosphorylation at Thr308, which is necessary for Akt activation, and by phosphorylation at Ser473, which is required for its maximal activity [43]. PI3K/Akt has been documented to be protective in heart I/R injury together with the inhibitory effect of KV on Sertoli cell line [44,45]. Furthermore, we, therefore, proposed that the cardioprotection produced by KV is mediated, at least partially, by PI3K/Akt activation and abrogation of apoptosis via inhibition of p38 MAPK and caspase expressions. In this study, compared with the control group, the KV group showed a significant increase in both total Akt and phosphorylated Akt, indicating its activation.

The generation of ROS from oxidative stress caused by reperfusion injury is known activated MAPKs signaling transduction with subsequent Caspase 3 and cleaved Caspase 3 activation [46]. MI/R injury has been reported to compromise myocardial function and contribute greatly to morbidity and mortality [32]. Necrosis and apoptosis are the two morphologically distinct pathways that contribute to MI/R injury. The induction of apoptosis has been shown to occur by at least two pathways, which are the extrinsic and intrinsic pathways [46]. The mitochondrion has been implicated as a major regulator of the intrinsic pathway [47]. The translocation of pro-apoptotic protein bax causes mitochondrial dysfunction and swelling and induces the efflux of cytochrome C to the cytosol. The release of cytochrome C to the cytosol has been documented to activate caspase apoptotic capacity [48].

Pre-treatment with KV inhibited the activation of p38 MAPK and promoted the activation of Akt in rats subjected to reperfusion injury. At the same time, cardioprotection by KV might be related to the inhibition of cardiomyocytes apoptosis signal via a reduction in the expressions of Caspase 3, cleaved Caspase 3 and cleaved PARP. Hence, KV offered cardioprotection via inhibition of pro-apoptotic pathway and up-regulation of survival pathway in isolated rat heart. Furthermore, the activation of the effector caspase, Caspase-3, is followed by the initiation of the multiple different stimuli that induce apoptosis. In addition, Caspase-3 is primarily responsible for the cleavage of PARP, a nuclear enzyme that is catalytically activated by DNA strand interruptions [46].

CONCLUSION

Taken together, KV abrogated pro-apoptotic pathways by inhibiting p38 MAPK, Caspase 3 and cleaved Caspase 3 and cleaved PARP. Pre-treated with KV significantly increased the expressions of survival proteins thereby attenuating apoptosis and improving recovery from reperfusion injury. Furthermore, the functional parameters also improved in isolated rat hearts of animals pre-treated with KV. Hence, the mechanism of cardioprotection of KV might be associated with antioxidant, anti-inflammatory and free radical scavenging activity of KV via up-regulation of survival pathway and abrogation death pathway. This study clearly demonstrates for the first time the cardioprotective effect of KV against reperfusion injury in isolated rat heart. These cardioprotective properties may be linked to the ability of KV to abrogate death pathway via phosphorylation of Akt and to scavenge ROS. From these results, we propose that KV could be a future therapeutic approach against myocardial dysfunction.

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Original Research

Screening for antibacterial and antibiofilm activities in *Astragalus angulosus*

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ABSTRACT

Aim: In a search for finding novel therapeutic agents, extracts from an endemic Lebanese plant, *Astragalus angulosus*, were evaluated for their potential *in-vitro* antibacterial and antibiofilm activities against three Gram-positive bacterial strains; *Staphylococcus epidermidis* (CIP444), *Staphylococcus aureus* (ATCC25923), and *Enterococcus faecalis* (ATCC29212); in addition to two Gram-negative strains, *Escherichia coli* (ATCC35218) and *Pseudomonas aeruginosa* (ATCC27853). **Materials and Methods:** The plant was collected in April of 2013 and divided into several different portions, then its extracts were obtained by maceration using two different solvents. Extract analysis followed directly where microtiter broth dilution method was employed to assess antibacterial activity, while antibiofilm potential was tested using colorimetric method. **Results:** Whole plant ethanolic extract showed the highest bacteriostatic effect at a concentration of 12.78 mg/ml and also was the most versatile exerting its effect against 3 different strains. Other extracts also exhibited an effect but at higher concentrations and each against a single strain. Regarding antibiofilm activity, the majority of the extracts were able to eradicate >50% of *S. epidermidis* preformed biofilm, where the highest activity was obtained with flower fraction extracted in water, achieving 67.7% biofilm eradication at 0.2 mg/ml. **Conclusions:** This plant possesses a promising potential in regard to eradicating bacteria and their biofilms and it is the first contributing step of establishing a library for the endemic Lebanese plants in this domain.

KEY WORDS: Antibacterial, antibiofilm, *Astragalus angulosus*, phytochemical screening, minimal inhibitory concentration, minimal bactericidal concentration

INTRODUCTION

Ever since ancient times, people relied on drugs from natural sources to cure their diseases [1], that is true since medicinal plants are considered to be the richest biological resource of drugs in traditional and modern medicine, food supplements, pharmaceuticals and intermediates for synthetic drugs, where it is estimated that 14-28% of higher plants are used medicinally [2].

The importance of plants as a source of medicine is depicted in a study by the World Health Organization, estimating that a substantial percentage of the population in developing countries relies on medicinal plants, where also people in developed countries are increasingly gaining interest in this domain [3].

Despite the fact that the pharmacological industries have produced a relatively limited number of new antibiotics in

the past few decades, microbial resistance to these drugs have increased [4]. This problem was further accentuated by the over prescription and misuse of traditional antibiotics [5]. Furthermore, modern synthetic medication poses a major risk to patients' health where such adverse drug reactions (ADRs) occur almost daily in medium sized hospitals and outpatient panels; this results in substantial morbidity and mortality in addition to the decreasing efficacy of these products [1,6]. In addition to the previous implications, drug resistance severely increases the cost of medical care where the estimated cost of treating a case of tuberculosis (including drugs, procedures, and hospitalization) increases from $12,000 for a drug-susceptible strain to $180,000 for a multidrug-resistant strain [7]. Therefore, the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs an encouraging need again [1].

Another concern of the work at hand focuses on bacterial biofilms which can be defined as a community of microorganisms attached to a surface and embedded in their self-produced matrix. Moreover, organisms in this state of existence will exhibit new phenotypic characteristics rather than that of the planktonic state [8]. More specifically in the current study, we will be dealing with *Staphylococcus epidermidis* biofilms, in which the strain used here (CIP444) was isolated from an infected implanted device [9]. We are interested specifically in the *S. epidermidis* biofilm since although it is usually described as a commensal species and a permanent colonizer of human skin, it is among the most common sources of infections on indwelling medical devices [10].

Biofilms can be very problematic in various aspects of our lives ranging from medical to industrial areas. In addition to their increased resistance to antimicrobial agents, biofilms can form on many medical implants such as catheters, artificial hips, and contact lenses. The most worrisome fact is that cells existing in a biofilm can become 10-1000 times more resistant to antimicrobial agents, mainly through the production of extracellular polymeric substance matrix that hinders the access of antibiotics to the bacterial cells. These infections can often only be treated by removal of the implant, thus increasing the trauma to the patient and the cost of the treatment. It has been estimated that biofilms are associated with 65% of nosocomial infections and that treatment of these biofilm-based infections costs >$1 billion annually [11].

Therefore, it is of utmost importance to seek a novel therapeutic agent that has a minimum impact on patient's health and is cost-effective. The concern to unravel better and safer ways to treat microbial infections and their consequences encouraged us to hunt for natural products that might provide such solution. To achieve our goal, we have relied on plant secondary metabolites such as phenols, saponins, flavonoids and many others that possess antimicrobial potential [2]. Our chosen plant in this work is a species endemic to Lebanon, *Astragalus angulosus*, whose extracts were used in antibacterial and antibiofilm testing in an aim to unravel a new therapeutic agent.

MATERIALS AND METHODS

Plant Material

A. angulosus was collected from Yammouneh in Bekaa (34.116046, 36.037830) during its flowering period in April (2013). It was identified in accordance with the two well-known guides of Lebanon's flora [12,13].

Preparation of the Extracts

The plant was rinsed and divided into several fractions; whole plant, flowers, leaves, green stems, and brown stems. The phytochemicals were extracted by maceration and performed over multiple stages where after finely chopping the plant portions, each fraction (50-100 g according to the abundance of each part) was fully submerged in a suitable volume of distilled water and ethanol (100%) in separate light blocking beakers and agitated at ambient temperature for 8-12 h then for the same duration at 37°C. They were then filtered and the process repeated for the obtained marks. Finally, the resulting fractions were concentrated using a rotary evaporator under reduced pressure at 60°C for aqueous and 40°C for ethanolic fractions, and finally, they were frozen at −80°C and freeze-dried using a lyophilizer (Christ Alpha 1-4 LDplus, Martin Christ, Germany) for 2-3 days at −20°C to obtain the powders to study. To assess the obtained powders, they were dissolved in sterile distilled water, and aliquots with a defined concentration were prepared for further analysis.

Phytochemical Screening

For the purpose of this test, aliquots from the ethanolic and water extract of each fraction of the plant were prepared and evaluated by phytochemical qualitative reactions for common plant secondary metabolites; these include tannins, resins, coumarins, saponins, alkaloids, phenols, terpenoids, volatile oils, and flavonoids [14-16]. The results were evaluated according to the response of the extract to these tests, mainly color change and/or precipitate formation.

Antibacterial Testing

Bacterial strains, media, and reagents

Five referenced strains belonging either to the American Type Culture Collection or to the Collection of "Institut Pasteur" were used in this study. Three Gram-positive strains, *S. epidermidis* (CIP444), *Staphylococcus aureus* (ATCC25923), and *Enterococcus faecalis* (ATCC29212); two Gram-negative strains, *Escherichia coli* (ATCC35218) and *Pseudomonas aeruginosa* (ATCC27853). CIP444 is a strain that was isolated from a patient with an infected implanted device in the Mignot hospital of Versailles, France [9].

This strain was identified and characterized for many features by Pr. Ali Chokr and deposited to be enclosed within the micro-organisms of the collection of "Institut Pasteur"

in 2007 [9,17-19]. All strains were stored at −80°C in glycerol stocks and used as required. Brain heart infusion broth/agar (BHI/BHA), tryptone soya broth (TSB) and Mueller-Hinton broth (MHB) were purchased from HIMEDIA (Mumbai, India) and then prepared as per indicated by the manufacturer.

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

MICs and MBCs were determined using the microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute [20]. The different bacterial strains were grown in BHI overnight and then bacterial suspensions of each were prepared using MHB at 5×10^5 CFU/ml (confirmed by viable count) to be used for inoculation. 100 μl of each extract was used to perform serial two-fold dilutions in MHB using a 96-well flat-bottom polystyrene tissue culture-treated microtiter plate (Corning®Costar® 3598; Corning, NY 14831, USA) then 100 μl of the previously prepared suspensions were inoculated into each well. Positive growth control lacking any plant extract and negative growth control lacking a bacterial inoculum were taken into account. The plates were then incubated at 37°C for 24 h, the MIC of each extract was the lowest concentration with no visible growth in its corresponding well. Then, all wells with no visible growth were plated on BHA to determine the MBC which is the lowest concentration able to reduce the initial bacterial inoculum by >99.9%. For comparison, these strains were tested using the same procedure against standard common antibiotics including tetracycline, nalidixic acid, polymyxin, rifampicin, and ampicillin (results not shown).

Antibiofilm Susceptibilty Testing against a Preformed Biofilm

Biofilm formation

Assay of biofilm formation in polystyrene plates was performed essentially according to a standard procedure [21], where *S. epidermidis* (CIP444) was grown in TSB medium overnight at 37°C, then a bacterial suspension of concentration 4.16×10^5 CFU/ml was prepared in TSB supplemented with 0.25% glucose. 120 μl of this bacterial suspension were inoculated into each well of a sterile 96-well flat bottom plate except for column 12 that was used as a control and filled only with sterile TSB medium; then, the plates were incubated for 24 h at 37°C. The biomass and any non-adherent bacteria were then discarded by gently washing the plates with 0.9% NaCl physiologic water and the remaining biofilm was fixed by incubating the plates for 50 min at 50°C [9].

Antibiofilm activity assay

After the fixation of the formed biofilm as previously described, each well of the microtiter plate was filled with 100 μl of sterile physiologic water for use as a diluent of our plant extracts. The serial 1:2 dilution was then performed with 100 μl of each plant extract, and the plates were then incubated at 37°C during 18 h. Tests were performed in quadruple. The wells were then washed 2 times with saline water, filled with 100 μl of crystal violet 0.1% and incubated at room temperature for 10 min. The stain is then discarded, and the wells are washed 3 times with saline water. Finally, they were filled with 100 μl of physiologic water and the OD490 nm is measured. One microtiter plate was skipped of treatment with the plant extracts and used as an untreated positive control.

Statistical Analysis

Antibiofilm tests were performed in quadruple, and the results were expressed as mean values ± standard errors of the means. Differences in the biofilm eradication potentials were evaluated by an unpaired non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's multiple comparisons test of each mean versus the mean of the untreated (positive) control. Approximate eradication efficiency (percentage) was calculated according to the following equation: (Average [control wells]−Average (treated wells)/Average (control wells)]×100. GraphPad Prism® Software (Version 6.05; GraphPad Software, Inc.) was used for statistical analysis, considering a $P < 0.05$ to be statistically significant.

RESULTS

Phytochemical Screening

Saponins, coumarins, and flavonoids were present in all fractions and portions of this plant as represented in Table 1. Other secondary metabolites were also present but in variable amounts with respect to the different fractions, and noticeably low abundance of volatile oils and complete absence of the tannins.

MIC and MBC Results

After testing the extracts according to the method specified earlier, the entire raw findings are displayed in Table 2. For some extracts, no MIC and/or MBC was obtained at the used concentration and hence the symbol (>) was used to indicate that a higher concentration might be needed to achieve an effect.

Among the 10 extracts, only 5 exhibited a bacteriostatic effect. The most significant being whole plant ethanolic fraction which exerted its effects on three bacterial strains (*S. epidermidis*, *E. coli*, and *P. aeruginosa*), with the lowest being 12.78 mg/ml for *S. epidermidis* as shown in Figure 1 and it is also notable that *P. aeruginosa* was the most sensitive organism where 3 fractions were able to suppress its growth (brown stem ethanolic, aqueous green stem and whole plant ethanolic fractions) with the lowest being that of whole plant ethanolic at 102.2 mg/ml. The other fractions' MICs ranged between 188.7 mg/ml for leaves ethanolic on *S. epidermidis* to 546 mg/ml of flowers ethanolic fraction on *S. aureus*.

Regarding the MBC, only the whole plant ethanolic fraction was active among the 10 extracts as seen in Figure 2, and against 3 of

Table 1: Results of phytochemical screening of *A. angulosus*

Plant part	Extraction solvent	Alkaloids	Tannins	Resins	Phenol	Saponins	Flavonoids	Terpenoids	Coumarins	Volatile oil
Flower	Ethanol	+	−	−	++	+	++	−	+	−
	Water	+	−	+	+++	+++	++	++	+	−
Green stem	Ethanol	++	−	+	++	+	++	++	+	−
	Water	−	−	++	+	−	−	−	++	−
Brown stem	Ethanol	−	−	++	−	+	+	+++	+	−
	Water	+	−	−	+	+++	+	−	+	−
Leaves	Ethanol	−	−	++	-	+	+	+	++	++
	Water	+++	−	+	+	++	+++	−	++	−
Whole plant	Ethanol	+	−	−	+	+	+	−	+	+
	Water	+	−	++	−	+++	+	−	+	−

−: Absence, +: Small amount, ++: Average amount, +++: Large amount

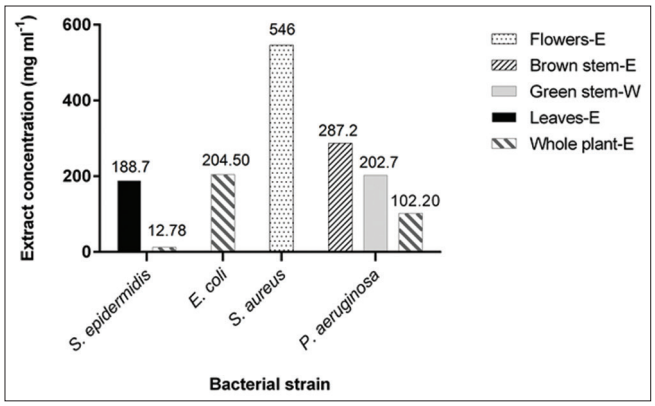

Figure 1: Significant minimal inhibitory concentration results of the tested fractions of *Astragalus angulosus* (E: Ethanol, W: Water)

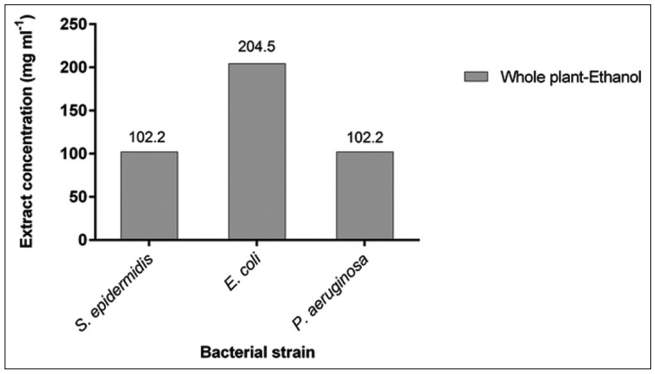

Figure 2: Minimal bactericidal concentration results of the active bactericidal extract of *Astragalus angulosus*

the tested strains, ranging from 102.2 mg/ml for *S. epidermidis* to 204.5 mg/ml for *E. coli* and *P. aeruginosa*.

Antibiofilm Activity Results

As demonstrated in Figures 3 and 4, in general, all our extracts were able to eradicate the *S. epidermidis* preformed biofilm but with variable patterns. For example, flower, brown stem and whole plant (water and ethanol fraction) also green stem and leaves (water fraction) extracts performed better under lower concentrations, and this was confirmed also by the statistical analyses performed. While green stem and leaves (ethanolic fraction) extracts had better eradication capacities at higher concentrations.

As shown in Figure 3, the highest overall significance belongs to the flower water extract which also achieved the highest eradication activity at a very low concentration (~67.7% at 0.2 mg/ml, $P < 0.0001$). Other extracts followed the same pattern such as flower ethanol (~62.5% at 0.4 mg/ml, $P < 0.01$), brown stem, and whole plant (both fractions) in addition to green stem and leaves (water fraction) but with a lesser impact and significance. By contrast, we noticed that green stem (ethanolic fraction) exerted best eradication efficiency at higher concentrations (~56.5% at 701.3 mg/ml, $P < 0.001$) and the same goes for leaves ethanolic fraction, but the overall test was insignificant according to the Kruskal-Wallis evaluation regarding the latter.

DISCUSSION

Increasing drug resistance to traditional treatments observed in several bacterial strains, in addition to harmful ADRs and substantial cost of treatment shifted the pace toward finding novel therapeutic agents, natural products such as plants and plant-derived compounds were the primary target due to their efficacy, safety, and lower cost.

Native to China, Astragalus has been used for centuries in traditional Chinese medicine. There are actually over 2,000 species of Astragalus; however, the two related species - Astragalus membranaceus and Astragalus mongholicus - are the ones primarily used for health purposes [22].

Historically, Astragalus has been used in traditional medicine. Usually in combination with other herbs, as antioxidant, to support and enhance the immune system, for chronic hepatitis, other viral infections and as an adjunctive therapy for cancer. It is also used as a folk or traditional remedy for colds and upper respiratory infections, and for heart disease [23-26].

The biological activities of the Lebanese plants are not well explored. Only a few studies on some biological activities of certain plant genera such as *Allium and Berberis* are available [27-29].

To the best of our knowledge, the activities of the endemic Lebanese plants are not yet studied.

In this study, we chose a Lebanese endemic species of *Astragalus* which is *A. angulosus* in an effort to find novel agents that

Table 2: MIC and MBC results of *A. angulosus* against 5 bacterial strains

Plant part	Extraction solvent	MIC (mg/ml)					MBC (mg/ml)				
		S. aureus	*E. faecalis*	*S. epidermidis*	*E. coli*	*P. aeruginosa*	*S. aureus*	*E. faecalis*	*S. epidermidis*	*E. coli*	*P. aeruginosa*
Flowers	W	>121	>121	>121	>121	>121	>121	>121	>121	>121	>121
	E	546	>546	>546	>546	>546	>546	>546	>546	>546	>546
Brown stem	W	>74.2	>74.2	>74.2	>74.2	>74.2	>74.2	>74.2	>74.2	>74.2	>74.2
	E	>287.2	>287.2	>287.2	>287.2	287.2	>287.2	>287.2	>287.2	>287.2	>287.2
Green stem	W	>202.7	>202.7	>202.7	>202.7	202.7	>202.7	>202.7	>202.7	>202.7	>202.7
	E	>350.6	>350.6	>350.6	>350.6	>350.6	>350.6	>350.6	>350.6	>350.6	>350.6
Leaves	W	>117	>117	>117	>117	>117	>117	>117	>117	>117	>117
	E	>188.7	>188.7	188.7	>188.7	>188.7	>188.7	>188.7	>188.7	>188.7	>188.7
Whole plant	W	>42.9	>42.9	>42.9	>42.9	>42.9	>42.9	>42.9	>42.9	>42.9	>42.9
	E	>204.5	>204.5	12.78	204.5	102.2	>204.5	>204.5	102.2	204.5	102.2

E: Ethanol, W: Water, MIC: Minimal inhibitory concentration, MBC: Minimal bactericidal concentration, *S. aureus*: *Staphylococcus aureus, E. faecalis*: *Enterococcus faecalis, S. epidermidis*: *Staphylococcus epidermidis, E. coli*: *Escherichia coli, P. aeruginosa*: *Pseudomonas aeruginosa*

could aid in fighting bacterial infections and their biofilms. In the present work, we assessed and tested for the first time the antibacterial and antibiofilm activities of water and ethanolic extracts from the flower, leaves, brown stem, green stem, and whole plant portions of *A. angulosus* against five bacterial strains.

MIC and MBC values indicate that not all extracts exhibited an activity. Regarding the MIC assay, only 5 out of 10 were able to inhibit bacterial growth, among which the whole plant ethanolic fraction was most significant where it achieved the lowest MIC and had an effect against multiple strains. We also noticed that one of our tested strains, *E. faecalis* was insensitive to all treatments used, that is, independently of the strain being a Gram-positive one but rather probably related to the strain itself or the nature of our extracts and their targets. It is worthy to note that the most successful extracts contained fair amounts of several secondary metabolites mainly flavonoids, coumarins, and volatile oils. Furthermore, it was evident that most of the active extracts were ethanolic fraction extracts, which are characterized by containing flavonoids and phenols that are known antimicrobial agents [30,31].

It is not surprising that bactericidal activity was also detected with our leading extract which is the whole plant ethanolic fraction, which was the only extract with bactericidal activity and that was against 3 strains as well. This could be due to a potential synergistic effect between the different phytochemical constituents found in the whole plant. The synergism effect in plant extracts was also highlighted in literature as in the study of Ncube *et al*., in 2012, on the antimicrobial synergism within plant extract combinations from three South African medicinal bulbs [32].

The other problem at hand is the infections caused by biofilm-related formation on indwelling medical devices, where the introduction of synthetic and artificial devices into body systems have provided the microorganisms with a way for evading host defenses and invading the host [33,34], where *S. epidermidis* is a major cause of these infections. This issue is not to be taken lightly where research has shown that an important cause of nosocomial infections is the catheter-associated bloodstream infections, with an estimated occurrence of 50,000-100,000 cases a year in the United States with the skin being the most common source of organisms causing catheter-related infections [35].

Our tests evaluated the ability of the extracts to disrupt and eradicate preformed biofilms, and the results were quite promising. We noticed a significant effect of some extracts, especially the flower water fraction in biofilm eradication and at low concentrations. Therefore, exposure to subinhibitory concentrations of some of our extracts could substantially affect the preformed *S. epidermidis* biofilms. We also observed that while some extracts showed better performance at higher concentrations, others were to some extent active irrespective of the used concentration.

By contrast to the antibacterial activity tests, the most successful extracts in the antibiofilm activity assays where

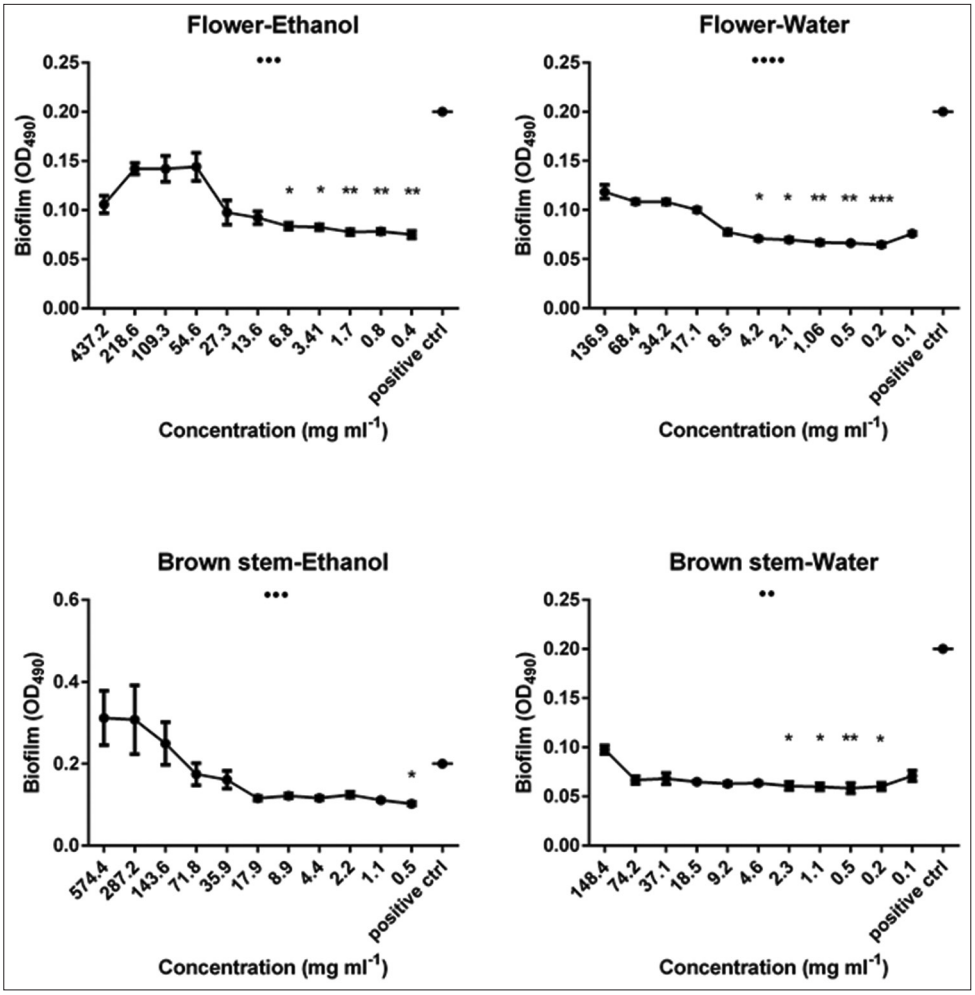

Figure 3: Biofilm eradication potential of 4 *Astragalus angulosus* extracts against a preformed *Staphylococcus epidermidis* biofilm assessed by spectrophotometric method. Results are displayed as mean biofilms plus standard error of the mean. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 (analysis of variance by Kruskal-Wallis test). *P<0.05, **P<0.01, ***P<0.001 (Dunn's post-test comparing the mean biofilm for each category (extract concentration) with that of the untreated control, OD490: Optical density at 490 nm

the water extracts that contained higher saponin content, which prompt us to hypothesize that it may have been acting as a kind of detergent against the preformed biofilms without excluding the effects of the other metabolites detected here such as alkaloids, resins, and phenols which are slightly more frequent in water extracts than in ethanolic ones. This is corroborated by the fact that our test involved preformed biofilm and as such the mode of action of the extracts in this test would be different, and that is, due to the absence of any bacteria in our biofilms where we were trying to combat the structure of the biofilm left by the bacteria and not the bacteria themselves.

This is supported by a study of Ye *et al*., in 2015, on the plant *Camellia oleifera*. They found that the extracts of this plant were rich in saponin identified as camelliagenin which shows significant inhibition on the biofilm of *E. coli* and *S. aureus* and it is related to the decrease of a component of the bacterial biofilm, the extracellular DNA. On the other hand, in 2015, Santiago *et al*. showed that *Acalypha wilkesiana* fraction extracts rich in saponins prevent the biofilm formation by methicillin-resistant *S. aureus* (MRSA). Bioactive fraction rich in saponins inhibited the production of MRSA biofilm by preventing the initial cell-surface attachment [36,37].

After observing the "disjunction" between the active extracts in antibacterial tests and antibiofilm tests, it was clear that some extracts could decrease the viability of preformed biofilms through mechanism(s) that are different from mere growth inhibition of bacterial cells in the planktonic form. Therefore, in our view, there are different compounds responsible for the results seen in our performed tests and further analysis should be done to try to pinpoint the exact effectors.

In end, we can say that the results obtained in this study, taken together, provide promising evidence on the antibacterial and antibiofilm potential and effectiveness of the Lebanese endemic plant A. *angulosus*. Further biochemical testing and the separation of the secondary metabolites by chromatography methods are required to identify the exact compounds responsible for the observed effects. The ability of the extracts to prevent biofilm formation rather than just its eradication,

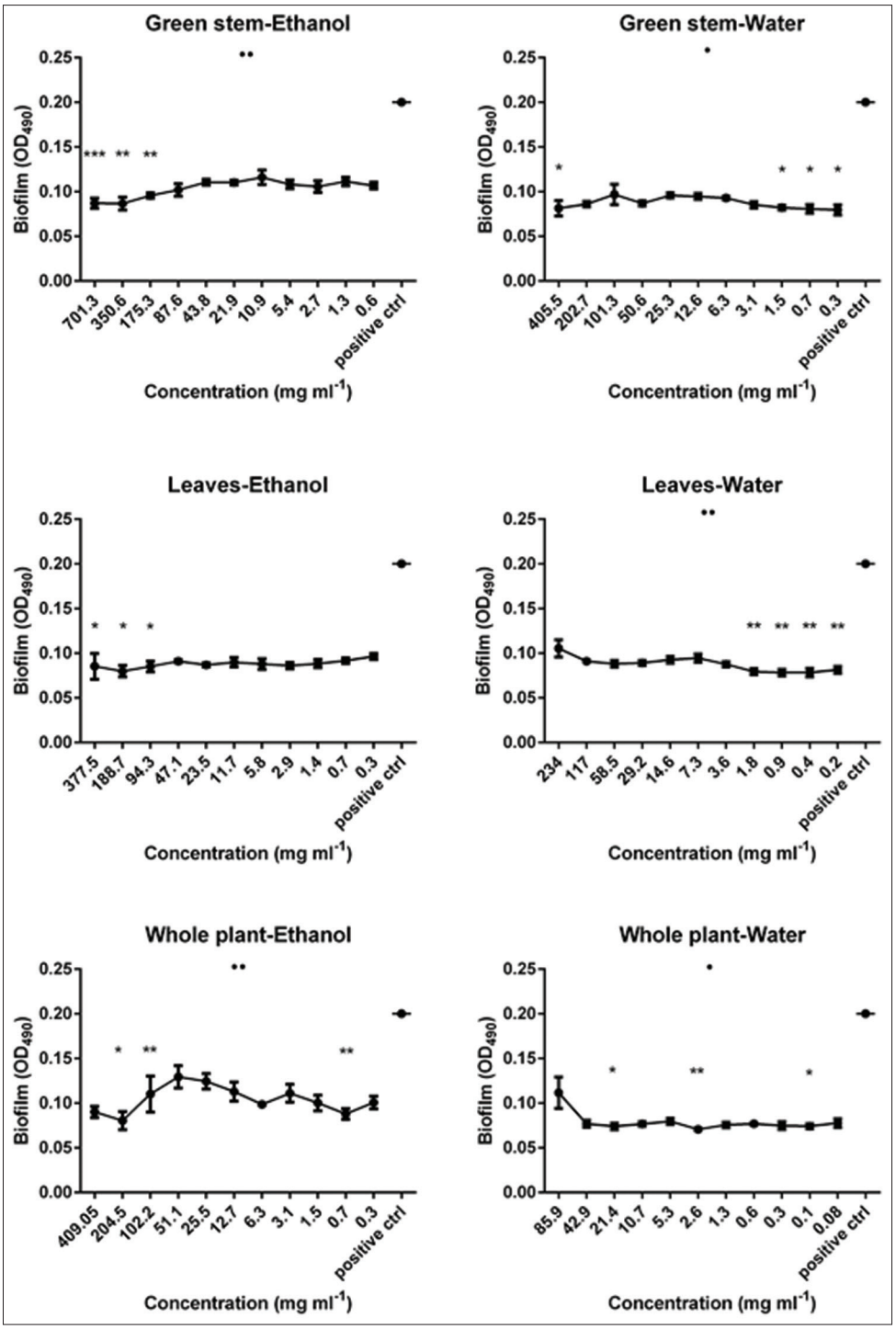

Figure 4: Biofilm eradication potential of 4 *Astragalus angulosus* extracts against a preformed *Staphylococcus epidermidis* biofilm assessed by spectrophotometric method. Results are displayed as mean biofilms plus standard error of the mean. *P<0.05, **P<0.01, ***P<0.001, ***P<0.0001 (analysis of variance by Kruskal-Wallis test). *P<0.05, **P<0.01, ***P<0.001 (Dunn's post-test comparing the mean biofilm for each category (extract concentration) with that of the untreated control, OD490: Optical density at 490 nm

then antioxidant, anticoagulant, and other biological activities, in addition to their toxicity on human cells remain to be further elucidated.

Our results showed that at relatively low concentrations some of our extracts displayed promising antibacterial and antibiofilm capabilities making them attractive for additional studies where we can hopefully label them as "novel therapeutic agents."

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Original Research

The adjuvant use of calcium fructoborate and borax with etanercept in patients with rheumatoid arthritis: Pilot study

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ABSTRACT

Objective: This study was designed to evaluate the effects calcium fructoborate (CFB) and sodium tetraborate (NTB) as supplements in Iraqi patients with active rheumatoid arthritis (RA) maintained on etanercept. **Materials and Methods:** A double-blind randomized placebo-controlled clinical trial with 60 days treatment period was carried out at Baghdad Teaching Hospital, Medical city, Baghdad, Iraq. Eighty RA patients were randomized into three groups to receive either 220 mg/day CFB, 55 mg/day NTB in capsule dosage form (equivalent to 6 mg elemental Boron), or placebo formula once daily. Only 72 patients completed the study. All patients were clinically evaluated utilizing DAS28-erythrocyte sedimentation rate (ESR), simple disease activity index-C-reactive protein (CRP), and clinical disease activity index scores at baseline, and at the end of the study. Venous blood was obtained at baseline and after 60 days, and utilized for the measurement of ESR, hemoglobin, in addition to evaluation of high-sensitivity CRP (hsCRP), tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α) and IL-6. **Results:** After 60 days, both types of boron significantly improve the clinical scores, in association with significant decrease in the serum levels of ESR, hsCRP, IL-1α, IL-6, and TNF-α with remarkable superiority for calcium fructoborate (CFB) over sodium tetraborate (NTB), compared to baseline and placebo-treated group. **Conclusion:** The use of boron, as adjuvant with etanercept, has potentiated therapeutic outcomes in RA patients, and may be a new strategy to improve treatment, and avoid the problems associated with biologics utilized in RA treatment.

KEY WORDS: Calcium fructoborate, etanercept, rheumatoid arthritis, sodium tetraborate

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disorder attributed to exaggerated and excessive implementation of inflammatory responses that finally predispose to synovial inflammation and destruction of joint tissues [1]. In medical practice, many therapeutic approaches are implemented and currently approved for clinical use in the treatment of RA, including disease-modifying drugs like methotrexate and biological agents like etanercept. However, the broad profile of adverse reactions and high cost burden limit the scope of effective and successful therapeutic use, especially in low-income communities [2,3]. Many consequences of inflammatory reactions, including oxidative stress and accelerated production of reactive oxygen species (ROS), can amplify the inflammatory response with consequent impact of increasing tissue damage and limited treatment outcomes [4,5]. Accordingly, the use of supplements from natural sources to attenuate the inflammation-induced oxidative damage, such as phytochemicals and trace elements, may provide further therapeutic benefits to the currently used antirheumatic agents [6,7]. Moreover, many of these natural supplements have the ability to potentially interfere with the inflammatory cascades, probably through attenuating the release of pro-inflammatory markers such as tumor necrosis factor-α (TNF-α) and other cytokines, which are clearly in many human and experimental animal studies [8,9]. The trace element boron is a necessary micronutrient for the proliferation of many biological systems [10]. Declaring various biological activities of boron as a regulator of many enzyme systems, membrane transporters, and biochemical processes represents the basis for developing new drugs and nutraceuticals that contain boron both as mineral and organic complex forms [11,12]. However, whether these supplements have clinical applications still represent a scientific dilemma in the practice of alternative therapy approach [13,14]. Current evidence indicated that boron concentration was significantly lower in the serum of RA patients and negatively associated with rheumatoid factor in those patients [15]. Accordingly, boron may have a relevant clinical role in the pathogenesis of RA, and suggest the importance of boron supplementation to

RA patients or to individuals who are at high-risk of developing RA [16]. This study was designed to evaluate the clinical benefits of CFB and borax, when used in pharmacological doses, as adjuvant with etanercept in the treatment of RA patients.

MATERIALS AND METHODS

A double-blind, randomized placebo-controlled clinical study was conducted with 8-week treatment period over 8 months (from December 2015 to August 2016) at the Rheumatology Unit, Baghdad Teaching Hospital, Baghdad. Of the 111 patients screened for eligibility, 80 patients with active RA maintained on etanercept were randomly selected and evaluated to participate. Only 72 patients completed the study [Figure 1]. The patients were randomly allocated to receive either CFB (220 mg/day; Futureceuticals, Momence, IL, USA), NTB (55 mg/day; Merck, Germany) specially prepared as capsule dosage (contain 6 mg elemental boron) as single dose and administered once daily after a meal, or a capsule formula filled with starch as a placebo (once daily after a meal). The boron-containing formulations were administered as an adjuvant with the regularly used etanercept regimen (50 mg/week; Amgen Inc., Thousand Oaks, CA). The patients were instructed to continue their regular drug treatment schedule and were clinically observed every four weeks for any unusual adverse effects. All participants provide signed informed consent form according to the principles of the Declaration of Helsinki. The local scientific ethics committee of Baghdad University, College of Pharmacy and Baghdad Teaching Hospital, Rheumatology Department approved the study protocol. All patients included had active RA, as defined by the American College of Rheumatology (ACR) 1987 revised criteria [17]. Active RA was proven by calculating either 28-joint Disease Activity Score (DAS28) or the simple disease activity index (SDAI). All included patients were maintained on etanercept treatment for at least three consecutive months before the time of inclusion. At screening time, patients with the following health disorders were excluded: Patients using nonsteroidal anti-inflammatory drugs 2 days before inclusion, hypersensitivity or severe adverse effects to boron containing formulas, renal or hepatic damage, pregnant and breastfeeding women, juvenile RA, patients using disease-modifying antirheumatic drugs other than etanercept or high dose steroids, missing medication for two consecutive days, coexistence of other connective tissue disorders, and mild or inactive RA. The clinical outcome of the treatment was evaluated using the DAS28 [18], SDAI [19], and the clinical disease activity index (CDAI) [20] at the start (baseline) and end of the 8-week study period. Blood samples were obtained from each patient by vein puncture at baseline and the end of the study. Of the blood collected, 3 ml was kept in an ethylenediaminetetraacetic acid tube to be used for measurement of erythrocyte sedimentation rate (ESR) and hemoglobin utilizing standard procedures. The remaining blood was kept in plain tube and left to coagulate at room temperature for at least 30 min, and then, centrifuged for 10 min at 4000 rpm to obtain serum. Using ready-made enzyme-linked immunosorbent assay kits, the resultant serum was utilized for the measurement of high-sensitivity C-reactive protein (hsCRP), TNF-α, interleukin (IL)-1α and IL-6 (Demeditec, Germany). All data were statistically analyzed using Graph Pad Prism 5.1 software (Graph Pad Software Inc., California, US). Continuous variables were presented as mean ± standard deviation and discrete variables presented as numbers and frequencies. The Chi-square and Wilcoxon-rank tests were used for independence to test the significance of the association between discrete variables. The paired *t*-test was used to evaluate the difference between pre- and post-treatment values. Moreover, one-way analysis of variance was used to test the significance of the difference in means of independent samples, and supported by Bonferroni's *post-hoc* analysis. A $P < 0.050$ was considered significantly different.

RESULTS

Table 1 indicates that the demographic data and baseline characteristics were not significantly different among the three groups of patients included in the study. Table 2 shows a

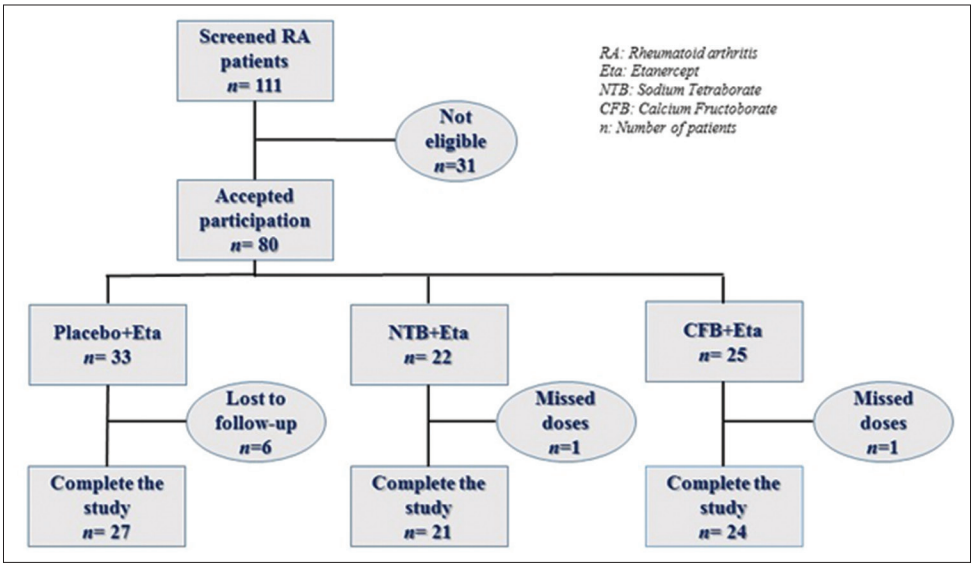

Figure 1: Flowchart of patient allocation and study follow-up

nonsignificant difference in tender joint counts (TJC) at the pretreatment level between NTB, CFB and placebo groups. At the end of the study, there was a significant decrease in TJC of NTB and CFB groups (37.14% and 33.96%) compared to placebo (12.3%). Meanwhile, there was a significant decrease in swollen joint counts (SJC) of CFB (40.54%) and NTB (30.23%) groups compared to baseline and placebo group (10.64%). Both CFB and NTB groups showed significant decrease in visual analog scale (VAS) (33.33% and 23.18%) compared to baseline and placebo group (10.45%). Moreover, both CFB and NTB groups showed significant decrease (35.38% and 23.8%, respectively; $P < 0.05$) in EGA compared to baseline and the placebo group; however, they are not significantly differ when compared with each other. Table 3 shows that all types of treatment significantly decreased the DAS28 score compared with baseline values. However, in CFB group 21.2% decrease in DAS28 score was achieved and represent a significant improvement compared to placebo group. Meanwhile, NTB group showed only 15.14% decrease in DAS28-ESR, which was also significantly different compared to placebo group. Table 3 also shows that CDAI score was not significantly changed in control group after 60 days of treatment. Meanwhile, both CFB and NTB groups showed a significant decrease in CDAI (32.75% and 25.35%) compared to both pretreatment value and placebo group, and CFB seems to be more effective in this regard although not significantly differ. Regarding the effect on SDAI score, the results of this study demonstrate no significant difference among three groups at baseline level, while at the end of treatment all types of treatment significantly decreased, the SDAI score compared with baseline values. The use of NTB produced 29.4% decrease in SDAI score, while using CFB resulted in 28.8% decrease in SDAI, and both represent a significant improvement in SDAI compared to pretreatment level and posttreatment value of the placebo group (10.8%) [Table 3]. Table also demonstrates that all types of treatments produced significant improvement in the duration of morning stiffness after 60 days compared with baseline values. The two forms of boron supplements (NTB and CFB) produced 39% and 61.3% decrease in the duration of morning stiffness, while the placebo formula resulted in 28.5% in this regard only. However, these effects are found nonsignificantly different when compared with each other. Regarding, the effect on the ESR, the results indicated that the placebo formula did not significantly change ESR compared with baseline value (11.8%). Meanwhile, both types of boron supplements (NTB and CFB) significantly decreased ESR value (72.8% and 32.5%, respectively) compared with baseline. However, when the three treatment approaches were compared among each other, the data reflects no significant differences in this regard [Table 4]. Table 4 revealed that the placebo formula produced nonsignificant decrease in hsCRP levels after 60 days (10.1%) compared with baseline values. Meanwhile, both NTB and CFB significantly decreased hsCRP levels compared with baseline values (47.1% and 47.8%, respectively). However, when posttreatment values of hsCRP were compared among each other, nonsignificant differences were reported in this respect. The data presented in Table 4 showed that treatment with placebo formula did not change TNF-α level significantly (0.6%) compared with the baseline values. Meanwhile, both boron supplements (NTB and CFB) significantly decreased serum TNF-α levels (20.3% and 35.0%, respectively) compared to baseline values. In addition, the effect of CFB was significantly greater than that produced by NTB in this regard. In this study, serum IL-1α levels were decreased significantly in all treated groups compared with baseline values, where placebo formula resulted in 15.7% decrease in these levels, while NTB and CFB produced comparable and nonsignificant decrease in this regard (25.0% and 37.1%, respectively); however, both of them are

Table 1: Demographic data and baseline characteristics of the RA patients

Parameters	Placebo group *n*=27	NTB group *n*=21	CFB group *n*=24	*P* value
Gender				
Male *n* (%)	2 (7)	3 (14.3)	1 (4.2)	0.36
Female *n* (%)	25 (93)	18 (85.7)	23 (95.8)	0.37
Age (years)	51.9±9.3	49.4±11.2	47.4±9.4	0.62
Body weight (kg)	84.6±18.6	89.2±14.2	77.6±12.1	0.10
BMI (kg/m^2)	33.6±6.7	35.5±6.3	31.2±4.4	0.12
Disease duration (month)	10.8±7.7	10.9±8.6	9.5±5.5	0.13
Etanercept treatment (months)	12.6±11.8	16.9±14.7	11.3±12.3	0.56
ESR (mm/h)	36.8±19.8	35.6±20.8	48.5±28.7	0.12
hsCRP (μg/ml)	6.9±5.2	8.7±5.9	9.2±7.2	0.38
DAS28 score (4 values)	5.7±0.60	5.6±0.84	5.9±0.61	0.48
SDAI score	29.01±5.8	28.9±8.4	28.7±5.7	0.98
Joint deformities *n* (%)	4 (14.8)	7 (33.3)	8 (33.3)	0.40
Associated diseases				
Hypertension *n* (%)	11 (40.7)	7 (33.3)	11 (45.8)	0.39
Diabetes mellitus *n* (%)	7 (26)	5 (23.8)	6 (25)	0.40
Smoking habits *n* (%)	1 (3.7)	1 (4.7)	1 (4.2)	0.34

Values are presented as mean±SD, *n*: Number of patients, NTB: Sodium tetraborate, CFB: Calcium fructoborate, SD: Standard deviation, SDAI: Simplified disease activity index, NTB: Sodium tetraborate, CFB: Calcium fructoborate, ESR: Erythrocyte sedimentation rate, hsCRP: High-sensitivity C-reactive protein

Table 2: Effect of treatment with NTB and CFB on different functional areas of DAS28 score of patients with active RA maintained on etanercept compared with placebo

Clinical score	Placebo (*n*=27)		NTB (*n*=21)		CFB (*n*=24)	
	Baseline	After 60 days	Baseline	After 60 days	Baseline	After 60 days
TJC	10.6±4.1	9.3±5.3[a]	10.5±4.7	6.6±5.4[a]*	10.6±3.8	7.0±3.3[a]*
SJC	4.7±2.5	4.2±1.6[a]	4.3±2.6	3.0±1.8[b]*	3.7±1.8	2.2±1.9[b]*
VAS (cm)	6.7±1.3	6.0±1.5[a]	6.9±1.7	5.3±1.7[a]*	7.2±1.3	4.8±1.0[b]*
EGA (cm)	6.3±0.8	5.6±1.1[a]*	6.3±1.3	4.8±1.5[b]*	6.5±1.0	4.2±1.0[b]*

Values are presented as mean±SD, *n*: Number of patients, *significantly different compared to pretreatment ($P<0.05$), posttreatment values with different superscripts [(a,b)]within each parameter are significantly different ($P<0.05$). SD: Standard deviation, NTB: Sodium tetraborate, CFB: Calcium fructoborate, TJC: Tender joint counts, SJC: Swollen joint counts, VAS: Visual analog scale

significantly greater than that produced by the placebo formula in this regard [Table 4]. Regarding, the effect on serum IL-6 levels, Table 4 revealed that the placebo formula produced nonsignificant decrease in IL-6 levels (3.7%) compared with baseline values. Meanwhile, both NTB and CFB significantly decreased IL-6 levels compared with baseline values (24.9% and 42.8%, respectively). However, when posttreatment values of IL-6 were compared among all groups, nonsignificant differences were reported in this respect [Table 4].

DISCUSSION

According to the currently available evidence related to the beneficial effects of supplementary boron in RA, the presented study was designed to evaluate the possibility of utilizing this concept in the clinical practice. Because, it is ethically unaccepted to use boron alone as a separate arm in such type of clinical trials, the principle of its adjuvant use with the biological agent etanercept was followed. Although baseline laboratory evaluations that include many biochemical markers are important for diagnosis and management of RA, clinical assessment with scored, standardized and reproducible tools are necessary both for scoring disease activity and treatment follow-up [21]. Accordingly, we utilized more than one type of internationally accepted disease activity indices to overcome the limitations that may be associated with any one of them. Assessment of tender and swelling joints is considered as one of the important parameters during evaluation and treatment decision making in RA [22]. In this study, both forms of boron produced significant decrease in TJC and SJC compared to baseline values; although the changes were remarkably greater than that reported in placebo group, they were not significantly different. This may be attributed to sample size limitation. These results were in tune with those reported previously regarding the use of boron as adjuvant in patients with knee OA, where inclusion of boron in the currently used treatment modulates the symptoms of arthritis and joint degeneration and improves the clinical scores [23,24]. The anti-inflammatory activity of boron was reported in animal models of inflammation [8,9]; however, no previous data declared its role as adjuvant with etanercept to improve the clinical outcome of RA treatment. The reported improvement may be due to long-term inhibition of the pro-inflammatory mediators, which may indirectly lead to reduction in the followed clinical scores. According to many epidemiological data, it is well-known that in countries with low quantities of boron in the soil there is much more arthritis, while the incidence decreases with the increase in soil boron content [25,26]. In addition, Al-Rawi *et al.* reported the correlation between disease activity and serum boron levels in Iraqi patients with active RA [15]. This may explain the achieved improvement in response to RA treatment by the addition of supplemental boron. Unfortunately, we failed to measure serum boron levels to add more support to the current idea, probably due to technical and financial limitations. In this study, although the reported improvement in pain severity of RA is well recognized with both forms of boron, it may be comparable with that reported in an open-label pilot clinical trial, where CFB improves mild and moderate pain in patients with osteoarthritis [27]. Many studies have addressed the benefits of boron as a therapeutic option in arthritic pain, which may be attributed to various effects including inhibition of the oxidative burst associated with the inflammatory reactions, improvement of the antioxidant defense systems and inhibition of the collagenase activity [8,16,28,27]. In this study, we rely on the outcome of two important clinical scoring systems for assessment of disease activity in RA, SDAI, and DAS28.

Table 3: Effect of treatment with NTB and CFB on different clinical scores of patients with active RA maintained on etanercept compared with placebo

Clinical Score	Placebo (n=27)		NTB (n=21)		CFB (n=24)	
	Baseline	After 60 days	Baseline	After 60 days	Baseline	after 60 days
DAS28-ESR	5.68±0.6	5.36±0.7[a]*	5.68±0.8	4.82±0.9[b]*	5.84±0.6	4.60±0.5[b]*
CDAI	28.3±5.7	26.9±6.0[a]	28.5±7.7	21.3±7.6[b]*	27.9±5.7	18.8±4.9[b]*
SDAI	29.0±5.7	25.9±7.5[a]*	28.9±8.4	20.5±9.1[b]*	28.7±5.7	20.4±5.6[b]*
MS time (min)	43.3±37.3	31.0±36.1[a]*	31.4±29	19.1±22[a]*	35.8±37.2	13.9±13.1[a]*

Values are presented as mean±SD, n: Number of patients, *significantly different compared to pretreatment ($P<0.05$), posttreatment values with different superscripts [(a,b)]within each parameter are significantly different ($P<0.05$). DAS: Disease activity score, CDAI: Clinical disease activity index, SDAI: Simplified disease activity index, MS: Morning stiffness, SD: Standard deviation, NTB: Sodium tetraborate, CFB: Calcium fructoborate, ESR: Erythrocyte sedimentation rate

Table 4: Effect of treatment with NTB and CFB on inflammatory markers of patients with active RA maintained on etanercept compared with placebo

Markers	Placebo (n=27)		NTB (n=21)		CFB (n=24)	
	Baseline	After 60 days	Baseline	After 60 days	Baseline	After 60 days
ESR (mm/h)	27.2±6.1	24.0±7.8[a]	28.3±8.3	20.4±9.2[a]*	27.8±5.6	18.8±4.8[a]*
hsCRP (μg/ml)	6.9±5.2	6.2±3.1[a]	8.7±5.9	4.6±2.8[a]*	9.2±7.2	4.8±4.3[a]*
TNF-α (pg/ml)	195.9±60	194.7±77[a]	177.1±44.4	141.6±38.6[b]*	152.4±20.6	99.0±38.2[c]*
IL-1α (pg/ml)	12.4±3.4	10.4±1.8[a]*	10.4±3.2	7.8±2.5[b]*	11.6±2.5	7.3±1.9[b]*
IL-6 (pg/ml)	13.6±2.8	13.1±3.1[a]	17.3±8.0	13.0±4.8[a]*	18.7±10.9	10.7±4.7[a]*

Values are presented as mean±SD, n: Number of patients, *significantly different compared to pretreatment ($P<0.05$), posttreatment values with different superscripts [(a,b,c)]within each parameter are significantly different ($P<0.05$). SD: Standard deviation, NTB: Sodium tetraborate, CFB: Calcium fructoborate, IL: Interleukin, TNF: Tumor necrosis factor, ESR: Erythrocyte sedimentation rate, hsCRP: High-sensitivity C-reactive protein

They are commonly linked with the assessment of CRP and ESR. These two acute phase reactants (ESR and CRP) provide reliable tools to discriminate between drugs that produce symptomatic relief only and those with more profound effects in RA. This study shows that both types of boron significantly decreased serum CRP levels compared with baseline values. This finding was consistent with that reported in patients with primary OA [29]. The ACR recommended the use of disease activity indices that include multiple variables, such as DAS28 or SDAI for accurate measurement of RA severity [30]. We showed, for the first time, that both forms of boron significantly improved the DAS28 scores and their effects are comparable. This result can be explained on the bases that DAS28 depends on different factors, including TJC, SJC, VAS, and ESR; so the effect of boron will be the result of the effects of all the above factors, which are highly modified as shown in boron administered groups. These effects can be attributed to the influence of boron on multiple sites within the inflammatory cascades beyond the types of the inflammatory initiator or grade of the inflammatory response [16]. Moreover, this study shows that both types of boron supplements significantly decreased SDAI score compared with baseline values and placebo. This was the first trial that evaluates the effect of boron supplements, when used as adjuvant with etanercept against placebo on SDAI score of RA patients. The results can be explained on the bases that SDAI depends on different factors, including TJC, SJC, VAS, EGA, and CRP levels [31]. Accordingly, this effect may represent the influence of boron on the above factors, which showed a high percent of changes in boron-treated groups. Since SDAI was shown to be superior over DAS28 in assessment of remission in RA patients [32], and even easier than DAS28 to calculate, it can be concluded that the use of SDAI may be better than DAS28 not only to follow-up RA patients but also to monitor response to particular therapy in clinical trials. These modest benefits of boron in Iraqi patients with active RA may be attributed to its pleotropic effects that antagonize many pathophysiological processes of RA, including immunomodulatory, anti-inflammatory, and antioxidant activities [16]. Meanwhile, other studies reported the dose-dependent antioxidant and anti-inflammatory activities of boron, and most of the pleotropic effects were produced using a relatively higher daily doses than the daily required amounts, which was similar to that used in this study [27,33]. This may explain partly why treatment with boron in this study produced statistically significant benefits when compared to placebo. This study declared the decrease in morning stiffness in boron-treated and placebo groups. This finding was expected because boron could improve the markers of inflammation that morning stiffness was correlated with such as ESR, SJC, pain, fatigue, tender joint and patient and physician global assessment of disease activity [34]. Meanwhile, this finding is consistent with previous data that reported placebo effect in this regard [35]. Many experimental animal and clinical data have demonstrated that boron decreases production of many inflammatory mediators [36,37]. In this study, the influence of boron on the inflammatory markers seems to be relatively in tune with the previously reported data, although they differ in the etiology of inflammation and the experimental model that may predispose to some differences in the change pattern. However, it confirms the expected anti-inflammatory role of boron, and furthermore shows a novel finding in RA model. In this study, although CRP levels significantly decreased in boron-treated groups compared with baseline values, they are found to be comparable with those reported in placebo-treated group. This can be attributed to the differences in baseline values among groups and the multifactorial etiology of CRP elevation. Although the current results represents the first finding regarding the influence of boron on CRP levels in patients with active RA maintained of biological therapy, they seem to be comparable with many previous reports that utilize other disease models [9,38]. TNF-α is important mediator of inflammation and tissue damage in active RA [39]. This study showed that both boron significantly decrease serum TNF-α levels compared with placebo, and CFB shows greater effect than NTB in this regard. This finding was consistent with that reported by others, where boron could significantly decrease the elevated levels of TNF-α produced during many exaggerated inflammatory responses [29,40]. Moreover, Newnham reveals the antiarthritic effect of boric acid in animals and some forms of arthritis in humans [38]. Similarly, Hunt and Idso indicated that joint damage was remarkably attenuated in adjuvant-induced arthritic rats that received supplemental boron [41], and conclude that boron may decrease the inflammatory response due to attenuating pro-inflammatory cytokines production by the activated inflammatory cells. These marked anti-inflammatory effects of boron could be attributed to various mechanisms, including the suppression of serine proteases released by inflammation-activated white blood cells, inhibition of leukotriene synthesis, reduction of ROS generated during neutrophil's respiratory burst, and suppression of T-cell activity and antibody concentrations [8]. Although the current results are consistent with many previously reported ones in other models of inflammation, the influence in RA can be considered as a new insight in this regard. Many experimental animal models and clinical studies have proved the role of IL-1 type cytokine in the pathogenesis of synovial inflammation and destruction of articular tissue [42]. Serum concentration of IL-1α was found to be substantially higher in RA patients compared with that of healthy control, and may provide clinically useful markers for the diagnosis of disease activity. The response of this marker to antirheumatic agents may be of value in monitoring response to treatment, especially when DMARDs are used in this respect [43]. In this study, NTB and CFB comparably decreased serum IL-1α levels, and the effect was significantly greater than that reported in control group. A relatively similar outcome was observed regarding the effects of boron supplements on serum IL-6 levels. The anti-inflammatory effects of NTB and CFB can be related to many different mechanisms, including suppression of serine proteases released by inflammation-activated white blood cells, inhibition of leukotriene synthesis, reduction of ROS generated during neutrophil's respiratory burst, suppression of T-cell activity, and antibody concentrations [44]. Although the current results are clear within the limitations of the trial, previously reported data raises many doubts about the effect of boron in this regard, where supplementation with dietary boron increases production of cytokines following stress, which indicates a role for boron in the immune system [45]. Accordingly, a mechanism beyond boron-induced reduction of cytokines might explain

the alleviation in the inflammatory symptoms in RA patient supplemented with boron as adjuvant with etanercept.

CONCLUSION

The use of elemental and organic complex forms of boron, as adjuvant with etanercept, improves the clinical scores and significantly decreases the inflammatory markers in RA patients. This improvement in therapeutic outcome supports the idea of utilizing this new strategy to improve the treatment and to avoid the problems associated with biologics utilized in RA treatment.

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Original Research

High dose of green tea infusion normalized spiral artery density in rats treated with the depot-medroxyprogesterone acetate

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ABSTRACT

Aim: The purpose of this study was to investigate the effects of green tea (GT) on the spiral artery density and endometrial thickness in female rats treated with the depot-medroxyprogesterone acetate (DMPA). **Material and Methods:** A total of 24 female rats were randomly divided into four groups (n = 6 each): The control group (no treatment), the DMPA-treated group, treated with DMPA and GT doses of 165 mg/kg of body weight/day, and treated with DMPA and GT doses of 330 mg/kg of body weight/day. Spiral artery density and endometrial thickness were subjected to histopathological analysis. **Results:** Spiral artery density decreased in the DMPA-treated group, despite the insignificant difference ($P > 0.05$). With regard to the administration of GT at doses of 165 and 330 mg/g of body weight/day, only GT at the high dose was capable of significantly preventing a decrease in spiral artery density ($P < 0.05$). At this dose, the spiral arteries achieved a density comparable to that of the control group ($P > 0.05$). Meanwhile, the administration of DMPA and/or DMPA with GT did not cause significant changes in endometrial thickness relative to the control group ($P > 0.05$). **Conclusions:** DMPA induced a decrease in spiral artery density, despite the insignificant differences, and these changes could be normalized by the administration of high doses of GT. Therefore, GT could be a candidate herb to prevent the adverse effects of the contraceptive DMPA.

KEY WORDS: Contraceptive, endometrium, histology, progestin, uterus, xenobiotics

INTRODUCTION

Depot-medroxyprogesterone acetate (DMPA) is a suspended solution of pregn-4-ene-3,20-dione,17-(acetyloxy)-6-methyl-(6α), which injected intramuscularly (150 mg once every 3 months) for long-term contraception effect. Once injected, a serum peak level of 1.0 ng/ml will be reached in 3 months and will be followed by a gradual decline. Unpredictable changes in menstrual patterns are often found among women who use DMPA. This constitutes the reason for discontinuation of DMPA as a contraceptive [1-3].

Changes in menstrual patterns of women using DMPA are associated with the local changes in the endometrial microvasculature. To date, the mechanism for the bleeding remains unknown. The underlying mechanism is not directly related to changes in the levels of endogenous and exogenous steroid hormones [4-7]. In addition to changes in the density of the spiral arteries, changes in endometrial thickness are also thought to be involved in the changes in the menstrual pattern of women using DMPA. A decrease in spiral artery density and an increase in endometrial thickness are found among women who use DMPA [8,9]. Increased thickness of the endometrium is proportional to the intrauterine administration of levonorgestrel relative to that of DMPA [10].

Green tea is a beverage derived from the plant *Camellia sinensis* (L). O. Kuntze. It contains a variety of catechins, such as -epicatechin (EC), (-)-epigallocatechin (EGC), (-)-EC-3-gallate (ECG), and (-)-EGC-3-gallate, are found in 30% of the dry weight of plant [11,12]. Previous studies showed that green tea (GT) was capable of affecting angiogenesis and inducing proliferation [13-17]. Thus, the researchers speculated that GT could be an adjunct to the administration of DMPA, as an herb capable of normalizing changes in spiral artery density and endometrial thickness. Thus, this study was to demonstrate the effects of the administration of GT on the number of the spiral arteries and endometrial thickness in DMPA-treated female rats.

MATERIALS AND METHODS

Animals

A total of 24 female Wistar rats were divided into four groups: The control group (no treatment), the DMPA-treated group and the group treated with DMPA and GT of various doses (165 and

330 mg/g of body weight/day, respectively). These mice were purchased with a body weight of 150 (200 g), from the Molecular Physiology Laboratory, Medicine Faculty of Brawijaya University, Malang, East Java, Indonesia. They were maintained in the laboratory conditions in an air-conditioned room at a temperature of 25 ± 1°C with a relative humidity of 65 (70%) and a cycle of dark and light per 12 h. Those rats were given drinking water and feed *ad libitum*. The feed given was in accordance with the standard recommendation from the American Institute of Nutrition.

DMPA Treatment

DMPA (Depo Progestin®) was administrated by intramuscular injection at a dose of 2.7 mg/rat/week for 10 weeks. Before injection, the drug was dissolved in 0.2 distilled water. This dose was determined on the basis the previous toxicity study [18].

Green Tea

The GT (Kepala Djenggot brand) that has been brewed with distilled water (15 min, 90°C) was administered by a feeding tube to each rat.

Histopathology

Spiral artery density and endometrial thickness were calculated from transverse sections of endometrial tissue. The tissue was then subjected to hematoxylin-eosin staining and photographed using a Dotslide Olympus Camera XC 10. Overall, an analysis was carried out on five fields at ×400 magnification.

Ethics

This study passed the ethical review of the Faculty of Medicine, Brawijaya University, Malang of East Java, Indonesia.

Statistical Analysis

All data were presented in mean ± standard deviation. Differences among groups were analyzed using ANOVA tests using the SPSS 15.0 statistical software package. Further tests were performed using the *post-hoc* tests when ANOVA found significant differences. A $P < 0.05$ was considered significantly different.

RESULTS

The density of spiral arteries is presented in Figure 1. The density of spiral arteries in the DMPA-treated group was lower than that of the control group, despite the insignificant difference ($P > 0.05$). With regard to the administration of GT at doses of 165 and 330 mg/g of body weight/day, only GT at the high dose was capable of significantly preventing a decrease in spiral artery density, reaching a density comparable to that of the control group ($P > 0.05$).

Figure 2 shows the values of endometrial thickness for the various treatment groups. There were no significant differences in endometrial thickness among the treatment groups ($P > 0.05$).

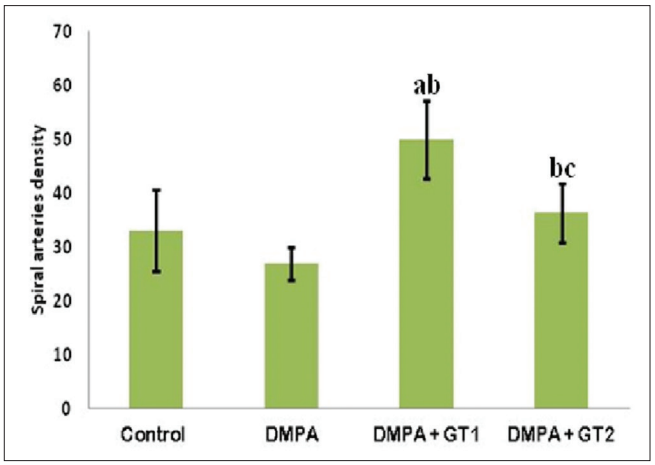

Figure 1: The density of spiral arteries in the control group and the treatment group. Note: Values are presented in mean ± standard deviation; [a]$P < 0.05$ is compared with the control group; [b]$P < 0.05$ is compared with the group administered with depot-medroxyprogesterone acetate (DMPA) without green tea (GT); [c]$P < 0.05$ is compared with the group administered with DMPA plus GT at dose of 330 mg/g body weight/day; DMPA: Depot-medroxyprogesterone acetate; GT: Green tea

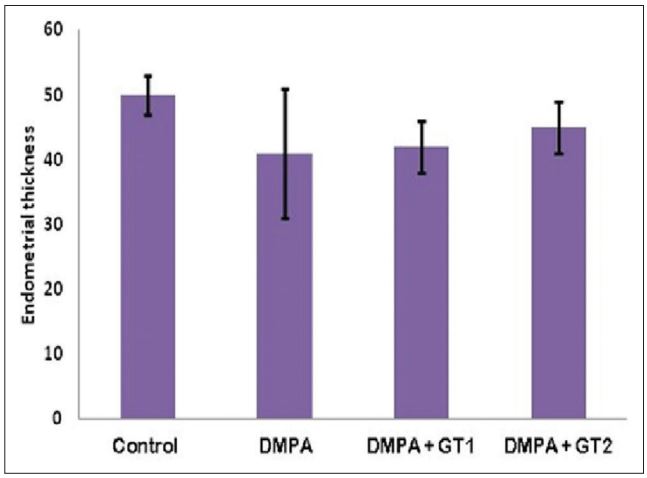

Figure 2: The thickness of the endometrium in the control group and the treatment group. Note: Values are presented in mean ± standard deviation; DMPA: depot-medroxyprogesterone acetate; GT: Green tea

DISCUSSION

The limitation in this study was not conducted analysis of active ingredients of GT infusion. Various literature states that GT contains polyphenolic compounds, minerals, and trace elements [19,20]. In detail, the chemical constituents of GT include polyphenols, caffeine (3.5%), theobromine (0.15-0.2%), theophylline (0.02-0.04%), lignin (6.5%), organic acids (1.5%), chlorophyll (0.5%), theanine (4%), and free amino acid (1-5.5%). Tea also contains flavonoids and flavanols [21-25].

This study showed that the density of spiral arteries tended to decrease in the DMPA-treated group relative to the control group ($P < 0.05$), despite the insignificant difference. This tendency was in accordance with previous studies, showing

that administration of high doses of DMPA was associated with decreased vascular density. Changes in endometrial vascular density are influenced by various factors including the types of hormone, dosage, and methods of administration [8]. Progestin-containing contraceptives will lead to atrophy as seen in vascular changes and characterized by the impaired development of the spiral arteries, dilation, and thin-walled blood vessels near the surface of the epithelium [26]. In this study, high doses of GT were capable of restoring the density of spiral arteries, reaching a value comparable to that of the control group ($P > 0.05$). On the contrary, low doses of GT significantly increased the density of spiral arteries relative to the control group and the DMPA-treated group ($P > 0.05$). This indicates that high doses of GT were capable of normalizing the changes in the spiral arteries as a result of DMPA administration. This is supported by previous findings that the catechins had fluctuating effects on angiogenesis based on the levels of vascular endothelial growth factor (VEGF-A) [27]. However, only ECG that inhibits binding of VEGF to its receptors. Meanwhile, EC, ECG, and EGC cannot inhibit binding of VEGF to its receptor [28].

In this study, endometrial thickness did not differ significantly among the treatment groups ($P < 0.05$). This study differed in the findings for women treated with DMPA, in which there was an increase in endometrial thickness, causing the endometrium to be fluffier and edematous [9]. Our study also extended the previous finding that there was no difference in endometrial thickness between patients treated with levonorgestrel-releasing intrauterine system (Mirena) and those treated with DMPA (Depo-Provera) [10].

CONCLUSION

DMPA induced a decrease in the density of the spiral arteries, despite the insignificant difference, and these changes could be normalized by the administration of high doses of GT. Thus, GT could be a candidate herb to prevent the adverse effects of the contraceptive DMPA.

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Original Research

Evaluation of the potential effect of *Allium sativum, Momordica charantia, Eugenia jambolana, Ocimum sanctum,* and *Psidium guajava* on intestinal p-glycoprotein in rats

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ABSTRACT

Aims/Background: This study was evaluated synergistic effect of a polyherbal formulation (PHF) of *Allium sativum* L., *Eugenia jambolana* Lam., *Momordica charantia* L., *Ocimum sanctum* Linn., and *Psidium guajava* L. on p-glycoprotein (Pgp) of intestine. These five herbs were traditionally used for diabetes. These herbs are commonly present in Ayurvedic product as antidiabetics in India. **Materials and Methods:** PHF was prepared by five indigenous herbs. Different doses (50, 100 and 200 mg/kg/day) of were orally administered to Sprague-Dawley rats of different groups for multiple weeks except control groups. Alteration in Pgp expression was evaluated by real-time-polymerase chain reaction and western blotting while modulation in activity of Pgp was evaluated using rhodamine 123 (Rh123) as transport substrate by *in-situ* absorption and everted gut sac method. **Results:** In PHF, pretreated group received 50, 100 and 200 mg/kg/day for 7 days, mRNA level decreased by 1.75, 2.45 and 2.37-fold, respectively, as compared to control. Similarly, when PHF at dose of 100 mg/kg/day was given consequently for 4 weeks, maximum decrease in Pgp expression level was observed only after 1 week and further increase in the treatment duration did not produce significant decrease compared to the 1st week treatment. Pgp mediated transport of Rh123 was significantly decreased with everted gut sac prepared from PHF pretreated rats (1 week) compared to those prepared from vehicle treated rats. **Conclusions:** We report that PHF pretreatment downregulated the expression of intestinal Pgp and this downregulated intestinal Pgp would result in decreased functional activity. In addition, this downregulated Pgp expression might affect the bioavailability of antidiabetic Pgp substrate drugs.

KEY WORDS: Everted-sac, herb-drug interaction, polymerase chain reaction, p-glycoprotein, western blot

INTRODUCTION

Diabetes mellitus becomes a growing problem in the contemporary world. It is the 7th foremost cause of death in the world. Approximately, 1.5 million deaths occurred in 2014 due to the Type 2 diabetes mellitus. This is a more common in developing countries. There were 366 million people suffering with Type 2 diabetes in 2011; these numbers will rise to 552 million by 2030 [1]. It is one of the intractable diseases identified by Indian council of medical research for which an alternative medicine is need for the treatment herbal remedies are safe and fascinating move toward alternative medicine for diabetes complementary medicine because their benefits are well documented from a historical viewpoint in the population of diabetes [2]. Most of the diabetics in India rely on the use of conventional medicines for the major health care needs [3]. However, the consumption of herbal preparation has been reported for their induction and inhibition potential on p-glycoprotein (Pgp) expression [4]. Pgp is an ATP-dependent multidrug efflux transporter and an absorption

barrier by transporting some drug from intestinal cells from the lumen [5,6]. Pgp is considered as one of the major barriers for bioavailability of orally administered drugs. Therefore, any alteration in expression and activity of Pgp will eventually result in increase or decrease in plasma concentration of Pgp substrate drug. Such herb-drug interaction may be hazardous if Pgp substrate drug levels reaching above toxicity thresholds or may change drug absorption, distribution, and elimination. Among various transporter proteins, Pgp is a major efflux protein that actively efflux the Pgp substrate in intestinal lumen.

Several compounds derived from the natural product and plant extracts have been reported to inhibit Pgp activity [7,8]. In this study, *Allium sativum* L., *Eugenia jambolana* Lam., *Momordica charantia* L., *Ocimum sanctum* Linn., and *Psidium guajava* L., which have been commonly used for the treatment of diabetes or consumed daily in Indian population [9-14], were monitored for their synergistic effect on intestinal Pgp efflux transporter. All these plants were used in the polyherbal formulation (PHF). The PHF has shown a significant antidiabetic effect on streptozotocin in induced diabetic rats (data are not shown in the manuscript). Here, we have estimated the effect of PHF on altering intestinal Pgp expression and its function. Alteration in Pgp expression was done using western blotting while modulation in the activity of Pgp was evaluated using rhodamine 123 (Rh123) transport study. To achieve these aims, we studied the effect of PHF administration on transcriptional level of Pgp and its functional activity in dose and time dependent manner.

The previous studies are only based on a single herbal active constituent. However, in Ayurvedic system of medicine practice, a combination of polyherbal preparations are prescribed but no such studies are documented in literature. Moreover, these formulations are also not told to prescribing physician by the patients. This may due to the ignorance of patients because these polyherbal preparations are not considered as a part of active medication. Prolong usage of these formulations may alter the expression of efflux transporters that finally leads to altered the bioavailability of antidiabetic or other Pgp substrate drugs that have narrow therapeutic index.

MATERIALS AND METHODS

Reagents and Chemicals

Phenylmethanesulfonyl fluoride (PMSF), Rh123, bovine serum albumin (BSA), protease inhibitor cocktail, Dulbecco's phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS), sodium pentobarbital, Triton-X, TEMED, acryl and bis-acrylamide, ammonium persulfate and Evans blue were purchased from Sigma-Aldrich (USA). High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Sigma-Aldrich (St. Louis, MI, USA).

Preparation of PHF

Five herbs, *A. sativum* L. (garlic), *E. jambolana* Lam. (Jamun) seeds, *M. charantia* L. (Bitter gourd) fruits, *O. sanctum* Linn. (Holy Basil) leaves, and *P. guajava* L. (guava) were purchased from the local vegetable market from the Lucknow, Uttar Pradesh, India. PHF was prepared by mixing 200 mg powder of each herb in single formulation. First, the selected herb materials were shade dried and grinded by mixer grinder. Prepared hydro-alcohol extracts of herbs were concentrated using rotary evaporator at 40°C temperature than extracts were freeze-dried at −20°C for 12 h afterward lyophilized using lyophilizer. The lyophilized extracted powders were placed in an airtight glass box and kept in the desiccator until used.

Animals

The animal experimental procedures were carried out in accordance with current legislation on animal experiments as per Institutional Animal Ethical Committee at King George Medical University, Lucknow (IAEC approval no IAEC/2013/44). Male Sprague-Dawley (SD) rats of weight between 220 ± 20 g were purchased from CSIR-IITR (India). Animals were maintained at 25°C temperature in steel cages with alternate 12 h of light and dark cycles and given a pallet diet and water. Before starting the experiment rats were acclimatized for 7 days then divided into two groups, PHF pretreated ($n = 5$), and vehicle treated control ($n = 5$). Rats in the pretreated group were administered orally with PHF (50, 100, and 200 mg/kg/day) 16-gauge gavage needle for 7 days and multiples of weeks accordingly. The PHF suspension was made in 0.5% sodium carboxymethyl cellulose for oral administration. The control group received the same volume of the vehicle for 7 days. Animals were allowed free access to food and water but before euthanasia, rats were overnight fasted to decrease the intestinal content. At the end of the experiment, rats were sacrificed by inhalation of anesthetic ether.

Dose Response and Time Dependent Experiment

To evaluate the effect of dose response of PHF on intestinal Pgp of rats were given 50, 100, and 200 mg/kg/day by oral administration for 7 days and multiple weeks. The control group received same volume of the vehicle for 7 days. During time-dependent, course of experimentation rats were given 100 mg/kg/day of PHF consequently for 7, 14, 21 and 28 days [15].

Effect of PHF Pretreatment on Gastrointestinal Pgp Expression and Activity

The effect of PHF administration on intestinal Pgp was evaluated at transcriptional and translational level. Similarly, the effect of PHF administration on intestinal Pgp activity was evaluated using everted gut sac methodology.

Real-time Polymerase Chain Reaction (RT-PCR)

mRNA isolation, cDNA synthesis, primer designing was performed as Singh *et al*. The forward and reverse primer sequence for ATP-binding cassette subfamily B member 1 (ABCB1) is 5'-TGATGCTTTCCCCAATGC-3' and 5'-TGTCCT CTCTCTGAAAAACTGTCA-3', respectively. Glyceraldehyde

3-phosphate dehydrogenase (GAPDH) was used as internal loading control and sequence is 5'-AGCTGGTCATCAATGGGAAA-3' and 5'-ATTTGATGTTAGCGGGATCG-3', respectively. Effect of pre-treatment of PHF on the mRNA expression of Pgp was examined by RT-PCR. For quantitative RT-PCR, cDNA was amplified using Light Cycler 480 (Roche Molecular Biochemicals, USA) using SYBR green kit (Fermentas, USA). The reaction was performed in 96-well white PCR plate. The reaction mixture was consisted of 1X SYBR green, 200-300 nM primers and 2 μl of cDNA in final reaction volume of 20 μl. The thermal cycle condition was 95°C for 15 min to activate Amplitaq Gold DNA polymerase, 95°C for 15 s and anneal/extension at 60°C for 1 min (40 cycles). GAPDH was used as internal loading control. Fold changes in mRNA level are derived after normalizing with GAPDH mRNA level [6,15].

Preparation of Crude Intestinal Membrane Fractions

Crude membrane fractions were prepared from the intestinal mucosa after every week in 28 days of successive PHF treated and control rats [16]. Intestine was immediately excised out and washed with ice-cold normal saline containing 1 mM PMSF as a protease inhibitor then snap frozen and stored at −80°C until analysis. Intestinal samples were homogenized in the 50 mM tris buffer (pH 8) containing 1% Triton-X-100, 1 mM PMSF and cocktail protease inhibitor (Sigma-Aldrich, USA). Centrifugation of homogenate was performed at 10,000 rpm for 20 min at 4°C. Protein in the supernatant was examined using the Lowry method with BSA as standard [17].

SDS-Polyacrylamide Gel (PAGE) and Western Blotting

About 50 μg of crude membrane fraction was boiled for 10 min in denaturing sample buffer (1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue, 10 mM Tris-HCl; pH 6.8). In the crude intestinal membrane fractions, protein was separated on 8% SDS-PAGE. Resolute proteins were electrophoretically moved to polyvinylidene difluoride membrane (Fermentas, USA). Membrane was blocked for nonspecific sites in phosphate buffer (containing 0.05% Tween 20 and 5% skimmed milk) for 2 h and washed 2 times in PBS tween (PBST) for 5 min then probed with antibodies for Pgp (1:700; Santa Cruz Biotechnology Dedham MA), and then re-probed with β-actin antibody (1:5000; Cayman Chemical Company, Michigan, USA) for loading correction. Then, the blots were washed three times in PBST with 0.1% Tween 20 and incubated with 1:10,000 dilution of secondary antibody (anti-immunoglobulin G-horseradish peroxidase conjugate) for 2 h at room temperature. After extensive washing in PBS, substrate solution was applied to the membrane, then incubation was carried out for 5 min at room temperature. Bands were examined and visualized on X-ray film with enhanced chemiluminescence western blot kit (ECL, Millipore), following the manufacturer's protocol.

In-vitro Transport of Pgp Substrate Rh123 across PHF Treated Rat Intestine

In-vitro transport of Rh123 across rat everted gut sacs was performed [18]. Briefly, at the end of pre-treatment of PHF, rats were sacrificed, and ileum was excised out, then washed with normal saline, and everted using a glass rode. Small intestine was segmented into 4-5 cm long segments that were tied at one end. The Rh123 solution (25 μM in TC199) was filled in the serosal side and tightly ligated to make a gut sac. Immediately, this intestinal everted sac was placed in 30 ml of TC 199 medium. The solution was gassed by 5% CO_2/95% O_2 and maintained at 37°C during the experiment. Transport of the Rh123 across the serosal to the mucosal side was calculated by sampling 1 ml of the external medium every 10 min up to 80 min. The rate of Rh123 transport was expressed as μM or percentage secreted per minute in the mucosal compartment. In some experiments, Pgp inhibitor (verapamil 100 μM) was added in the mucosal and the serosal sides at the same concentration. This model was validated with animals pre-treated with dexamethasone (100 mg/kg/day for 2 days) by oral route [19].

Assay Method for Rh123

The determination of Rh123 concentrations in the samples was performed immediately after the experiment. For plasma samples, an equal volume of methanol was added to 100 μl aliquots of plasma to precipitate proteins. The mixture was vortexed for 2 min and centrifuged at 9000 ×*g* for 10 min at 4°C and supernatant thus obtained was used for analysis. However, the perfusate and samples from everted sac were directly used. 100 μl aliquots of the samples (supernatant or perfusate and everted sac samples) were then added to 96-well black plates, and readings were taken at an excitation wavelength of 485 nm and emission wavelength of 527 nm on multimode microplate reader (Tecan, USA). Rh123 concentration in the samples was derived from standard curve of Rh123.

Excretion Study of Rh123 Using *In Situ* Perfusion Method

Total two group of animals, *viz*., control (5 rats) and two PHF treated (5 rats) were prepared. Each group of rats was fasted overnight for at least 12 h and on the 8th day, animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). They were positioned in a supine position on a heating pad to maintain constant normal body temperature under a surgical lamp. The upper jejunum (15 cm) of the intestinal lumen was catheterized with an inlet silicon tube (4.2 mm i.d.). This was perfused with PBS containing 25 mM glucose into the intestinal lumen in a single perfusion manner at a flow rate of 0.5 ml/min [18]. After 30 min of perfusion for stabilization, 0.2 mg/kg of Rh123 was injected via the femoral vein. The intestinal perfusate was collected every 15, 30, 45, 60, 75, 90, 105 and 120 min. Samples were stored at 4°C after collection. Rh123 excreted at each time point and cumulative amount of Rh123 was determined and used as function of Pgp activity.

Statistical Analyses

All data were presented as mean with standard error. Statistical analyses of data were performed using ANOVA and if applicable followed by the Student Newman–Keuls multiple range tests.

Statistical analysis of data was performed using *t*-test followed by unpaired test. The level of significance was set a priori at $P > 0.05$.

RESULTS

Effect of PHF Pretreatment on Gastrointestinal Pgp Expression

The ABCB1 mRNA expression profile of intestine of PHF pre-treated and the vehicle pre-treated group was compared. In PHF pre-treated group that received 50, 100 and 200 mg/kg/day for 7 days mRNA level decreased by 1.75 ($P > 0.05$), 2.45 ($P < 0.05$) and 2.37 ($P < 0.05$) fold respectively as compared to vehicle pre-treated group. Similarly, when PHF at dose of 100 mg/kg/day was given accordingly for 4 weeks maximum decrease in ABCB1 expression level was observed only after 1 week and further increase in the treatment duration did not decrease significantly compared to the 1st week treatment [Figure 1].

Western blotting was carried out for approving the mRNA data with protein level. Results showed the decreased intensity of bands from PHF treated rats as compared to control rats. Intensity of the bands revealed that Pgp expression has been downregulated with PHF treatment [Figure 2].

Effect of PHF Pretreatment on Intestinal Pgp Activity

In-vitro everted gut sac methodology was first validated using a known inducer of Pgp expression. The measurement of Rh123 transport was calculated in the absence and presence of Pgp inhibitors in everted gut sac prepared from PHF pre-treated and control rats. In absence of verapamil, the transport of Rh123 was decreased by 55 ($P < 0.01$) and 60% ($P < 0.01$) at 100 and 200 mg/kg/day of PHF for 7 days compared with control rats [Figure 3]. On the other hands, no significant alteration was observed at 50 mg/kg/day of PHF dose.

However in the presence of verapamil, Rh123 transport across the everted gut sacs was significantly decreased by 59% ($P < 0.05$), 61% ($P < 0.05$) and 73% in control, 100 and 200 mg/kg/day of PHF when administered for 7 days, respectively [Figure 3]. The Pgp-mediated transport of Rh123 was significantly decreased by 62% ($P < 0.05$) and 72% ($P < 0.01$) at 100 and 200 mg/kg/day of PHF dose for 7 days, respectively, compared with the control group [Figure 3].

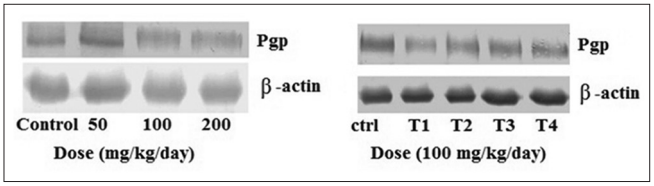

Figure 1: Dose and time dependent effect of PHF administration on intestinal ABCB1 mRNA level. In dose response experiment rats were orally gavaged with 50, 100 and 200 mg/kg/day of PHF extract for 1 week. To evaluate time dependent changes in ABCB1 mRNA expression rats were gavaged with PHF (p.o 100 mg/kg/day) consecutively for 28 days and any alteration in mRNA expression was assessed after every 7 days. IT1, IT2, IT3 and IT4 represent treatment for 7, 14, 21 and 28 days, respectively. Control group received the same volume of blank formulation for same time duration. Each value is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P*<0.05, ***P*<0.01) from control.

In the time-dependent study evaluation of the effect of treatment duration, PHF (100 mg/kg/day) was administered orally for 4 weeks and Rh123 transport across everted gut sac was evaluated after every week. Basal transport of Rh123 (without inhibitor) was significantly ($P < 0.01$) decreased by 52%, 55%, 58% and 56% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control. No significant difference was obtained between different treated and control groups.

Similarly, in the presence of verapamil, Rh123 transport across the everted gut sac was considerably decreased by 47%, 51%, 49% and 49% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control. The Pgp mediated transport of Rh123 was significantly ($P < 0.01$) decreased by 63, 65, 68 and 70% after every week of PHF treatment, respectively, up to 4 weeks in comparison to control [Figure 4].

In exsorption study with Rh123, PHF (100 mg/kg/day) and vehicle were orally administrated in treated group and control group, respectively for 7 days and excretion of Rh123 from intestinal lumen was calculated after designated time interval. It was observed that intestinal lumen excretion of Rh123 was decreased at each time point in PHF treated rats as compared to control. Similarly, the cumulative excretion of Rh123 after 120 min was significantly decreased by 34% ($P < 0.05$) in PHF treated rats in comparison to control and this excreted amount of Rh123 over 120 min in intestine of control, and PHF treated rats was 0.72 ± 0.02 and 0.46 ± 0.02 μg/mL, respectively [Figure 5].

DISCUSSION

Intestinal Pgp is the major efflux transporter that confines the bioavailability of orally administered drugs. Any alteration in level and/or activity of this protein would ultimately result in the altered pharmacokinetics of substrate drug. Among various factors, consumption of herbal preparation is a major factor that can modulate the expression and/or activity of Pgp. Various herbal preparations have been reported to alter the expression and activity level of this protein, e.g., St. John Wort consumption increased the expression of Pgp that eventually decreased the bioavailability of cyclosporin resulting in rejection of tissue implant in the patient. Other clinical drug-drug and herb-drug interactions were reported in the literature between digoxin (Pgp substrate) and other Pgp substrates such as quinidine verapamil, talinolol, clarithromycin, traconazole, erythromycin, and propafenone [20]. Herbs in PHF that has been used from ancient times to improve glycemic control. However, no reports are available regarding the synergistic effect of this type formulation on Pgp. Therefore, in this study, we evaluated *in-vivo* effect of PHF administration in dose and time

dependent manner in male SD rats. One possible method of estimating the induction/inhibitory potency of any compound to protein is measuring mRNA and protein levels. However, the relationship between mRNA and protein levels has not been established yet, and using mRNA and protein levels alone may misleading to the contribution of Pgp on the pharmacokinetics of drugs. Assessing functional activity for induction/inhibition potential of any compound is supposed to be best because it is only the functional activity that comes to play the following treatment. However, in this study, we used all three methods to analyze the induction/inhibition potential of PHF. In our data, we obtained a decrease in mRNA and protein level of Pgp in intestine of rats with PHF treatment. The reason behind this phenomenon might be attributed to the overall exposure of intestine, which is more exposed to oral components compared to liver, kidney, and other parts of the body. Therefore, intestinal

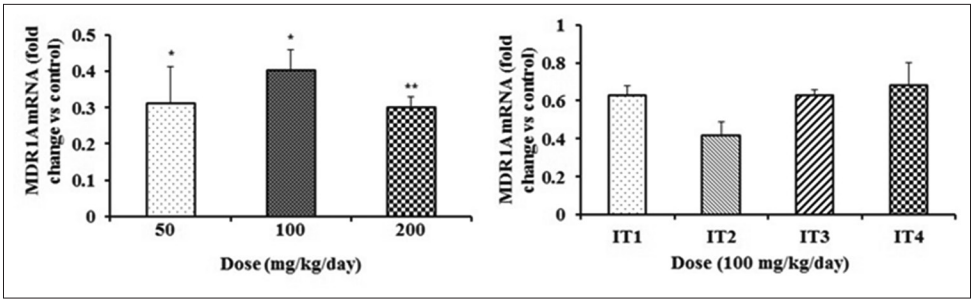

Figure 2: Dose and time-dependent effect of PHF administration on intestinal Pgp protein level. In dose response experiment rats were orally gavaged with 50, 100 and 200 mg/kg/day of PHF extract for 1 week. In time response study rats were gavaged with PHF (100 mg/kg/day) consecutively for 28 days and any alteration in mRNA expression was assessed after every 7 days. T1, T2, T3 and T4 represent treatment for 7, 14, 21 and 28 days, respectively. Control group received the same volume of blank formulation for same time duration.

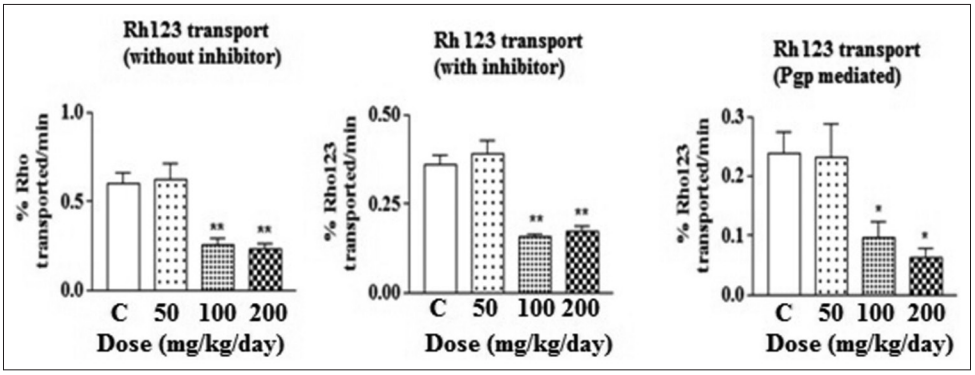

Figure 3: Dose response of PHF administration on intestinal Pgp activity. Rh123 transport was performed in absence and presence of inhibitor (verapamil 100 μM) across the everted gut sac obtained from control and PHF treated rats. Each value (percent of Rh123 transported per min) is expressed as mean ± standard error from three independent experiments. Asterisks indicate significant differences (**P*<0.05 and ***P*<0.01) from control.

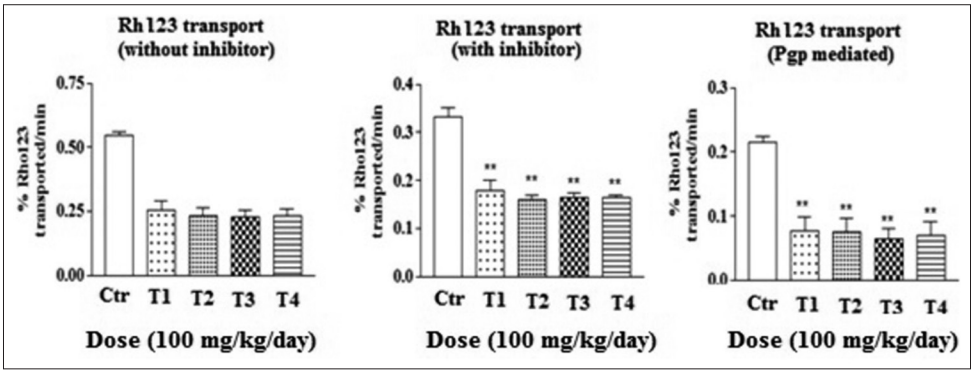

Figure 4: Time dependent effect of PHF administration on intestinal Pgp activity. Rh123 transport was performed in absence and presence of inhibitor (verapamil 100 μM) across the everted gut sac obtained from control and PHF treated rats. C, T1, T2, T3 and T4 represents control group, one, two, three and four week treated group, respectively. Values are expressed as percent of Rh123 transported per min and are mean ± standard deviation of three replicates.

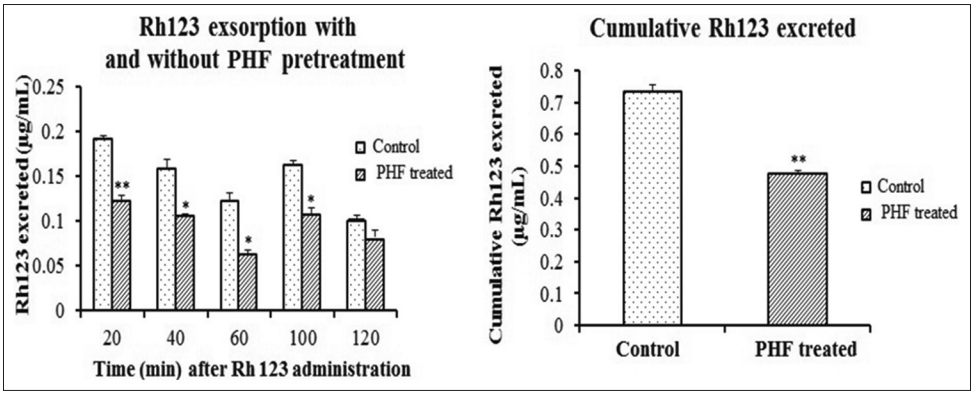

Figure 5: Exsorption of Rh123 in intestinal lumen with and without PHF pretreatment. Rats were treated with PHF (100 mg/kg/day) for one week and exsorption study with Rh123 was evaluated as described in material and methods. Each value is expressed as mean ± standard deviation of three independent experiments. Asterisk ** represents significant difference from control with $p< 0.01$

Pgp is considered a more important in determining the oral bioavailability of Pgp substrate drugs.

There is a not true measure of functional activity of Pgp either increase or decrease in mRNA and protein; we measure the functional activity both *in-vivo* and *in-vitro* following the PHF pretreatment using Rh123, as a typical Pgp substrate. The fluorescent dye Rh123 has been extensively used as index of Pgp-mediated transport in rodents and various tissue culture models [21]. Tian *et al.* (2002) reported that the measurements of Rh123 using a spectrofluorometer were as same as those using HPLC method in a transport study using the rat everted-sac method [4]. Therefore, the use of spectrofluorometer in our study as a convenient tool can be rationalized.

Rh123 has been reported to be metabolized by intestinal esterase and some of its metabolite also has fluorescence intensities [4]. However, it has been considered that intestinal esterase during the study had lesser effect on the fluorescence intensity of Rh123 in everted gut sac method because intestine was washed several times with normal saline before use and everted sac is in direct contact with only Rh123. Similarly, *in-situ* perfusion method also has much less effect on intestinal esterase activity because intestinal lumen was constantly perfused with buffer solution and samples were immediately analyzed.

In our study, Pgp mediated transport of Rh123 was significantly decreased with everted gut sac prepared from PHF pretreated rats (1-week pretreatment) compared to those prepared from vehicle treated rats. However, this transport inhibition due to the presence of PHF components in intestine was denied because the experiment was conducted after the 24 h of last PHF dose with fasting of rats for 8-12 h. This duration is sufficient for normal washout of intestinal contents. Therefore, a decrease in Pgp mediated transport of Rh123 was attributed to decrease in intestinal Pgp expression. This decrease in Pgp mediated transport was in agreement with decreased protein expression measured with mRNA level and protein level with western blot. However, we found that protein as well as mRNA levels were decreased. There are some studies that reported decrease in Pgp protein expression due to post-transcriptional modification. These post-transcriptional modifications can alter the expression of Pgp by two ways: One at glycosylation level and other at the trafficking of membrane protein. In the first scenario, the synthesized Pgp needs to be glycosylated to reach plasma membrane. An alteration at the glycosylation level would result in increased degradation of native protein that causes a decrease in Pgp expression. In the second case, the reduction of Pgp movement from cytoplasm to plasma membrane would also result in downregulation of Pgp expression. Such kind of regulation that occurred at trafficking level has been reported [21].

To validate this hypothesis, we performed *in-vivo* functional activity assay (exsorption of Rh123). The result obtained in this experiment revealed that PHF pretreatment decreased the exsorption of Rh123 into intestinal lumen in comparison to control. These data support our hypothesis that increase in tissue concentration of Pgp substrates was due to downregulation in expression and activity of intestinal Pgp. In addition, Rh123 is not a CYP3A substrate, therefore, the modulation in distribution of Rh123 was attributed to decrease in Pgp expression only. These *in-vivo* results were in good agreement with the results, we obtained in western blot and *ex-vivo* everted gut sac. Therefore, the results obtained could be significantly attributed to downregulation of intestinal Pgp. Alteration in the expression and activity of Pgp with herbal constituents could also be a useful strategy to increase the oral bioavailability of Pgp substrate, in particular, to develop the oral formulations of antidiabetic drugs such as sitagliptin, glibenclamide, rosiglitazone and troglitazone, transported by Pgp transporter [22]. However, further investigations to estimate quantitatively Pgp in human cell line like Caco-2, intestinal epithelial as well as pharmacokinetic studies with Pgp substrates are required to validate their effect to clinical implementation.

CONCLUSION

This study demonstrates that PHF pretreatment downregulated the expression of intestinal Pgp, and this downregulated intestinal Pgp would result in decreased functional activity. Based on these results, PHF inhibits the efflux activity of

Pgp transporter which could play a key role in modulating the systemic availability of Pgp substrate drugs *in vivo*. It has been reported in literature that regular consumption of herbal preparation could alter the expression of Pgp in intestine and other tissues. In addition, this downregulated Pgp expression might affect the bioavailability of antidiabetic Pgp substrate drugs.

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AUTHORS' CONTRIBUTIONS

Experiment is conceived and designed by DK and RKD. DK and NT performed experiments and participated in data collection, interpretation and statistical analysis. DK wrote the paper.

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Original Research

Ethnomedical survey and safety evaluation of traditional eye medicines used in Misungwi district, Tanzania

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ABSTRACT

Aim: This study aimed at documenting products used as traditional eye medicine (TEM) in Misungwi district, Tanzania, and performing preliminary analysis on safety status. **Methodology:** Ethnomedical study was conducted in Misungwi district. Information was sourced by face-to-face interview with traditional healers, traditional medicine vendors, and knowledgeable people guided by a well-structured questionnaire. Safety was evaluated by determining pH using pH meter and mineral analysis using the Delta, Portable X-ray fluorescence equipment, and qualitative chemical tests. **Results:** A total of 23 TEM products were recorded from botanical (79%) and zoological (21%) sources including animal excreta. Liquid preparation ranked highest among dosage forms. Safety evaluation showed that only one product possessed the pH value of 7.4 as recommended for topical ophthalmic medicines. Fourteen minerals were detected and quantified in three samples; some of these minerals are known for their negative effects to the eyes, of medical interest is strontium used for the management of benign eye tumors. Information providers were unaware of health risks associated with the use of TEM. **Conclusion:** This study has revealed the common use of TEM in Misungwi district. The majority of the products are from the botanical source. Although literature provides supporting data for the application to some of the recorded TEM, safety evaluation by pH and mineral analysis in this study have indicated possible ophthalmological medical problems that could result from using such products. Extensive scientific studies including animal experiments and identification of bioactive compounds are essential to develop safe TEMs.

KEY WORDS: Ethnomedical, minerals, Misungwi district, safety, pH, Tanzania, traditional eye medicine

INTRODUCTION

Eyes are delicate sensory organs that make our everyday life comfortable. When it comes to treatment, eyes require special attention to avoid medical problems including partial or total vision disability. In Tanzania, modern health-care services are inadequate and majority of population especially in rural areas still rely on traditional methods including the use of traditional eye medicine (TEM). Various studies have associated TEM with medical eye problems based on diagnosis made by medical practitioners/ophthalmologist, for example, a study conducted in Tanzania showed that 25% of corneal ulcers resulted from the use of TEM [1] and a similar research conducted in Malawi showed 33% of patients with corneal disease in rural Malawi reported to have used TEM [2]. In the same country, 72% of the interviewee on self-treatment used TEM with no clear borders between biomedical and herbal medicines, i.e., used side by side [3].

TEM products are obtained from botanical, zoological, and mineral sources, and routes of administration are local application to eyelids, eye drops, instillation into the conjunctival sac, or taken orally [4]. Negative consequences of using TEM could be minor such as temporary irritation and pain or major such as permanent loss of vision if not timely and well managed. Examples include the following; *Calotropis procera* latex reported to cause significant ocular morbidity, including painless corneal edema, permanent endothelial cell loss with morphological alteration after intracorneal penetration ending up with keratitis [5-7] and a case study reported in Nigeria where man suffered from ocular discomfort and eventually blindness after applying the raw cassava extract [8]. Recently, Tanzanian plants and other products used for the treatments of eyes diseases/conditions were published in a systematic manner to enable quick search for further scientific research to prove their efficacy and safety [9].

Tanzania is among the African countries rich in natural resources that provide medicinal substances for traditional health care. Misungwi district mainly populated by the Sukuma tribe is a place where traditional medicine is still valued, especially among old people and the rural population. It involves the use of all sorts of natural resources and supernatural powers. It is one of the seven districts of Mwanza region in Northern part of Tanzania situated in the savannah grassland about 156 km from the Serengeti national park at 02°51′S 033°05′E. Its map

is given in Figure 1. The area has tropical climate, the summers have a good amount of rainfall, while the winters have very little. According to the Köppen-Geiger climate classification, the temperature averages 23°C and the rainfall averages 901 mm [10].

This study aimed on documentation of TEM products in Misungwi district as a way conservation of such knowledge and performing preliminary safety evaluation by pH determination and mineral analysis. Our findings are expected to stimulate researches on TEM products on various aspects to enable preparation of standardized TEM product/identify useful bioactive compounds for the development of ophthalmic products.

METHODOLOGY

Study Design

This study was conducted in two phases as follows:

i. Ethnomedical study comprised oral interview and field work done on alternate days
ii. Safety evaluation included laboratory work.

Ethnomedical Study

Study period, study site, and information providers (IPs)

The study was carried out for 14 days in December 2014 in Misungwi district and covered Mapilinga, Mwaniko, Nange, and Ng'ombe villages plus the Misungwi street in town. Interviewee included traditional healers and knowledgeable people (KP) found at homes in the villages whereas traditional medicine vendors were found at the business center of the town.

Data and material collection

The purpose of the study was explained, and informed consent was obtained from each of the participants. Face-to-face oral interview was conducted in Swahili (national) and Sukuma (local language) to obtain reliable information using a well-structured questionnaire (Annex I), which was translated to Swahili language. The data on TEM including sources, dosage forms, preparation methods, route of administration, knowledge, and awareness of IPs on risks associated with TEM were immediately recorded in the questionnaire. Field work involved collection of plant material for herbaria preparation, pH determination, and mineral analysis. Samples of animal excreta were bought from IPs. Identification/authentication of collected plants using herbaria specimen was done in the Botany Department at the University of Dar Es Salaam where the voucher specimen were deposited.

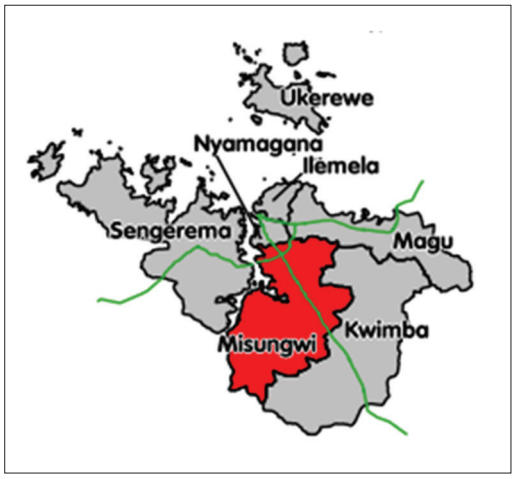

Figure 1: Map of Misungwi district [11]

Quantitative ethnomedical data analysis

The collected information was quantitatively analyzed using an index of relative frequency citation (RFC) as,. $RFC = \frac{FC}{N}$ This index shows the local importance of each product and it is given by the frequency of citation (FC), the number of informants mentioning the use of the product (species) divided by the total number of informants participating in the study (N) [12].

Safety Evaluation

This was performed in February 2015. The pH determination was done in the Laboratory of Pharmacognosy Department at Muhimbili University of Health and Allied Sciences and mineral analysis in the Geology Department at University of Dar es Salaam.

Determination of pH values

Determination of pH values employed the JENWAY 3035 pH meter, made in the UK by Jenway Felsted Dunmow, Essex CM6 3LB. Sample preparation was done according to the method previously described by Maregesi *et al.* [9] with minor modification at room temperature of 25°C. In brief, 1 g of the powdered material of each test sample was weighed in triplicate then macerated with 20 mL distilled water with occasional shaking for the period of 12 h. The filtrates were used for pH determination. The pH of each sample was obtained by taking the average value from triplicate analysis.

Mineral analysis

Mineral analysis was carried out using Portable X-ray fluorescence (XRF) spectrometer, and the application note by Innov-X systems, 2003, was adopted. Each test sample was ground to a very fine powder and then passed through a sieve of 250 μm mesh. About 100 mg of each sample was weighed and then transferred into respective XRF test cups which were then covered tightly with nylon material. The XRF test cups containing samples were then subjected to the analyzer (XRF spectrometer) for spot analysis (Innov-X systems XRF testing guideline). Additional mineral analysis

was done using standard procedures/reagents for qualitative tests of salts.

RESULTS

Ethnomedical Study

Eighteen IPs participated in the study whose demographic data are presented in Table 1. Number of males and KP were higher than females and others groups, respectively. We afforded to record twenty-three TEM products and their methods of preparation, dosage forms, administration routes, FC, and RFC as summarized in Column A of Table 2. Frequency of mention of treated disease/conditions is presented in Figure 2. All IPs were neither aware of any health risks associated with the use of TEM nor making follow-ups on patient progress. In case of treatment failure, patients could opt to report back to get an alternative medicine or seek treatment from other sources including modern medicine.

Safety Evaluation

All tested products were alkaline in nature with the pH values ranging from 7.3 to 10.0 as shown in Table 3. Fourteen minerals detected/quantified in three TEM products are given in Table 4. Hyena excreta ranked highest for the total number of detected minerals.

Table 1: Demographic data of information providers

Demographic data	*N* (%)
A. Gender	
Sex	
Female	7 (39)
Male	11 (61)
B. Source of information	
Groups of information providers	
Traditional medicine vendors	2 (11)
Traditional healers	5 (28)
Knowledgeable people	11 (61)
C. Age	Adults above 30 years
D. Education level	Primary school

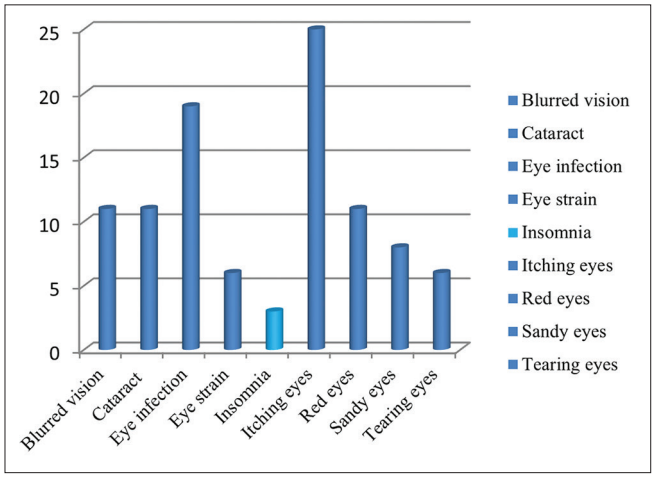

Figure 2: % Frequency of mention of the treated eye disease/condition

DISCUSSION

Ethnomedical Study

The majority of the recorded TEM products (79%) are obtained from plants with half of them prepared from leaves. Animal products comprised of honey (4%) and animal waste (17%). The suitability of the later for use as eye medicines is doubted since microbial contamination with pathogenic microbes is most likely as evidenced by lizard droppings [9]. The majority of reported products are liquid preparations applied as eye drops (91%) and matching with topical ophthalmic solution most commonly used and accepted dosage forms in modern medicine [46]. Most of the recorded TEM products were mono-component except for the following: (i) *Ficus glumosa*, *Ficus natalensis*, and *Nicotiana tabacum*; (ii) lizard and hyena excreta; and (iii) lemon juice and salt – a containing common and easily available substances.

Aloe vera leaf juice (latex) was the most mentioned product with the RFC of 0.61 followed by *Euphorbia hirta* and *Vernonia amygdalina* each with RFC of 0.43. About 61% of the recorded TEM products had previously been reported in Tanzania or other parts of the world for treatment of the same or different eye problem(s). This is a good indication on reliability of the information obtained from IPs. Previous data including those of related species are summarized in Column B of Table 2.

Itching, sand, and tearing eyes are common symptoms of allergic conjunctivitis [45]. In this context, allergic conjunctivitis is the most common treated eye disease/condition constituting 39% of all cases. It is normal that common diseases in a particular society tend to have more attention and various medicines. Allergic conjunctivitis is likely to be the most common eye problem as it relates well to the climate of Misungwi district and especially during the dry season with blowing winds that carry various dust particles capable of causing some allergies.

Chemical constituents of TEM products render therapeutic effects through various biological/pharmacological activities such as antimicrobial, anti-inflammatory, analgesic, and wound healing. For example, (i) honey is used against eye infection and eye itching, the former can be related to the antimicrobial activity of honey in aerobic conditions brought about by the osmotic effect of its sugar contents, hydrogen peroxide produced by the action of the enzyme glucose oxidase in diluted honey and phytochemicals found in the nectar including flavonoids and aromatic acids [47]; (ii) management of wounds resulting from conjunctivitis/irritation may be linked with the wound healing activity of *E. hirta* whose probable mechanism of action is promotion of collagen biosynthesis [24]; and (iii) curative effect of *Cocos nucifera* shell charcoal could be due to adsorption of foreign substances causing red eyes or minerals dissolved in the aqueous solution.

An interesting observation from this study is the use of related species for treatment of a particular eye disease/condition as reported for *Crotalaria* and *Ficus* species. Based Based on

Table 2: TEMs used in Misungwi district

A. Current ethno medical data TEMs from botanical sourcex						B. Data from previous studies
Plant and family names, voucher specimen no	Vernacular name	Plant part, preparation and route of administration	Eye disease/ condition	Frequency of citation	Relative frequency of citation	TEM uses, biological/ pharmacological/phytochemicals and related species
Aloe vera (L.) Burm. f. Aloaceae JM2014 (1)	Magaka (Sukuma) Makaka (Kerewe/Jita)	One drop of leaf juice used as eye drop twice a day	Cataract eye discharge, itching eyes	14	0.61	The *A. vera* gel is useful for dry skin conditions, especially for treatment of eczema around the eyes. It had shown antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*[13] as well as anti-inflammatory and antiseptic properties [14]
Cassia siamea (Lam.) Irwin et Barneby. Caesalpiniaceae JM2014 (2)	Mchongoma (Sukuma)	Steam from boiled leaves is allowed to enter the eye	Sandy eyes, itching eyes	6	0.26	*C. siamea* leaf juice is used to treat eye stye, conjunctivitis, and other minor eye problems [15]. In addition, a formulation prepared from leaves is used for treatment of bacterial/fungal eye infection [16]
Citrus limon (L.) Burm.f. Rutaceae JM2014 (3)	Limao (Swahili)	Salted lemon juice used as eye drops. 1 drop every morning until recovery	Cataract	6	0.26	*C. limon* juice had shown antibacterial activity against *S. aureus, Escherichia coli,* and *Klebsiella pneumoniae* among others [17]
Cocos nucifera L. Arecaceae JM2014 (4)	Makala (Sukuma) Mkaa (Swahili)	Very fine powder of the shell charcoal is directly used or dissolved in water to obtain the filtrate which is used as eye drops three times a day	Red eye	2	0.09	*C. nucifera* shell charcoal was recently, reported to treat eye problems among many other ailments [18]
Crotalaria laburnifolia L. Papilionaceae JM2014 (5)	Lupi (Sukuma)	Fresh leaf juice is used as eye drops (2 drops) three times a day	Itching eyes	4	0.17	Related species including *Crotalaria retusa* is used against eye infection [9], *Crotalaria pallida* and *Crotalaria assamica*
Crotalaria rogersii Baker f. Papilionaceae JM2014 (6)	Matulo (Sukuma)	Fresh leaf juice is used as eye drops. 2 drops three times a day	Itching eyes	5	0.22	possess anti-inflammatory effect due to flavonoids and pterocarpanoid [19] and *Crotalaria pusilla* have both analgesic and anti-inflammatory properties [20]
Datura stramonium L. Solanaceae JM2014 (7)	Malongelonge (Sukuma)	One tablespoon of dried seed powder is soaked in a full glass of hot water for at least 6 hours and taken orally at night only	Insomnia	7	0.30	*D. stramonium* seeds are used to induce sleep and treatment of insomnia. Alkaloids are present in varying concentrations in different organs of *Datura* plant, e.g., scopolamine and atropine classified as anticholinergics [21,22]
Euphorbia hirta L. Euphorbiaceae JM2014 (8)	Kashono (Sukuma)	Fresh latex is applied as eye drops three times a day	Itching eyes	10	0.43	*E. hirta* latex and leaf juice are used to treat eye problems including sore eyes, conjunctivitis, and eyelid stye [23,24]. Extracts of aerial parts exhibited anti-inflammatory activity [25] and antimicrobial activity against *P. aeruginosa*, *S. aureus* and others [26]
Ficus glumosa Delile Moraceae JM2014 (9)	Ngumo (Sukuma)	Fresh stem fibers mixed with fresh tobacco leaves and fresh bark of *F. natalensis* are macerated overnight. The filtrate is used as eye drops twice a day	Blurred vision	4	0.17	*F. natalensis* is used against cattle blindness, conjunctivitis and related ocular infections [27], eye tumor and cataract [9]

(Contd...)

Table 2: *(Continued)*

A. Current ethno medical data TEMs from botanical sourcex						B. Data from previous studies
Plant and family names, voucher specimen no	Vernacular name	Plant part, preparation and route of administration	Eye disease/ condition	Frequency of citation	Relative frequency of citation	TEM uses, biological/ pharmacological/phytochemicals and related species
Ficus natalensis Hochst Moraceae JM2014 (10)	Numbaga (Sukuma)	Fresh stem fibers, fresh barks of *F. glumosa* and tobacco leaves are macerated in water for overnight. The filtrate is used as eye drops twice a day	Blurred vision, eye infection	6	0.26	
Indigofera colutea Burm.f. Papilionaceae JM2014 (11)	Mburulambuli (Sukuma)	Root bark decoction is used as eye drops (3 drops) three times a day	Sandy eyes, itching eyes	3	0.13	*I. colutea* shoot had shown antibacterial activity against *Bacillus cereus* and *S. aureus* [28]
Jatropha curcas L. Euphorbiaceae JM2014 (12)	Makale (Sukuma)	Two drops of latex used as eye drops three times a day	Eye infection, tearing eyes	3	0.13	*J. curcas* latex is traditionally used to heal wounds and stem bark exhibited antimicrobial activity against *S. aureus, P. aeruginosa,* and *E. coli* [29,30]
Manihot esculenta Crantz Euphorbiaceae JM2014 (13)	Kayeba (Sukuma)	Leaf juice mixed with *Jatropha curcas* juice and python feaces. One drop applied into the eye three times a day	Sandy eyes and eye style	6	0.26	*M. esculenta* leaf juice is used to treat conjunctivitis and sore eyes [31]. It has anti-inflammatory, antimicrobial, and analgesic activity [32,33]
Nicotiana tabacum L. Solanaceae JM2014 (14)	Tumbaku (Swahili)	Fresh leaves mixed with *F. natalensis* and *F. glumosa* roots. The filtrate is used as eye drops twice a day	Blurred vision	7	0.30	The decoction of *N. tabacum* leaves is applied for muscle relaxation and relieving pain. Nicotine in its zinc complex isolated from leaves showed the antibacterial activity against ten different strains of Gram-positive and Gram-negative bacterial strains [34]
Ocimum canum Sims Labiate JM2014 (15)	Manung'u (Sukuma)	Fresh leaf juice is used as eye drops. 2 drops three times a day	Itching eyes	4	0.17	*O. canum* shoot was reported to treat conjunctivitis and possess antibacterial and antifungal activities against *S. aureus* and *Aspergillus* species. It contains essential oil rich in linalool along with several other compounds [35,36]
Solanum incanum L. Solanaceae JM2014 (16)	Matura (Sukuma)	Sun dried leaf powder is mixed with cooking oil. The paste obtained is topically rubbed on inflamed eye veins	Tearing eyes	5	0.22	*S. incanum* leaf juice is used against eye disease [37]
Vernonia amygdalina Delile Compositae JM2014 (17)	Mbarizi (Haya, Ha)	Liquid oozing from plant leaves at night is used to wash the eyes (with infection) prior to the application of other eye medicine	Eyes infection	10	0.43	*V. amygdalina* leaf is used to treat cataract [9], possess antimicrobial activity against *S. aureusa* and *P. aeruginosa* [38,39], drug resistant bacteria viruses and have anti-inflammatory activity. It contains alkaloids, tannins, saponins, phenolics, glycosides, and phlobatannins that may account for the therapeutic effects [40,41]
Vitex mombassae Vatke Verbenaceae JM2014 (18)	Nsungwi (Sukuma)	Two drops of the fruit juice is used as eye drops three times a day	Red eyes	5	0.22	The related species *Vitex doniana* is used for treatment of eye disease and possess anti-inflammatory activity [42]

(Contd...)

Table 2: *(Continued)*

TEMs from animal source					
Product name and source Vernacular name	Preparation and route of administration	Eye disease/condition	Frequency of citation	Relative frequency of citation	TEM uses
Honey bees Bhuki (Sukuma)	2-3 drops of raw honey is applied as eye drops twice a day	Eye infection and itching eyes	7	0.30	Honey is used since ancient times in treatment of various diseases including wounds and prevention of corneal scarring due to measles [43,44]. It is a common eye medicine for minor trauma, redness, pain, itching, crusting, and vision sharpening in Pakistan [45]
Hyena feaces Hyaenidae Mashi-Gambiti (Sukuma)	Equal parts of the powdered of lizard (white portion) and hyena feaces are macerated. The filtrate is used as eye drops twice a day	Eye strain and red eyes 6	6	0.26	None
White part of lizard feaces Lacertilia Mashi-Gakuli (Sukuma)				0.26	*Python feaces*, lizard droppings, and snail shells have been reported for cataract treatment [9]
Sea snail shells molluscs Shilungu (Sukuma)	Powdered shell is soaked in in water for at least six hours. The filtrate is used as eye drops twice a day	Blurred vision, eye infection, cataract and itching eyes	5	0.22	
Python faeces Python Mashi-Ganogwasato (Sukuma)	Powder is soaked in hot water. The filtrate is applied as eye drops three times a day	Cataract	4	0.17	

TEM: Traditional eye medicines

Table 3: pH of traditional eye medicine products

Plant/product	pH
V. amygdalina leaf extract	7.6
Python excreta micella	7.4
Hyena excreta micella	8.1
Sea snail shell micella	8.6
Lizard droppings (white portion) micella	7.3
Charcoal/water suspension	10.0
Distilled water	6.9
Prepared 0.9% NaCl	7.3

V. amygdalina: Vernonia amygdalina

chemotaxonomy, i.e., related species contain same/related phytochemicals thus likely give the same therapeutic effects as reported by the IPs. Another observation was that the concept of eye diseases was not clear among few IPs who regarded insomnia as an eye problem simply because it involves closure of the eyes by reporting *Datura stramonium* to treat this condition which is in accordance with the known use of the plant [22]. In addition, IPs were able to specify different eye diseases/conditions as compared to those from previous studies, who gave generalized information.

The lack of awareness and follow-up of the patient is a drawback on this particular traditional medical service. The fact that treatment the failure oblige patients to the seek treatment from modern medical services is in agreement with observation made in studies conducted in Tanzania and Malawi [1,2] and elsewhere. The unfortunate part is that the harm could have reached an irreversible stage by the time patients consult the medical practitioner/ophthalmologist due to switching from one or more TEM until desperation.

Safety Evaluation

Among the six analyzed samples, the python excreta micella was the only TEM with the recommended pH of ophthalmic products of 7.4 which is the same as that of the lacrimal fluid due to isotonicity importance. However, pH values of 7-9 are tolerated by the eye without marked irritation. Acidic and too alkaline products are corrosive to the eye [48]. Regarding the pH of *V. amygdalina* leaf extract, it was alkaline (pH of 7.6) compared to the acidic pH of 5.6 in our previous work [9]; at this point, no definitive comment can be made but just to speculate the causes such as unspecified age of leaves collected from different locations.

Table 4: Mineral contents of the excreta and sea snail shells

Mineral	Hyena excreta (%)	Python excreta (%)	Sea shells (%)
Ca	21.794±0.19	42.886±0.24	0.28±0.02
K	1.385±0.07	ND	6.291±0.18
P	8.723±1.68	ND	ND
S	1.10±0.23 (ppm)	ND (ppm)	ND (ppm)
Ti	489±30	ND	ND
Mn	100±9	31±8	9±0.3
Fe	2728±86	375±43	ND
Zn	217±12	21/7	ND
Rb	36±4	60±5	26.5±1.6
Sr	569±21	1239±45	20.2±1.6
Zr	179±10	ND	ND
As	ND	ND	13.4±1.8
Cl	ND	4425±8.32	1130±10.4

Qualitative analysis showed the presence of CO_3. *ND=Not detected

With regard to minerals, some metals causes toxicity by their action on the retina and optic nerves and are implicated in structural and physiological damage in the mammalian eye [49]. Some of the negative health effects of the detected metals and salts include vision impairment by manganese [50], chemical burn of eyes and skin by rubidium hydroxide formed from the reaction between rubidium and skin moisture [51], redness, pain and inflammation by calcium carbonate [52], skin and eye irritation by zinc salts particularly the carbonate [53], granuloma caused by zirconium and arsenic being carcinogenic [49]. Thus, frequent use of TEM containing these minerals could lead to medical eye problems. On the other hand, strontium has medical application for management of benign tumors of the eye [54].

This study and literature data show the role of TEM in various communities despite reported harmful consequences of some TEM products [5-9] and lack of evident scientific support to justify their uses. However, the use of TEM is inevitable due inadequate modern health services, thus a need to educate the public about health risks associated with TEM through the media and other means. In parallel, scientists should focus their research to produce standardized, safe TEM products and/or identify bioactive compounds for the development of modern medicine.

CONCLUSION

The findings of this study showed that majority of the recorded TEM products (79%) are botanical products while animal products (21%) comprised of honey and animal wastes. Some of the recorded products have previously been reported in Tanzania/other countries, suggesting that the information given by IPs is reliable but this not a guarantee for efficacy and safety. IPs and the public as a whole need to be informed on risks associated with the use of TEM. Results from safety evaluation, though preliminary, necessitate a very comprehensive study to identify safe TEM products as well as getting evidence for public declaration of unsafe products. Animal experiments to establish the safety status of some botanical TEM products are in progress.

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ANNEX

Annex I: English version - questionnaire for ethnomedical study on traditional eye diseases/conditions7

1. Source of information:
   i. Name of traditional healer/herbalist/ others ……
   ii. Age and education level: …….
   iii. Sex: ……..
   iv. Date: ……..…….
   v. Address: ……..……
   vi. Level of education: ……..
   vii. Tribe: ……
2. Do you treat any eye diseases/condition? Yes/No: …….
3. If yes, what are they? .....…..……...
4. Which of the mentioned diseases do you treat and have obtained positive results?
   (i) …..……
   (ii) …..……
   (iii) ……...
5. Which plant(s) and plant part(s) do you use? ……
6. Do you use a one or a mixture of plants in treating your patients? ……
7. Besides plant material(s) do you use other products like minerals or animal products? Yes/No: ……..
8. If yes what are they? (Vernacular names: ……
9. Do you have any specific time/season for collection and storage conditions for you plant material? ……
10. How to you prepare your medicine? (e.g., soaking/boiling in water, powdering, juice, latex, etc.) ……
11. How do you administer your medicine to your patients (e.g., orally, topical application, eye drop, etc.) ……..
12. What amount/quantity of medicine do you administer to your patient at one time? ……..
13. How many times per day is the medicine to be taken? ……
14. With regard to the amount of medicine given, do the age/weight matter? Yes/No: ……...
15. If yes, how do you determine the amount to be given to your patients? ……
16. How long does the patient use the medicine? ……
17. Are you aware of any side effect such as blindness that can be caused the medicine you supply to the patients? ……
18. Do make any follow-up of your patient to see if they fully recovered? …….....
19. Are you willing to show me the plant(s) so that we can carry out some scientific research to confirm their efficacy? Yes/No ……
20. If no, what reasons do you have for that? If yes, what are your future expectations from scientific finding ……
    ……

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Original Research

Ethnobotanical survey and toxicity evaluation of medicinal plants used for fungal remedy in the Southern Highlands of Tanzania

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ABSTRACT

Background/Aim: Some of the antifungal drugs used in the current treatments regime are responding to antimicrobial resistance. In rural areas of Southern Tanzania, indigenous people use antifungal drugs alone or together with medicinal plants to curb the effects of antibiotic resistance. This study documented ethnobotanical information of medicinal plants used for managing fungal infections in the Southern Highlands of Tanzania and further assess their safety. **Materials and Methods:** Ethnobotanical survey was conducted in Makete and Mufindi districts between July 2014 and December 2015 using semi-structured questionnaires followed by two focus group discussions to verify respondents' information. Cytotoxicity study was conducted on extracts of collected plants using brine shrimp lethality test and analyzed by MS Excel 2013 program. **Results:** During this survey about 46 plant species belonging to 28 families of angiosperms were reported to be traditionally useful in managing fungal and other health conditions. Among these, *Terminalia sericea*, *Aloe nutii*, *Aloe lateritia*, *Zanthoxylum chalybeum*, *Zanthoxylum deremense,* and *Kigelia africana* were frequently mentioned to be used for managing fungal infections. The preparation of these herbals was mostly by boiling plant parts especially the leaves and roots. Cytotoxicity study revealed that most of the plants tested were nontoxic with $LC_{50} > 100$ which implies that most compounds from these plants are safe for therapeutic use. The dichloromethane extract of *Croton macrostachyus* recorded the highest with LC_{50} value 12.94 μg/ml. The ethnobotanical survey correlated well with documented literature from elsewhere about the bioactivity of most plants. **Conclusions:** The ethnobotanical survey has revealed that traditional healers are rich of knowledge to build on for therapeutic studies. Most of the plants are safe for use; and thus can be considered for further studies on drug discovery.

KEY WORDS: Ethnobotanical, fungal, brine shrimp test, medicinal plants, traditional medicine

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INTRODUCTION

The history of mankind has continuously remained interlocked to the surrounding environment. The first civilizations realized that there were plants with healing potential. The value of plants has a long history in saving human beings cutting across different cultures in the world [1]. Utilization of medicinal plants by individuals lies on the knowledge accumulated through the interaction of people with the environment and the diffusion of information, traditionally transmitted orally through subsequent generations [2]. In the contemporary world of conventional medicine, the practice of herbal medicine has attracted more attention and is becoming accepted globally [3]. Traditional medicine is not well documented in most African societies [4]. However, the practices and resources have been orally transferred from one generation to another thus limiting its reliability.

Documenting the indigenous knowledge through ethnobotanical studies is important for sustainable utilization of medicinal plants in drug discovery. Several active compounds have been discovered from plants based on ethnobotanical information, some used directly as therapeutic drugs [3]. Therefore, the focus of the study was to collect and document information on the use of antifungal medicinal plants and their therapeutic practices among the Hehe and Kinga tribe in Southern Highland of Tanzania. The information could further help scientific research in drug development.

MATERIALS AND METHODS

Study Area

The study was conducted in Mufindi District found in Iringa Region and Makete District based in Njombe Region. Makete District is one of the six districts of Iringa Region and is located in the Southern Highlands of Tanzania about 115 km from the regional headquarters (Figure 1). It is situated within 9°15'0" S 34°10'0" E [5]. Mufindi district on the other hand lies between 08°35′40″S 035°17′20″E. Both districts are dominated by Hehe, Kinga and Bena ethnic tribes. Furthermore, these districts experience high levels of migration and mobility (61.4%) caused by seasonal workers to numerous plantations in the areas and being a logistical hub for transport infrastructural facilities by road and railway (Tanzania-Zambia route) [6]. These unique dynamics increase the risk for HIV transmission in the communities. Most of the livelihoods are from agriculture which is the major source of subsistence, occupying about 80 % of the households in the districts [5]. Other activities include livestock keeping, timber production, and petty businesses at small scale. Most household members are thus compelled to engage in multiple jobs and activities to make a living [5].

During the ethnobotanical survey that was done between July 2014 and September 2015 semi-structured questionnaire was used as data collection tools to interview traditional health practitioners, elders and selected villagers who have knowledge on medicinal plants. This study employed a purposive sampling, in which selection of respondents do only focus to people who are considered by the community as having exceptional knowledge about the use of plants such as traditional healers, herbalists and elders. The questionnaire aimed to collect and document ethnobotanical information of plants that are used to treat various infections including fungal infections. Documentation of plants, parts used and their preparations whenever possible was done. Focus group discussion was employed to validate information collected using questionnaire method.

Collection of Plant Materials

Identification of plant species was done by the botanist from the Department of Botany, University of Dar es Salaam, Tanzania, and all voucher specimens were deposited at the Institute of Traditional Medicine, Muhimbili University of Health and Allied Sciences. Collection of the identified plants was aided by the traditional health practitioners and elders. Decision on which plant and/or part of plant to be collected for further studies was mainly influenced by the information given by respondents in the field validated first by focus group discussion and by literature.

Reagents

Absolute ethanol, dichloromethane, and petroleum ether were purchased from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands), dimethyl sulfoxide (DMSO) was purchased from Sigma® (Poole, Dorset, UK) while sea salt was prepared locally by evaporating water collected from the Indian Ocean, along the Dar es Salaam Coast.

Extraction and Concentration

Plant materials from the field were cut into small pieces, air-dried and ground using a machine grinder consequently soaked, sequentially using petroleum ether, dichloromethane, and ethanol for 48 h for each solvent. The method of percolation was employed during extraction process. The crude extracts were obtained by concentrating the filtrate in vacuo using a rotary evaporator with the bath temperature maintained at 40°C. The crude extract obtained was placed in the refrigerator for few hours and then subjected to freeze drier to remove solvent that could have remained.

Brine Shrimp Lethality Test

The brine shrimp lethality assay was used as an indication for bioactivity of different tested plant extracts as well as investigation for toxicity [7,8]. Artificial seawater was prepared by dissolving 3.8 g of sea salt in 1 L of distilled water. Brine shrimp eggs (2 g) were added and left for 24 h to hatch in light condition. Stock solutions (40 mg/mL) of all extracts were dissolved in DMSO. Different levels of concentrations (240, 120, 80, 40, 24, 8, 4.5, 3, 1.5 and 1 μg/ml) were prepared by drawing different volumes from the stock solutions and then added into vials, each containing ten brine shrimps larvae. The volume was adjusted with the prepared artificial seawater. Each level of concentration was tested in duplicate. The negative control contained brine shrimp, artificial seawater and DMSO (0.6%) only. The vials were incubated under light for 24 h. The dead larvae were counted and mean percentage mortality calculated.

Data Analysis

The mean percentage mortality was plotted against the logarithm of concentrations and the concentration killing 50% of the larvae (LC_{50}) were determined from the graph using Microsoft Excel 2013 computer software. Regression equation obtained enabled calculation of lethal concentrations, i.e., LC_{50}, LC_{16}, and LC_{84}. The 95% confidence interval was then calculated using method reported by Litchfield and Wilcoxon [9]. The results were used to document safety and cytotoxicity activity of plant extracts.

RESULTS

Ethnobotanical Survey

During the ethnobotanical survey, a total of 40 respondents (traditional healers, herbalists, and elders) were interviewed from the selected regions. 5 different villages in Njombe and Iringa regions were visited for the survey including three villages; Tambalang'ombe, Mayale, Kingege, and Ifwagi from Mufindi, Iringa region as well as Lupalilo and Maliwa villages of Makete district in Njombe region. These villages were chosen based on the information of registered or known traditional health practitioners obtained from the District Medical offices.

A total of 46 plant species used by the Hehe, Bena and Kinga tribe for the treatment of various microbial related ailments were documented [Table 1]. The plants represent about 28 families with the most prominent families being Euphorbiaceae (6 species), Combretaceae, and Rubiaceae (4 species each) and followed by Rutaceae, and Fabaceae (each with 3 species). Most of the ethnobotanical information were related to fungal infections since the study focused on documenting plants that were used in managing fungal infections among these ethnic groups. Out of 46 reported plant species, 14 (32%) had similar cited antifungal activity while 8 (18%) of plant species traditionally used for managing other nonfungal infections in Mufindi and Makete districts were reported by the literature to have antifungal activity [Table 1].

Brine Shrimp Lethality Assay

The brine shrimp test is used as a preliminary test for testing toxicity of a plant and anticancer activity after a single dose administration. In this study, the LC_{50} values were clustered per Moshi *et al*., [95]. The LC_{50} of <1.0 μg/ml is considered highly toxic; LC_{50} 1.0-10.0 μg/ml is toxic; LC_{50} 10.0-30.0 μg/ml - moderately toxic; LC_{50} > 30 < 100 μg/ml - mildly toxic and LC_{50} > 100 μg/ml as nontoxic. Studies done by Moshi *et al*., [96,97] provided the evidence that plant extract with the LC_{50} <20 μg/ml could be a source for anticancer compounds. The results from this study revealed that most (77.1%) of the plants tested were nontoxic with LC_{50} value <100 [Table 2]. The present findings imply that most compounds from these plants were safe for therapeutic use. Among the tested plant extracts dichloromethane extract of *Croton macrostachyus* had moderate toxicity with LC_{50} value 12.94 μg/ml.

DISCUSSION

Ethnobotanical Survey

Plant-based traditional medicine system continues to play an essential role in primary health care for the wider communities irrespective of the locality. This work has revealed the potential herbal medicines used in managing fungal infection in Njombe and Iringa Regions which are leading in spread of HIV infection in Tanzania with about 14.8% and 9.1% HIV prevalence, respectively [6,94]. Association of opportunistic fungal infections and HIV have been reported from the early days of the HIV/AIDS pandemic in Tanzania and worldwide [98]. The majority of the people living with HIV/AIDS are susceptible to fungal and bacterial opportunistic infections due to immunity suppression [37]. Availability of fungal herbal medicines may subsidize the effect of antifungal drugs resistance and availability to patients due to recurring fungal infections. The findings showed that remedies used in these communities consisted of one or a combination of two or more plant species. According to the traditional health practitioners, combinations of different plant species increases the efficiency of medicine and improves the cure's power which could be due synergistic effects in treatment of various diseases. Most of plant species collected have been documented to be used in different African communities for the treatment of skin diseases [12]. Furthermore, the study noted that there was a wide use of the leaf part which could be considered as a good sign for the conservation of the environment and ensures sustainable utilization of plants.

Among the frequently mentioned plants, included *Terminalia sericea*, *Aloe nutii*, *Aloe lateritia*, *Zanthoxylum chalybeum*, *Zanthoxylum deremense*, and *Kigelia africana*. The claims on these plants have a special merit as they are also recorded in the literature to be useful in managing various microbial infections. Pharmacological studies by several authors have demonstrated the potency of the mentioned plants in terms of antifungal activity [12,16,21,27,30,81,99,100]. However, the proportion of claims made by traditional health practitioners in Makete and Mufindi districts concerning some of the plants documented in this study and which are supported by literature evidence of proven biological activity or similar ethnobotanical uses elsewhere is remarkable. The results also confirmed the supportive role of traditional health practitioners in offering health-care services to local communities in addition to available conventional medical cares.

Brine Shrimp Lethality Assay

Apart from efficacy, safety of herbal medicines is of paramount importance as little is documented about many plants that are used in traditional medicine. Findings from various studies have recommended brine shrimp assay as one of the methods for preliminary investigations of toxicity. This assay is also used in screening bioactive compounds from medicinal plants popularly used for several purposes and for monitoring the isolation of such biologically active compounds [101-103]. This work present few results from plant extracts that were tested for toxicity against brine shrimps. However, not all collected plant samples were screened for toxicity since during extraction yield was very little or none for some samples to be used for the testing. Findings obtained in this study showed that 77.1% of plant tested to be nontoxic supporting the popular use of medicinal plants by communities since they are regarded as safe therapeutic agents. Unlike other plants, *C. macrostachyus* exhibited high toxicity level that suggests its potential for anticancer agents. The LC_{50} of *C. macrostachyus* (12.94 μg/ml) is not statistically different to the standard anticancer drug

Table 1: List of medicinal plants reported for managing various diseases in Iringa and Njombe regions

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
Acanthaceae	*Dicliptera laxata* (Hehe)	Leaves and roots	Roots are chewed as a stomach pain and coughs remedy Leaf decoction is drunk to treat fever, headache, rashes and itching	Antimicrobial Inflammatory Antinociceptive	No report	05	[10]
Aloaceae	*Aloe lateritia* Litembwembwe (Hehe) Lyusi (Kinga)	Leaves	Combined with other plant roots and use for washing the wounds for 7 days Leaves can be boiled and drunk or applied topically for fungal infections Leaves can also be used against typhoid and wounds	Antimicrobial	Alkaloids, phenolic compounds, tannins, terpenoids	25	[11,12]
	Aloe nutii Litembwetembwe (Hehe)	Leaves and roots	Grind the leaves and soak, for roots grind into powder and take a tea spoon. It can also be mixed with *Toddalia asiatica* and mngalanga to stop diarrhea for HIV/AIDS patients The juice from leaves rubbed on the skin to treat ringworm Leaves decoction for diarrhea	No report	Alkaloids, phenolic compounds, tannins, terpenoids	17	[11]
Anacardiaceae	*Sorindeia madagascariensis* Muzingilizi (Bena)	Leaves, stem barks and roots	Grind the stem barks and smell for headache Root used for treatment of tuberculosis	No report	No report	10	No report
Apocynaceae	*Rauvolfia caffra* Mveriveri (Hehe)	Roots and stem barks	Roots decoction used for management of mental case and epilepsy Stem barks decoction used for rheumatism and chest pains	Antioxidant Antimicrobial	Alkaloid resperine, serpentine	09	[13,14]
Asteraceae	*Bidens pilosa* Lipuli (Hehe)	Leaves, roots and seeds	Leaves grounded and soaked to be gargled in the mouth-oral infection Decoctions of leaf powder for kidney problems, headache and blood clotting Leaves prepared as poultice for wounds and cuts	Anti-inflammatory, antifungal, antibacterial, antimalarial, antitumor Antihyperglycemic, antihypertensive, antiulcerogenic, hepatoprotective, antipyretic Immunosuppressive, antileukemic, antioxidant	Tannins, flavonoids, phlobatannins, terpenoids and cardiac glycosides	10	[10,11,15]

(*Contd...*)

Table 1: (*Continued*)

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
Bignoniaceae	*Kigelia africana* Mfumbi (Hehe) Sausage tree (English)	Fruits, leaves and stem, root barks	Take the fruit sap apply over the wounded part for acute wounds Leaves and stem barks decoction used for treatment of STDs Fruits and barks decoction and powder for skin, fungal infections	Antibacterial Antioxidant Antiulcer Antifungal Antipyretic	Iridoids, flavonoids, naphthoquinones, meroterpenoid coumarin derivatives, lignans, sterols, furanones, furonaphthoquinones	16	[15-17]
Caesalpinaceae	*Ximenia caffra* Mtundwa (Bena) Mpingipingi (Hehe)	Roots and leaves	Roots decoction for treatment of hypertension, chest pain, infertility bilharzia and epilepsy A decoction of leaves is used as a remedy for malaria, coughs, toothache Pounded leaves are used as poultices for wounds and boils	Anti-infammatory Antigonococcal Antibacterial	Gallic acid, catechin, quercetin, kaempferol, terpenoids	07	[18,19]
	Hymenaea verrucosa Gaerth	Stem bark		No report	Terpenes	05	[20]
Celastraceae	*Elaeodendron buchananii* Muhulamwiko (Hehe)	Stem bark	Stem barks powder used for topical application against fungus	Antifeedant	Buchaninoside, glycoside, dihydroagarofuranoid sesquiterpene (mutangin)	08	[21,22]
Chrysobalanaceae	*Parinari curatellifolia* Msaula/msawola (Hehe)	Roots, stem barks and leaves	Leaves decoction for treatment of anemia Barks powder are used for vaginal douches, treatment of itching scalp	Antioxidant Antibacterial Antidiabetic	Phenols, flavoniods, sterols, terpenoids, carbohydrates and saponnins	12	[23,24]
Clusiaceae	*Garcinia buchananii* Mduma/mfilafila (Hehe)	Roots stem barks	Stem barks powder used against abdominal discomfort, pains An infusion from roots used as aphrodisiac and lotion for sores	Antidiarrheal Anti-inflammatory Antipropulsive motility Antiperistaltic	Biflavanones, flavonoids, steroids, alkaloids, tannins and phenols	15	[25,26]
	Garcinia acutifolia Baker Mfilafila/ Mduma (Hehe)	Leaves, stem barks and roots	An infusion from the roots is used as an aphrodisiac and as a lotion for lotion Stem barks decoction for venereal diseases and powder massaged for abdominal discomfort	No report	Xanthones, friedelin, stigmasterol	01	No report

(*Contd*...)

Table 1: (*Continued*)

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
Combretaceae	*Terminalia sericea* Mpululu (Hehe)	Leaves, stem barks and root barks	Roots decoction for washing and apply on wounds and drinking-fungal infection Dried leaves and powdered to make decoction for dysentery Roots and leaves decoction for CD_4 boosting, syphilis, gonorrhea	Antimicrobial Anti-inflammatory Antioxidant	Anolignan B, Saponins, Glycoside, triterpene sericoside, β-sitosterol, β-sitosterol-3-acetate, lupeol, and stigma-4-ene-3-one	15	[27-31]
	Combretum zeyheri Mnavasenga (Hehe)	Roots and leaves	Roots and leaves used against ameba infections and abdominal Boil the roots and drink for Oesophageal candidiasis	Antifungal Antibacterial Antioxidant	Flavonoids	10	[30,32,33]
	Terminalia mollis Mupululu (Hehe)	Leaves, barks Roots	Roots, leaves or roots decoction for bilharzia, coughs, measles, rectal prolapse, and stomachache, HIV	Antioxidant Antimicrobial Antiplasmodial Anti-HIV	Tannins, triterpenes, flavonoids, gallic acid and saponins	14	[34-38]
Cucurbitaceae	*Cumumis dipsaceus* Mtango mwitu (Swahili)	Leaves and roots	Leaves and roots pounded and used as poultice for wound treatment	Antioxidant	Phenolics, flavonoids, tannins	09	[39,40]
Euphorbiaceae	*Psorospermum febrifugum* Mfwifwi (Hehe)	Leaves and roots	Leaves dried then grounded and smear on affected part-fungus Roots grounded and soaked in water for oral infection-as a mouth wash or gargle for tonsillitis	Antifungal Antitumor Cytotoxic Anticonvulsant	Steroids, acetylvismione F, prenylated bianthrone and 1, 8-dihydroxyanthraquinone	12	[41-44]
	Clutia abyssinica Mvuruku (Pare)	Leaves and roots	Roots prepared as hot infusions for kidney cleansing and roundworms	Antimicrobial	No report	05	[45]
	Eurphorbia candelabrum/trucalli Mlangali (Hehe)	Roots and latex	Latex is used against sexual impotence, warts, epilepsy, toothache, hemorrhoids, snake bites A poultice of the roots or stems is applied to nose ulceration, hemorrhoids, and swellings	Antimicrobial Antioxidant Antiviral Hepatoprotective	No report	13	[46]
	Uapaca kirkiana Mguhu (Bena)	Roots	Roots are boiled and the decoction is used as a remedy for indigestion and intestinal problems	No report	No report	02	[47]

(*Contd...*)

Table 1: (*Continued*)

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
	Drypetes natalensis Hark	Leaves	Leaves decoction used against fever and malaria infections	Antitrypanosomal Antileishmanial	No report	01	[48]
	Croton macrostachyus Mulugu (Hehe) Liwurungu (Bena)	Leaves, stem and root barks	Stem barks decoction used for bathing babies against skin infections. Leaf decoction used against abdominal discomfort, sores and ring worms.	Antidiabetic Antimicrobial Purgative Anti-inflammatory Antiplasmodial	saponin, phenolic compound, tannins, anthocyanins, steroids, triterpens, alkaloids, coumarins, antraquinones, glucosides and essential oils	13	[49-52]
Fabaceae	*Dichrostachys cinerea* Mgegele/ mgegera (Hehe)	Leaves, stem and root barks	Grind the leaves and dress the wounds Roots decoction used for TB, infertility, venereal diseases, abdominal ulcers	Antidiarrheal Antibacterial Antioxidant Nephroprotective Immunostimulant	Terpenoids, tannins	07	[53-55]
	Albizia harveyi Msisina (Hehe)	Roots and leaves	Roots and leaves boiled then wash the affected parts and drink, fruits active for scabies, fungus and other skin diseases	Cytotoxic	Alkaloids, glycosides, saponins, Terpenes and flavanoids	11	[56,57]
	Cassia abbreviata Mulimuli (Hehe)	Roots, stem barks and leaves	Dry and powder the roots then take 1 tea spoon in water 3 times a day for strong fever, tooth ache, abdominal pains, back pains and feet pains	Antimicrobial Antimalaria Anti-HIV	Flavonoids, sterols, triterpenoids and anthraquinones	04	[58,59]
Hypoxidaceae	*Hypoxis hemerocallidea* Munyunyu (Hehe)	Roots-potato	Young tuber eaten fresh for diarheal, peptic ulcers and vomiting	Antimicrobial Antioxidant Anticancer Anti-HIV	Hypoxoside, rooperol, phytosterols, laectins, levoglucosan	01	[60,61]
Linaceae	*Hugonia castaneifolia* Ngaze (Hehe)	Root barks	Root barks used as a remedy against intestinal worms, malaria, fungus.	Antifungal Cytotoxic Larvicidal Antibacterial Antioxidant	Terpenoids, lignans	04	[62,63]
Loganiaceae	*Strychnos spinosa* Li/Mtangadasi (Hehe)	Leaves, stem barks and root barks	Sap from leaves used against snake bites	Acaricidal Antitrypanocidal Antimicrobial	Alkaloids, terpenoids, glycosides, flavonoids and tannins	12	[64-66]
Meliaceae	*Azadirachta indica* Mwarobaini (Kinga)	Leaves, stem and roots	Boil the roots and drink for treatment of syphilis	Antimicrobial	Tetranortriterpenoid, protolimonoid	15	[67,68]
Moraceae	*Ficus sycomorus* Mkuyu (Swahili)	Barks and whole plant	Barks powder used for body rashes	Antifungal Antibacterial Antioxidant Insecticidal Acaricidal	Quercetin, gallic acid, Rutin	05	[69-71]
Myrtaceae	*Eugenia capensis subsp. nyassensis* Kivengi/ Mkangaa (Hehe)	Roots	Powdered roots and sniff for -Head ache, flu and chest diseases	No report	No report	07	No report
Oleaceae	*Olax obtusifolia* De Wild Mtungapwezi	Roots	Leaves powder for treatment of pains	No report	No report	01	No report

(*Contd...*)

Table 1: (*Continued*)

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
Rosaceae	*Prunus africana* Mwiluti (Hehe)	Roots	Boil the roots decoction and drink	Anti-inflammatory, Antispasmodic, Anticancer	Glycosides, terpenoids, sterols, fl-sitosterol, lauric acid, myristic acid, n-docosanol, ferulic	03	[72,73]
Rubiaceae	*Gardenia jovis-tonantis* Kilekamahame (Hehe)	Roots and leaves	Grind the roots make decoction drink and smelled for migraine Leaves for wounds	Cardio-relaxant Antisickling Antibacterial Antioxidant Antimalarial	Terpenoids, saponins,		[47,74,75]
	Breonadia salicina Ngwina (Bena)	Leaves, stem barks and roots	Roots decoction drunk as purgative Stem barks decoction for stomach-ache	Antimicrobial Antidiarrheal	No report	01	[76]
	Multidentia crassa Muwewe (Hehe)	Leaves and roots	Leaves are pounded, soaked in water and the juice applied into ears for ear infection Roots used for stomachache	No report	No report	11	Not reported
	Catunaregum spinosa Mpongolo (Hehe)	Roots barks, stem barks and leaves	Roots decoction for treatment of skin diseases, HIV, epilepsy, oral infection Grind the barks to make decoction and feed that child with convulsions. Its roots combined with *Dovyalis abyssinica* roots boiled and drink 3 times in 7 days for syphilis.	Cytotoxic Anthelmintic Antioxidant Sedative	Saponins, coumarins, Terpenoids, carbohydrates, glycosides, phytosterols, phenolic compounds, tannins and mucilage	08	[57,77-79]
Rutaceace	*Zanthoxylum chalybeum* Lungulungu (Hehe)	Leaves and roots	Drink the roots/leaves decoction-oral sores and ulcer	Antimicrobial	Isoquinoline alkaloids, protoberberines	18	[80,81]
	Toddalia asiatica Lutono (Hehe)	Leaves and roots	Leaves and roots decoction used for treatment of microbial diseases Hot infusion from barks for cancer and toothache	Antimalarial Anti-inflammatory Analgesic Sedative Antimicrobial Antioxidant Fungicide Inhibit HIV-reverse transcript tase	Flavanoids, alkaloids, tannins, steroids, phytosterols, saponins, glycosides, coumarins, carbohydrates coumarins, quinoline, nitidine	06	[82-84]
	Zanthoxylum deremense Engl Mkunungu-Hehe	Stem	A decoction of bark and roots is used as a remedy for malaria, generalized body pains, coughs, body swellings, anemia, and as a gargle for toothache Bark and root powder is mixed with oil and applied as liniment for pains and sprains Root bark is powdered and added to tea oral, two cups are taken twice daily	No report	No report	05	No report

(*Contd...*)

Table 1: (*Continued*)

Family	Botanical/common name	Part of the plant	Ethnobotanical preparation and use	Reported ethnopharmacology activity	Reported phytochemical profile or compound	Frequency of mention during FGD	Supporting literature
Santalaceae	*Osyris lanceolata* Mdunula (Hehe)	Stem and root barks	Stem barks decoction for treatment of Sexual Transmitted Diseases (STDs) A decoction of the bark and heartwood is used to anemia Leaves and roots used against backbones and stomach pains, fungus and typhoid	Antioxidant Antimicrobial Antifungal	Phenols, flavonoids sesquiterpenes and pentacyclic triterpenoids	17	[85-87]
Smilaceae	*Smilax anceps* (Mkwangasale)	Leaves	Leaves powder used for body rashes	Antimicrobial	Alkaloids and saponins	04	[88]
Solanaceae	*Solanum anguivii* Kumkalanga (Hehe)	Roots and fruits	Combined with leaves of Mkiringiti then use the decoction to wash the body Fruits chewed for coughs and chest pains	Antioxidant Antihypertensive	Saponins, phenols, flavanoids	14	[89,90]
	Solanum incanum Musufi/mtula (Hehe) Ndulele (Swahili)	Roots, leaves and fruits	Grind leaves and pressed the juice/ ointment over the affected tooth-teeth infections Fruits used for skin infections. Treatment of painful menstruation	Acaricidal effect Cytotoxic Anticancer Hypoglycemic Antimicrobial Antischistosomal Antinociceptive Antipyretic Antispasmolytic Anorexic	Anthraquinones, flavonoids, glycosides, carbohydrate and steroids	07	[21,64,91-94]

Table 2: Brine shrimp toxicity results of medicinal plants used in Southern Highland regions

Plant name	Part of plant	Solvent used	LC_{50} (μg/ml)	95% Confidence interval
Cyclophosphamide	NA	NA	16.3	10.6-25.1
Bidens pilosa	Leaves	Ethanol	107.15	69.94-164.15
Brachystegia spiciformis	Leaves	DCM	151.81	82.28-280.69
Cassia abbreviata Oliv.	Roots	Ethanol	140.89	108.21-183.44
Commiphora africana	Roots	Ethanol	122.04	75.28-197.84
Croton macrostachyus	Leaves	DCM	12.94	6.71-24.95
Diospyros usambarensis	Roots	Ethanol	>1000	-
		DCM	420.83	247.72-714.91
	Leaves	Ethanol	547.09	306.81-975. 46
Drypetes natalensis	Leaves	Ethanol	93	64.95-132.85
Eledendrum buchananii	Stem barks	DCM	>1000	-
Garcinia acutifolia	Leaves	Ethanol	54.18	25.16-46.44
Garcinia spp.	Stem barks	Ethanol	82.73	64.08-106.77
Hymenaea verrucosa	Stem barks	Ethanol	41.47	30.64-56.11
Kigelia africana	Roots	DCM	424	281.73-638.12
		Ethanol	557.92	315.52-986.35
	Stem barks	Ethanol	>1000	-
Lantana viburnoides	Stem barks	DCM	191.27	119.64-305.8
Leonotis lepetifolia	Leaves	Ethanol	>1000	-
Mucuna stans	Leaves	Ethanol	>1000	-
		DCM	488.05	281.63-845.79
Olax obtusifolia	Roots	Ethanol	77.09	60.15-98.81
Parinari curatellifolia	Stem barks	DCM	476.67	258.11-880.41
	Roots	Ethanol	>1000	-

(*Contd*...)

Table 2: (*Continued*)

Plant name	Part of plant	Solvent used	LC_{50} (μg/ml)	95% Confidence interval
	Leaves	Ethanol	175.05	119.61-256.2
		DCM	>1000	-
	Stem barks	Ethanol	>1000	-
	Roots	DCM	43.43	36.9-51.11
	Stem barks	Ethanol	>1000	-
Solanum incanum	Leaves	Pet. ether	>1000	-
Strychnos spinosa	Leaves	Ethanol	>1000	-
		Pet. ether	592.4	332.89-1054.24
		DCM	>1000	-
Terminalia sericea	Leaves	Ethanol	113.4	70.05-183.57
Zanthoxylum chalybeum	Roots	Ethanol	38.51	32.50-45.63
Zanthoxylum deremense	Stem barks	Ethanol	78.69	52.48-118

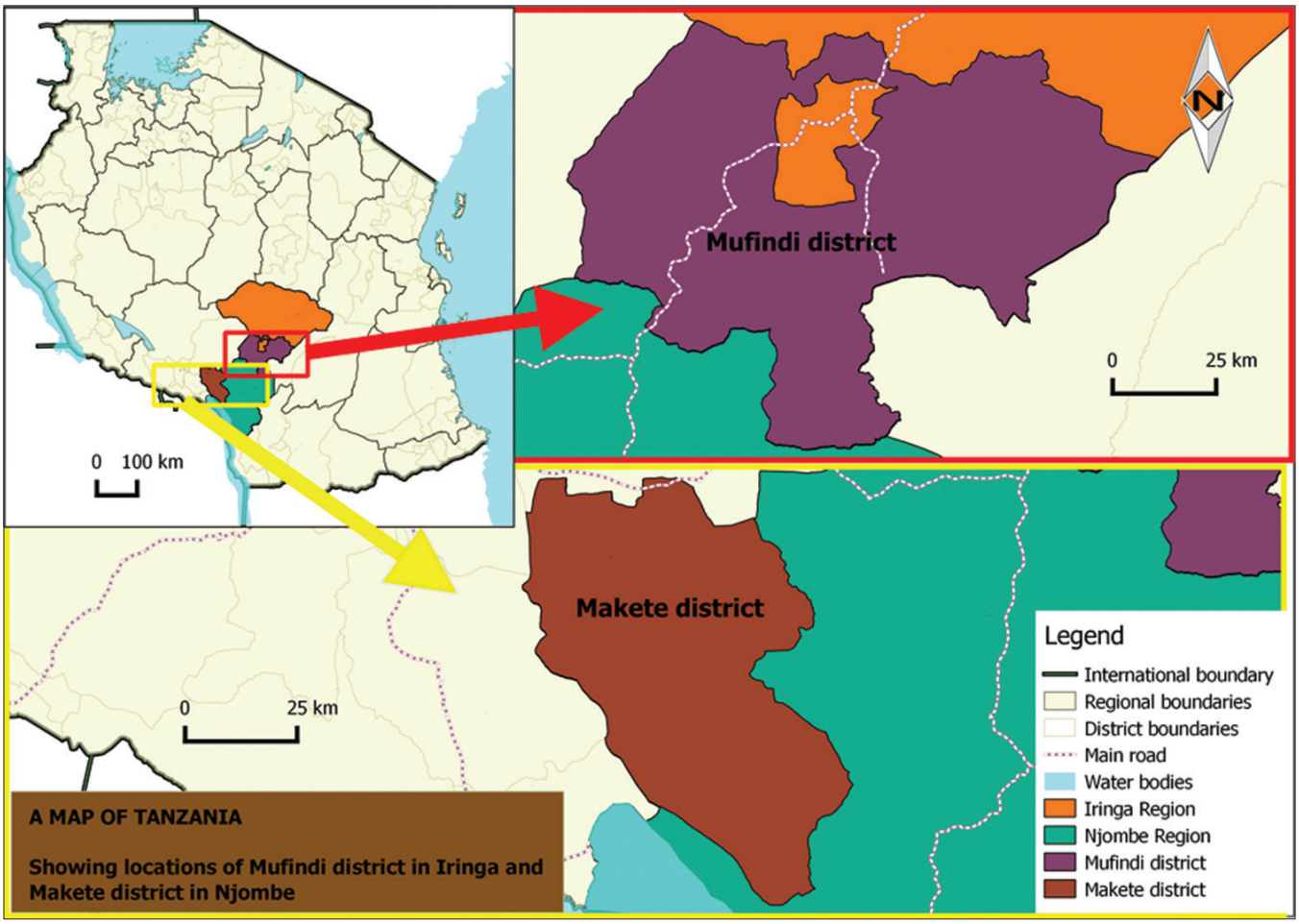

Figure 1: Map of Tanzania showing the study areas (Mufindi and Makete Districts) Ethnobotanical survey

cyclophosphamide (16.3 μg/ml). Other similar study undertaken on stem barks of this plant to evaluate cytotoxicity and acute toxicity in mice demonstrated the toxicity of the plant resulting in mortality of tested organisms [104]. The genus *Croton* has been reported to demonstrate moderate to high toxicities with proven the anticancer activity [51]. This knowledge triggers the use of plant products as complementary and alternative therapies both as direct and adjuvant remedy. A growing body of literature suggests the cancer preventive and therapeutic potential of phytochemicals and a lot of research has focused on the cellular mechanisms by which these phytochemicals interfere with the carcinogenic process. With the ability to target a variety of signaling pathways, phytochemicals are considered to be promising therapeutic agents against tumors with limited toxicity to normal cells.

CONCLUSION

The ethnobotanical survey has revealed that traditional health practitioners are rich in knowledge of fungal medicinal plants in these areas. These plants though have received little attention from modern biomedical research could be a promising source

of knowledge for the discovery of useful remedies if this wealth is preserved through proper documentation and research. Most of the plants collected were ascertained to be safe for use and hence could be considered for further scientific studies. The reported species may be used for the development of new, affordable, and effective herbal formulations for antifungal health-care management or used in drug discovery.

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Original Research

An active principle of *Nigella sativa* L., thymoquinone, showing significant antimicrobial activity against anaerobic bacteria

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ABSTRACT

Aim/Background: Thymoquinone (TQ) is the major active principle of *Nigella sativa* seed (black seed) and is known to control many fungi, bacteria, and some viruses. However, the activity of TQ against anaerobic bacteria is not well demonstrated. Anaerobic bacteria can cause severe infections, including diarrhea, aspiration pneumonia, and brain abscess, particularly in immunodeficient individuals. The present study aimed to investigate the *in vitro* antimicrobial activity of TQ against some anaerobic pathogens in comparison to metronidazole. **Methods:** Standard, ATCC, strains of four anaerobic bacteria (*Clostridium difficile, Clostridium perfringens, Bacteroides fragilis,* and *Bacteroides thetaiotaomicron*), were initially isolated on special *Brucella* agar base (with hemin and vitamin K). Then, minimum inhibitory concentrations (MICs) of TQ and metronidazole were determined against these anaerobes when grown in *Brucella* agar, using serial agar dilution method according to the recommended guidelines for anaerobic organisms instructed by the Clinical and Laboratory Standards Institute. **Results:** TQ showed a significant antimicrobial activity against anaerobic bacteria although much weaker than metronidazole. MICs of TQ and metronidazole against various anaerobic human pathogens tested were found to be between 10-160 mg/L and 0.19-6.25 mg/L, respectively. **Conclusions:** TQ controlled the anaerobic human pathogenic bacteria, which supports the use of *N. sativa* in the treatment of diarrhea in folk medicine. Further investigations are in need for determination of the synergistic effect of TQ in combination with metronidazole and the activity of derivatives of TQ against anaerobic infections.

KEY WORDS: *Brucella* blood agar, metronidazole, minimum inhibitory concentrations, *Nigella sativa*, thymoquinone

INTRODUCTION

Thymoquinone (TQ) is the major active principle of *Nigella sativa* L. seed. This seed is commonly named as "Al-Habbah Al-Sawda" in Arabic and "black seed" in English language [1]. Black seed is a commonly used herbal medicine for many ailments in Arab countries, Middle Asia, and the Indian Subcontinent [2].

TQ is known to have many pharmacological activities, to include anticancer, anti-inflammatory, antiasthmatic, antidiabetic, antihypertensive, and hypolipidemic, and antimicrobial effects [2-4]. The antimicrobial activity of TQ and various extracts of *N. sativa* has been reported against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*, and *Listeria monocytogenes* [5-8].

Microorganisms are becoming resistant to many antibiotics. Therefore, there is need to find new remedies against pathogenic microbes [9]. Black seed extracts were found to be effective against some resistant microorganisms, such as *S. aureus* and *P. aeruginosa* [9,10].

Anaerobic bacteria can cause serious infections, particularly in immunocompromised individuals, for example, elderly, diabetics, and those suffering from HIV infection and using anticancer chemotherapy, immune suppressant drugs, or broad spectrum antibiotics. Anaerobes have been reported to cause aspiration pneumonia, lung abscess, and emphysema [11-13]. Moreover, they have been shown to cause brain abscess and bacterial meningitis [14-16]. They are generally resistant to many antibacterial drugs and are known to develop biofilm around them [17]. Metronidazole is considered as a drug of choice for the treatment of anaerobic infections but can cause agranulocytosis [18].

Because of the scarcity of studies for the activity of black seed or its active components against anaerobic bacteria, the present study has been designed to investigate the activity of TQ, *in vitro*, against anaerobic human pathogenic strains, including *Clostridium difficile*, *Clostridium perfringens*, *Bacteroides fragilis*, and *Bacteroides thetaiotaomicron* by standard antimicrobial assay and compare it with that of metronidazole.

METHODS

Microorganisms

Standard, ATCC, strains of *C. difficile*, *C. perfringens*, *B. fragilis*, and *B. thetaiotaomicron* were purchased from Danat Alajiyal for Medical and Scientific Equipment (Saudi Arabia). These strains were initially grown on special *Brucella* agar base (with hemin and vitamin K), supplemented with 5% laked or defibrinated sheep blood in Petri plates and identified by conventional methods.

Chemicals

The materials for the culture media used in the study were purchased from Micromaster and Himedia (Saudi Arabia), TQ from Sigma-Aldrich (Saudi Arabia) and anaerobic jar, anaerobic gas pack and indicator from Becton Dickinson (Saudi Arabia). Metronidazole IV fluid (Flagyl from Pfizer, USA) was obtained from the Pharmacy Department of Prince Abdulaziz Bin Mosad Hospital, Arar, Saudi Arabia.

Stock Solutions and Serial Dilutions

Stock solution of TQ 64 mg/ml was prepared in DMSO and water. From the stock solution, serial dilutions of TQ 32, 16, 8, 4, 2, 1, 0.5, and 0.25 mg/ml were prepared in 5 ml sterile test tubes. Then, 100 μl from each diluted concentration of TQ was added to 20 ml of molten *Brucella* agar base (with hemin and vitamin K), supplemented with 5% defibrinated sheep blood, giving final concentrations of TQ 160-1.25 μg/ml (160, 80, 40, 20, 10, 5, 2.5, and 1.25 μg/ml) in the Petri plates (three plates for each concentration level).

Stock solution of metronidazole (Flagyl) contained 500 mg of metronidazole in100 ml of water (5 mg/ml), which was serially diluted down to 0.035 mg/ml (5, 2.5, 1.25, 0.625, 0.31, 0.15, 0.07, and 0.035 mg/ml) in 5 ml sterile test tubes. Then, 100 μl from each diluted concentration of metronidazole was added to 20 ml of molten *Brucella* agar base (with hemin and vitamin K), supplemented with 5% defibrinated sheep blood, giving final concentrations of metronidazole 25-0.195 μg/ml (25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 μg/ml) in the Petri plates (three plates for each concentration level).

The ranges of serial dilutions of TQ and metronidazole in *Brucella* agar given above were chosen from the results of the pilot study. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines for the *Brucella* agar method, metronidazole $\leq$8 μg/ml is considered as sensitive, 16 μg/ml as intermediate, and $\geq$32 μg/ml as resistant.

Minimum Inhibitory Concentration (MIC) Value Determination Assay

The MICs of TQ and metronidazole against the tested strains were determined by the standard method recommended by the CLSI. In each Petri plate (Either containing TQ 160- 1.25 ug/ml, or metronidazole, 25-0.195 ug/ml), the standard inoculum of (1 μl) of 0.5 MacFarland (10^5 CFU) was spot inoculated. Three Petri plates containing 20 ml *Brucella* agar (with supplements) without TQ or metronidazole were also inoculated with the standard inoculum of each test strain as controls. All plates were incubated anaerobically for 42-48 h and the bacterial growth was observed.

RESULTS

The results of the antibacterial activity of various concentrations of TQ are depicted in Table 1 and Figures 1-4, which reveal that *C. difficile* was the most sensitive among the anaerobes tested, with intermediate sensitivity to TQ 10 and 20 μg/ml and completely sensitive to TQ 40 μg/ml, giving an MIC of 40 μg/ml. Whereas, *C. perfringens*, *B. fragilis* and *B. thetaiotaomicron* were relatively less sensitive to TQ, with MICs of 160 μg/ml.

The results of the antibacterial activity of various concentrations of metronidazole are given in Table 2, which reveal that *C. difficile* was again the most sensitive to metronidazole (MIC 0.78 μg/ml), followed by *B. fragilis* and *B. thetaiotaomicron* (MICs 3.12 μg/ml), while *C. perfringens* was least sensitive (MIC 6.25 μg/ml).

Table 1: Antibacterial activity of thymoquinone against anaerobic human pathogenic strains

Reference strains	Thymoquinone (μg/ml)							
	160	80	40	20	10	5	2.5	1.25
C. perfringens ATCC 13124	S	I	R	R	R	R	R	R
C. difficile ATCC 700057	S	S	S	I	I	R	R	R
B. fragilis ATCC 25285	S	R	R	R	R	R	R	R
B. thetaiotaomicron ATCC 29741	S	R	R	R	R	R	R	R

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: Bacteroides thetaiotaomicron, S: Sensitive, I: Intermediate sensitivity, R: Resistant

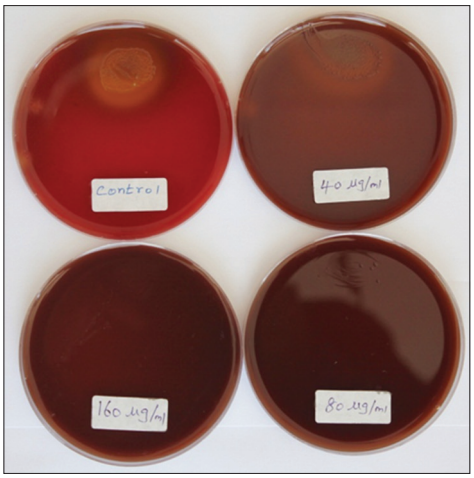

Figure 1: Growth of *Clostridium perfringens* in different concentrations of thymoquinone in Brucella agar

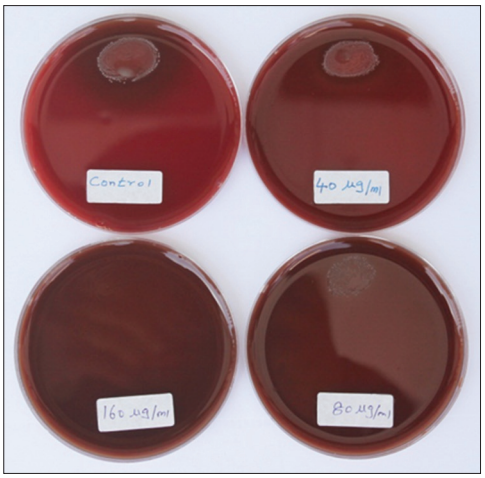

Figure 3: Growth of *Bacteroides fragilis* in different concentrations of thymoquinone in *Brucella* agar

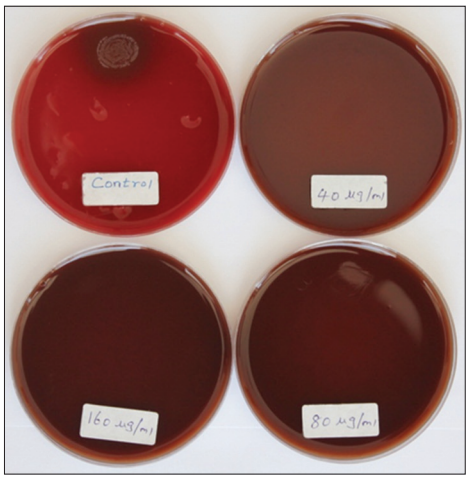

Figure 2: Growth of *Clostridium difficile* in different concentrations of thymoquinone in *Brucella* agar

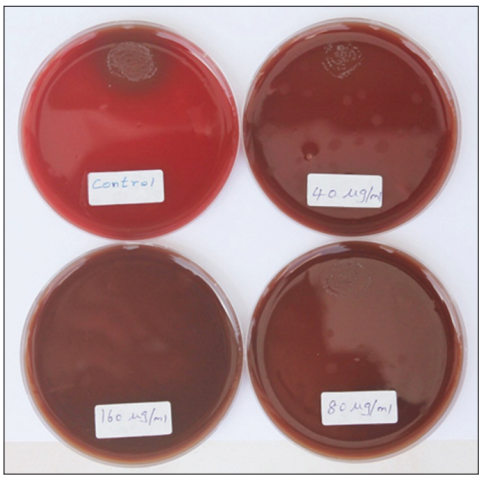

Figure 4: Growth of *Bacteroides thetaiotaomicron* in different concentrations of thymoquinone in *Brucella* agar

A summary of the results of estimated MICs for TQ and metronidazole against test anaerobes is given in Table 3. TQ showed significant antibacterial activity against anaerobic bacteria used in the study, particularly against *C. difficile*, although much weaker than metronidazole.

DISCUSSION

Anaerobic bacteria are normal commensals and reside in human skin and mucous membranes, thus may cause endogenous infections, such as diarrhea, aspiration pneumonia, lung abscess, brain abscess, and meningitis [11-16]. Metronidazole is very effective and commonly used for the treatment of anaerobic infections but unfortunately is relatively more toxic and can cause serious adverse effects, including agranulocytosis [18]. Besides metronidazole, other effective antibiotics against anaerobic bacteria are the carbapenems (imipenem and meropenem), chloramphenicol, the combinations of penicillin and beta-lactamase inhibitor (ampicillin plus sulbactam, ticarcillin plus clavulanate, and piperacillin plus tazobactam), tigecycline, and clindamycin [19]. Unfortunately, like other bacteria, anaerobes are gradually becoming more resistant to antibiotics. The most frequently isolated antibiotic-resistant anaerobe is *B. fragilis*, but the *Clostridium* species and other anaerobes are also becoming increasingly resistant [20]. In the present study, also, MICs of both TQ and metronidazole against *B. fargilis* were relatively higher than against *C. difficile*.

Because of the limited published work for the antibacterial activity of TQ against anaerobic human pathogens, we could not find similar studies to compare our results. However, there was one study reported in the literature regarding the effect of TQ on foodborne anaerobic bacteria and the results of our study were not much different from that (MIC of TQ against *Clostridium* species was from 5 to 10 μg/ml in the former study while, in our study, it was from 10 to 40 μg/ml for *C. difficile* [21].

The activity of TQ against anaerobic human pathogens is much less than metronidazole. However, derivatives of TQ could be prepared and tested for their activity against

Table 2: Antibacterial activity of metronidazole against anaerobic human pathogenic strains

Reference strains	Metronidazole (μg/ml)							
	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19
C. perfringens ATCC 13124	S	S	S	I	R	R	R	R
C. difficile ATCC 700057	S	S	S	S	S	S	I	R
B. fragilis ATCC 25285	S	S	S	S	I	R	R	R
B. thetaiotaomicron ATCC 29741	S	S	S	S	I	R	R	R

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: Bacteroides thetaiotaomicron, S: Sensitive, I: Intermediate sensitivity, R: Resistant

Table 3: MIC of thymoquinone and metronidazole against anaerobic human pathogens

Microorganisms	Thymoquinone (μg/ml)	Metronidazole (μg/ml)
	MIC	MIC
C. perfringens ATCC 13124	80-160	1.56-6.25
C. difficile ATCC 700057	10-40	0.19-0.78
B. fragilis ATCC 25285	80-160	0.78-3.12
B. thetaiotaomicron ATCC 29741	80-160	0.78-3.12

C. perfringens: Clostridium perfringens, C. difficile: Clostridium difficile, B. fragilis: Bacteroides fragilis, B. thetaiotaomicron: *Bacteroides thetaiotaomicron*, MIC: Minimum inhibitory concentrations

anaerobes and might be as effective as metronidazole, because some derivatives of TQ were demonstrated to be much more effective than TQ itself against cancer cell lines. For example, some analogs of TQ, when tested for their biological activity against pancreatic cancer cell lines, were found to be more potent than TQ in terms of inhibition of cell growth, induction of apoptosis, and modulation of transcription factor [22].

C. difficile is one of the most important microorganisms causing health-care-associated diarrhea, i.e., diarrhea secondary to the use of broad-spectrum antibiotics, cancer chemotherapy, and immune suppressant drugs. Unfortunately, data reporting the decreased effectiveness of metronidazole in the treatment of severe disease due to *C. difficile* have also been published [23]. In the present work, TQ was found to be relatively more active against *C. difficile* and further studies could be designed to investigate effectiveness of the combination of TQ with metronidazole for the control of health-care-related diarrhea.

CONCLUSIONS

TQ, the most abundant active principle of *N. sativa*, was shown to possess a reasonable activity against anaerobic human pathogens. TQ was, particularly, more effective against *C. difficile*, which is relatively common to cause diarrhea in immunocompromised individuals and those taking broad-spectrum antibiotics. Regular use of black seed could prevent infection from *C. difficile*. The study also supports the use of black seed in the treatment of diarrhea in folk medicine.

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Original Research

Pregnancy detecting plants used in Remo and Ijebu areas of Ogun State, Nigeria

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ABSTRACT

Aim/Background: Plants and plants extracts are employed in cultures for religious purposes, as beauty therapies, in the detection and management/treatment of diseases. **Materials and Methodology:** In this study, an ethnobotanical studies of plants used in detecting pregnancy in Ijebu and Remo areas of Ogun State were carried out using semi-structured to obtain demographic data, local names of plants, the morphological parts used. Furthermore, a phytochemical analysis of two of the identified plants was performed. Topical and urine tests of plants in detecting pregnancy were designed to mimic procedures used in traditional medicine for detecting pregnancy. **Results:** Five plant species were identified belonging to the families Araceae, Asteraceae, Convolvulaceae, Nyctaginaceae, and Rubiaceae in the survey. The identified plants had the use value (UV) of 0.25 (*Culcasia scandens*), 0.17 (*Ipomoea mauritiana*), *Boerhavia diffusa* while *Launea taraxacifolia* and *Chassalia kolly* had the UV of 0.08, respectively. *B. diffusa* L, *C. kolly* (Schumach) Hepper tested positive for the presence of flavonoids, alkaloids, and tannins. The onset and duration of symptoms of both *B. diffusa* and *C. kolly* leaves at 2000 and 1000 mg were dose-dependent. The hexane, ethyl acetate, and ethanol extracts of *B. diffusa* and *C. kolly* exhibited pruritus and restlessness in the *in vivo* model while the urine of pregnant women caused black spots on the leaves of *L. taraxacifolia* (Willd) Amin Ex. C. Jeffrey. **Conclusion:** This study reports a rare knowledge of using plants in detecting pregnancy in the Remo and Ijebu areas of Ogun State, Nigeria.

KEY WORDS: Detection, extracts, plants, pregnancy, Remo and Ijebu areas

INTRODUCTION

Procreation is an essential aspect of every culture and each of these cultures have people who have mastered the traditional obstetrics and gynecology particularly treatment of women during pregnancy and child birth. There are some studies who have reported the use of plants and plant materials in women health care particularly during pregnancy and child delivery [1].

In the African culture, particularly South West Nigeria, from the time of detecting pregnancy to the delivery of the child, the use of various plants and plant products are administered. This is because it is believed that these various herbs or plant products will assist the expecting mother in gaining strength, for blood formation as well as ease labor and believed it will enable mother and child to be healthy.

Although, there are knowledge about the use of plants in detecting pregnancy, this has been shown in the ancient Egyptian culture where the wheat and barley test is used and claimed to be 70% accurate, the use of pregnancy test strips and assay has eroded the traditional knowledge of the plants used in various culture in detecting pregnancy. The knowledge on the use of plants in detecting pregnancy in South West Nigeria is an art that is going extinct while in some cases, the plants used are no longer known. This gap of knowledge regarding traditional practice of detecting early pregnancy has, therefore, led to this study. This study aims at carrying out ethnobotanical studies on plants used in detecting early pregnancy, in the Remo and Ijebu areas of Ogun State, Nigeria. It will also carry out the phytochemical screening of two of the identified plants as well as investigate their pregnancy detecting potentials in designed *in vivo* and *in vitro* models.

METHODOLOGY

Study Area

The study was carried out in the Remo and Ijebu areas of Ogun State comprising Sagamu (6°50′N 3°39′E), Ikenne (6°52′N 3°43′E), Isara (7°00′N 3°41′E), Ago Iwoye (6° 57' N 3° 55' E), and Ijebu-Oru Ijebu Igbo (6° 58' N 4° 00'E).

Data Collection

Data were collected according to the Ethics of the International Society of Ethnobiology (http://www.ethnoniology.net/code of ethics) [2].

The study was carried out by administering semi-structured questionnaires [3,4] which were filled through a face-to-face interview [2,5]. The respondents were herb sellers, traditional medical practitioners (TMP), and midwives. The consent of each respondent was obtained before administering the questionnaire.

The demographic features of the respondents, vernacular names of the plants, mode of usage, and features expected were obtained. The plants mentioned by the respondents were collected and authenticated at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu Campus. The Botanical names of the plants were verified from literature and the International Plant Database (www.ipni.org).

Plant Materials

Collection and authentication

The leaves of *Culcasia scandens*, *Ipomoea mauritiana*, *Boerhavia diffusa*, and *Launea taraxacifolia* were collected from Sagamu while the leaves of *Chassalia kolly* were collected from Ago-Iwoye in Ijebu North Local Government area of Ogun State. The plants were authenticated by Mr. Owolabi Ogunlana and voucher specimens of the plants are deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu Campus.

Phytochemical Screening

The phytochemical screening of two of the identified plants was carried out using the methods of Harborne [6].

Extraction

The leaves of *B. diffusa* and *C. kolly* were air dried and powdered in an electric blender. The leaves of the plant species were extracted by cold maceration successively with hexane, ethyl acetate, and ethanol. The extracts were each dried under reduced pressure in a rotary evaporator and stored for further studies.

Urine Test

In conjunction with the Department of Obstetrics and Gynecology, Olabisi Onabanjo University Teaching Hospital, Sagamu, women who had their pregnancy confirmed using pregnancy tests were included in this study. Apart from the pregnancy test carried out for the women in the laboratory, pregnancy was further confirmed using the pregnancy touch strip.

The leaf of *L. taraxacifolia* was used in the *in vitro* assay. The urine of women in their first trimester and who visited the Antenatal clinic for the first time were used. The consent of each of the pregnant women was obtained, and they were explained to the purpose of the study.

Urine of the women was collected in universal bottles. The method adopted is that used traditionally in which urine is poured in a container on the leaf. The development of spots on the leaves is believed to be a confirmation of pregnancy. This method is similar to the "wheat and barley" method used in the ancient Egypt for detecting pregnancy.

In this study, pregnancy test strips were used to confirm pregnancy before the "spot method" was used.
Group 1: Pregnant women at antenatal clinic for the first time (first trimester pregnancy).
Group 2: Nonpregnant females.

This study observed the appearance of dark spots and how long it took spots to appear on the leaves in the urine test.

Topical Test

Animals used in this study were handled according to the International Guidelines on animal care.

The topical test model used was designed to mimic that used traditionally in human beings. In traditional medicine, the female could be asked to crush the leaves of *B. diffusa* or *C. kolly* or wash her hands with some of the aqueous extracts. Signs of itching and burning sensation are usually used traditionally to confirm pregnancy.

Two of the plants identified in this ethnobotanical study (*B. diffusa* and *C. kolly*) were used for the *in vivo* model.

Female rats of about 12 weeks old were used ($n = 35$). The females were allowed to mate and pregnancy determined within first 7 days by microscopic method.
Group A: 2000 mg of extract was applied topically on the ears and around the mouth of pregnant female rats.
Group B: 1000 mg of extract was applied topically on the ears and around the mouth of pregnant female rats.
Group C: 2000 mg of extract was applied topically on the ears and around the mouth of nonpregnant.
Group D: Water applied topically on the ears and around the mouth of pregnant rats.

The rats were observed for the time it took the animals to first exhibit itching or any reaction as well as how long the reaction lasted.

Statistical Analysis

The demographic information of the respondents is presented as percentage while the onset and duration of symptoms are presented as mean ± standard deviation. The use value (UV) of the species identified were determined [5,7] using the formula:

UV = Um/n

Um = Total number of use report per species
n = Total number of informant interrogated for each given plant.

RESULTS

The respondents used in this study were Traditional Medical Practitioners (TMP), Herb sellers and traditional midwives from Isara, Ijebu Oru, Ago-Iwoye, Ijebu-Igbo, Ikenne and Sagamu in the Remo and Ijebu areas of Ogun State, Nigeria. Figure 2.

Five plants species from the families Araceae (Culcasia scandens), Asteraceae (Launea taraxicifolia), Convolvulaceae (Ipomoea mauriiana), Nyctaginaceae(Boerhavia diffusa) and Rubiaceae (Chassalia kolly) were identified as used in the studied areas for detecting pregnancy. (Table 1)

DISCUSSION

In every culture, particularly in Africa, conception and procreation are important because they are considered as part of indices of success in marriage as well as generations to whom traditions and norms can be passed on to. The knowledge of which is becoming eroded and lost because of the modern methods of detecting pregnancy.

The area studied was the Remo and Ijebu areas of Ogun State [Figure 1] and questionnaires were administered in six towns to TMP, herb sellers and traditional midwives [Figure 2]. 73% of the respondents were women and most of the respondents (42%) have primary school leaving qualification. About 67% of the respondents claimed that they obtained information on plants used in detecting pregnancy through learning by apprenticeship [Figure 3].

Five plant species from five families whose leaves are used for detecting pregnancy were identified in this survey. *C. scandens* had the highest UV of 0.25 while *C. kolly* and *L. taraxacifolia* had the lowest UV of 0.08 [Table 1].

The phytochemical screening of *B. diffusa* revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins, and the absence of cardiac glycosides [Table 2]. This is similar to previous phytochemical studies carried out on the leaves of *B. diffusa* obtained from Kerala, India [8] while *C. kolly* showed the presence of alkaloids, tannins, cardiac glycosides and lacked saponins, anthraquinone, flavonoids, and steroids. This is also similar to reports of

Figure 1: Map of studied areas

Table 1: Plants used in detecting pregnancy in Remo and Ijebu areas of Ogun State

Family	Botanical name	Common name	Vernacular name	Morphological part used	UV
Araceae	*Culcasia scandens* P. Beauv	Climbing arum	Agunmona	Leaf	0.25
Asteraceae	*Launea taraxacifolia* (Willd) Amin Ex. C. jeffrey	Wild lettuce	Yanrin	Leaf	0.08
Convolvulaceae	*Ipomoea mauritiana* Jacq	Natal cotton plant/giant potato	Atemole	Leaf	0.17
Nyctaginaceae	*Boerhavia diffusa* L	Red Spiderling	Etiponla	Leaf	0.17
Rubiaceae	*Chassalia kolly* (schumach) Hepper		Isepe agbe	leaf	0.08

Table 2: Phytochemical screening of *Boerhavia diffusa* and *Chassalia kolly* used in detecting pregnancy

Plant	Saponins	Alkaloids	Anthraquinone	Flavonoids	Tannins	Cardiac glycosides	Steroids
Boerhavia diffusa	+	+	-	+	+	-	+
Chassalia kolly	-	+	-	-	+	+	-

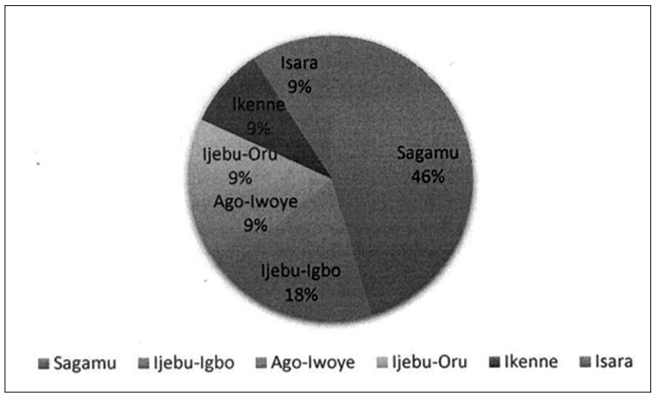

Figure 2: % distribution of respondents in the ethnobotanical study

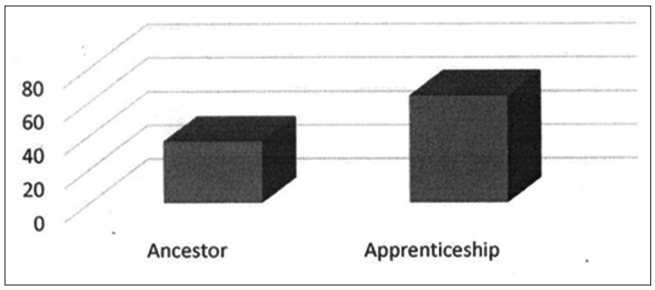

Figure 3: Respondents source of information on plants used in detecting pregnancy

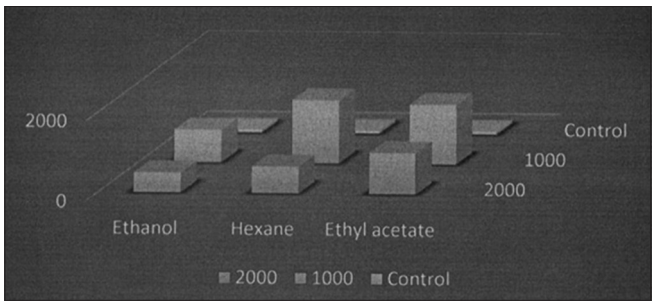

Figure 4: Onset of symptoms of *Boerhavia diffusa*

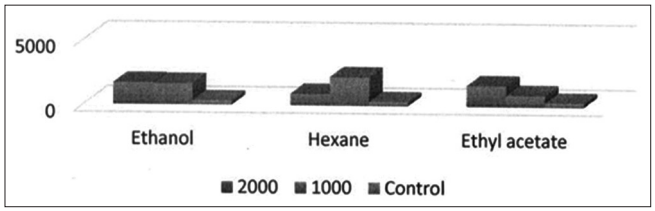

Figure 5: Duration of symptoms of *Chassalia kolly* on pregnant rats

Onocha and Ali [9]. Although, the phytochemical analysis of *L. taraxacifolia* was not carried out in this study, previous studies had reported the presence of alkaloids, tannins, and flavonoids [10].

In the topical test, the hexane, ethyl acetate, and ethanol extracts were used. The onset of symptoms of the hexane extract of *B. diffusa* was the shortest compared to that of the ethanol extract, however, the duration of the symptoms of the ethanol extract at 2000 mg and 1000 mg were shorter than that of the hexane and ethyl acetate extracts [Figure 4]. The onset of symptoms exhibited by the hexane extract of *C. kolly* is similar in terms of the time of onset and duration of symptoms to that of *B. diffusa* [Figure 4 and 5].

Traditionally in pregnant women, the symptoms often observed when these plants extracts are used include severe pruritus, itching, and restlessness which can be managed traditionally by applying palm oil to affected areas. In this study, the ethanol and hexane extracts caused immobility on application of the extracts on the animals shortly before itching commenced. Other symptoms are tremor and standing up of fur while in the ethyl acetate extract, immobility occurred in the animals after itching had stopped.

The leaves of *C. kolly* caused itching, twitching, restless, tremor, standing erect of furs, and whiskers as well as pink coloration of the eyes which were dimmed.

The symptoms exhibited on the topical application of the extracts of both *B. diffusa* and *C. kolly* are similar to those caused by abnormal discharges in the brain. This study shows that the extracts of both *C. kolly* and *B. diffusa* are easy absorbed and transported to the brains to cause such effect.

Furthermore, the *in vitro* assay used in this study is similar to the "wheat and barley" test used in detecting pregnancy in women in ancient Egypt.

The minimum time it took the black spots to appear on the leaves of *L. taraxacifolia* was 10 min [Figure 6].

Although, the exact mechanism of action responsible for these reactions in the studied plants is unknown, *B. diffusa*, *C. kolly*, and *L. taraxacifolia* all possess alkaloids, tannins and flavonoids. It, therefore, shows the possibility of one or a combination of these secondary metabolites reacting with the HgC hormone in pregnant women.

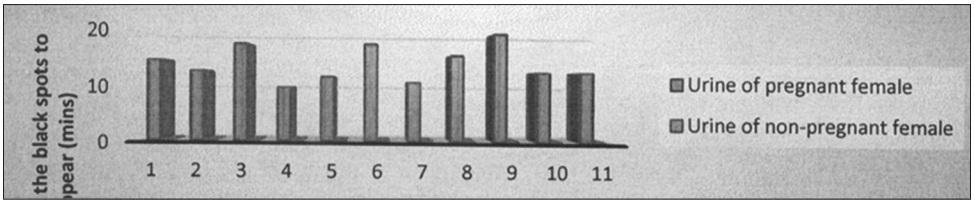

Figure 6: Black spot effect of urine of pregnant and nonpregnant females on *Launea taraxacifolia* leaves

This study has, therefore, been able to document plants used in detecting pregnancy in the Remo and Ijebu areas of Ogun state. Further studies are, however, ongoing to validate the constituents in these plants responsible for the activities.

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Original Research

Antioxidant activity, phenolic-flavonoid content and high-performance liquid chromatography profiling of three different variants of *Syzygium cumini* seeds: A comparative study

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ABSTRACT

Background: The medicinally important phytochemicals present in *Syzygium cumini* seeds probably accounts for its wide use in traditional systems of medicines in India, like Ayurveda, Unani, and Siddha. **Aim:** The aim of the study was to determine the antioxidant potential of three different geographical variants of *S. cumini* seeds and to compare the phenolic profiling to know the effect of geographical variation in phenolic composition. **Materials and Methods:** Total phenolic and flavonoid content of *S. cumini* seeds were analyzed. Antioxidant activities in terms of 2,2-diphenyl-1-picrylhydrazyl, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), nitric oxide and superoxide radical scavenging assays were performed. The most active fractions were subjected to high-performance liquid chromatography (HPLC) profiling to identify the phenolic composition. **Results:** Among all the fractions, 70% methanol fraction of S. cumini seed showed significant antioxidant potential. There existed a linear correlation between phenolic content and antioxidant activity. HPLC profiling of 70% methanol (ME) fractions of all the variants revealed the presence of phenolic compounds with high concentrations of ellagic acid and gallic acid. The differences in phenolic concentration due to geographical changes might be the reason for higher antioxidant potential showed by 70% ME of Trivandrum variant. **Conclusion:** 70% methanolic fraction of *S. cumini* can act as a novel source of natural antioxidant.

KEY WORDS: Antioxidant activity, high-performance liquid chromatography, total flavonoid content, total phenolic content, *Syzygium cumini*

INTRODUCTION

The recent trend in the knowledge of free radicals and biology is producing a medical revolution that ensures a new age of health and disease management. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the predominant by-products of cellular redox processes. These free radicals possess both toxic and beneficial effects. ROS and RNS exert beneficial effects on cellular responses and immune function at low or moderate level. They generate oxidative stress at high concentrations, which is a deleterious process that can damage cell structure and function [1]. Oxidative stress is an important factor in the progression of chronic degenerative diseases including coronary heart disease, cancer, and arthritis [2]. The human body can counteract this oxidative stress with the support of exogenous antioxidants and by producing various endogenous antioxidants. For the past few decades, the secondary metabolites from plants have been well known for their antioxidant potential.

Medicinal plants are an important source of antioxidants. Phenolic compounds from natural products are gaining importance because of their relatively safe and wide acceptance by consumers. The increasing interest in the search for natural replacements for synthetic antioxidants has led to the antioxidant evaluation of a number of plant species. Antioxidants have the ability to counteract the damaging effects of free radicals inside our body. If free radicals formed are left unchallenged, they would eventually lead to the etiology of a wide range of diseases.

Plant phenolics are the most fascinating antioxidants which include predominantly phenolic acids, flavonoids, and tannins. Researchers and food manufacturers have become more engrossed in polyphenols due to their potent antioxidant properties, their richness in the diet, and their ability to prevent

various oxidative stress associated diseases [3]. The inverse relationship between the dietary intake of fruits and vegetables and the chance of oxidative stress associated diseases has been partially accredited to phenolics [4]. The phenolic compounds in plants are reported to have antidiabetic, anticancer, anti-inflammatory, antimutagenic, antimicrobial, and other activities.

Syzygium cumini (L.) Skeels, belonging to the family Myrtaceae, is one of the best-known species and is often distributed in Asia (East India, Malaysia, and China). *S. cumini* is widely used in traditional systems of medicines in India, such as Ayurveda, Unani, and Siddha. Different parts of *S. cumini* are reported to have several medicinal properties like antidiabetic [5], antimicrobial [6], anti-inflammatory [7] and free radical scavenging potential [8,9]. The seeds have been reported to possess compounds such as jambosine, gallic acid (GA), ellagic acid, corilagin, 3,6-hexahydroxy diphenoylglucose, 1-galloylglucose, 3-galloylglucose, quercetin, β-sitoterol and 4,6 hexahydroxydiphenoylglucose [10,11].

The phytochemical content of active fraction is subject to large variations due to variety, age, maturity of the plants used, season, geo-agro-climatic conditions, agronomical practices, post-harvest handling, storage, processing, etc. The active principle thus can vary tremendously and that, in turn, would affect the biopotency. It is in this context that chemical profiling of the plants is important to produce products with consistent quality. Therefore, the aim of this present study was to compare the antioxidant activity and profiling of *S. cumini* seeds collected from three different geographical locations.

MATERIALS AND METHODS

Chemicals

GA, chlorogenic acid (ClA), caffeic acid, syringic acid, coumaric acid, ferulic acid (FA), ellagic acid, cinnamic acid, catechol, myricetin, quercetin, kaempferol, apigenin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), sodium carbonate, aluminum chloride, potassium acetate and all other chemicals and biochemicals unless otherwise noted were from Sigma (St. Louis, MO, USA). All the positive controls used were of high-performance liquid chromatography (HPLC) grade. Folin Ciocalteau reagent, Methanol and acetic acid of HPLC grade were supplied by Merck, Germany. All other chemicals used were of the standard analytical grade.

Plant Material

The fully mature *S. cumini* fruits were collected from Trivandrum - TVM (8° 29' N, 76° 59' E), Trichy - TCH (10° 48' N, 78° 41' E) and from Malampuzha - MPA (10.7° N, 76.6° E). The samples were authenticated by Dr. E. S. Santhosh Kumar, Technical Officer, Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Trivandrum, Kerala and voucher specimens (Collection No: SC-APNP-CSIR-100, 101 and 102) were deposited in the herbarium of JNTBGRI, Palode, Trivandrum, Kerala, India. Samples were collected during the month of April and stored at −80°C until processed.

Preparation of Plant Fractions

S. cumini seeds were separated from fruits and washed well using distilled water. The seeds thus obtained were dried in the oven at 40°C for 24 h, seed coats were removed, and seeds were coarsely powdered using a motor and pestle. 2 kg dry powder was fractioned sequentially with hexane (HE), ethyl acetate (EA), methanol (ME), 70% ME, and water (WE) at room temperature (27 ± 1°C). The extraction process was repeated till each solvent became colorless. These fractions were filtered through Whatman No. 1 filter paper. The fractions were evaporated in rotavapor and stored at 4°C, protected from light and humidity for further analysis.

Yield of Extracts

The extract (1 mL) was pipetted out to a pre-weighed petri dish and kept in the oven for 4 h at 100°C. The weight of the petri dish was then measured. The petri dish was kept in the oven till the weight become constant. The difference in weight of the petri dish gave the yield of extract in 1 mL.

Determination of Total Phenolic Content (TPC)

The TPC was determined by Folin–Ciocalteau method [12] with slight modifications. Briefly, different concentrations of fractions (20-100 μL) were taken, and 80 μL of Folin–Ciocalteau reagent and 200 μL sodium carbonate (20%) were added, made up to 700 μL using distilled water and incubated at ambient temperature (25-27°C) for 90 min. The color developed was measured at 760 nm using a multimode reader (Biotek, USA). The phenolic contents were calculated using a standard curve for GA, and the result was expressed as mg GA equivalents (GAE) per gram dry weight of fraction (mg GAE/g). All measurements were performed in triplicates.

Determination of Total Flavonoid Content (TFC)

The TFC was estimated using standard procedures described by Chang *et al*. [13] with slight modifications. Different concentrations of fractions were diluted with 150 μL of ethanol. Further, 10 μL of 10% aluminum chloride solution and 1 M potassium acetate (10 μL) was added and made up to 280 μL using distilled water. The final solution was mixed well and incubated at room temperature for 40 min. The absorbance was measured at 415 nm using a multimode reader (Biotek, USA). Quercetin was used as a standard, and results were expressed as mg quercetin equivalents (QE) per gram dry weight of fraction (mg QE/g).

Based on the antioxidant assays, TPC and TFC, the active fraction of all the variants was selected for further studies.

Antioxidant Assays

DPPH scavenging activity

The DPPH scavenging activity of different fractions was evaluated according to the method of Brand-Williams *et al.* [14]. 1 mL of 0.1 mM DPPH solution in ME was mixed with 1 mL of each fraction at varying concentrations. The corresponding blank sample was prepared, and GA was used as reference standard. Mixture of 1 mL ME and 1 mL DPPH solution was used as control. The mixture was shaken well and incubated for 30 min in the dark. The reaction was carried out in triplicate, and the decrease in absorbance was measured at 517 nm after incubation using a multiplate reader (Synergy 4 Biotek. USA). The scavenging activity was expressed as IC_{50} (μg/mL). The % inhibition was calculated using the formula:

$$\text{DPPH radical scavenging activity}\,(\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Nitric oxide (NO) scavenging activity

NO scavenging activity was estimated according to the method of Marcocci *et al.* [15] with slight modification. The reaction mixture contained 1 mL of 10 mM sodium nitroprusside (SNP), phosphate buffered saline (pH 7.4) and various concentration of fractions in a final volume of 1.1 mL. After incubation for 150 min at room temperature, pipetted out 100 μL into wells plate and 100 μL of Griess reagent was added. The mixture was incubated for 10 min at 25°C. The pink chromophore generated was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed in triplicates. Ascorbic acid was used as the standard. The percentage inhibition of NO radical generation was calculated using the following formula:

$$\text{NO radical scavenging activity}\,(\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Superoxide radical scavenging activity

Superoxide radical scavenging activity of different fractions was measured by the reduction of NBT according to a previously reported method [16]. The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals, which reduce nitro blue tetrazolium (NBT) to a purple formazan. The 1 mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), NADH (73 μM), NBT (50 μM), PMS (15 μM) and various concentrations of the sample solution. After incubation for 5 min at room temperature, the absorbance at 562 nm was measured against an appropriate blank to determine the quantity of formazan generated. Quercetin was used as the standard, and the percentage radical scavenging capacity was determined using the formula:

$$\text{Superoxide radical scavenging activity}\,(\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

ABTS scavenging activity

ABTS scavenging potential of each fraction were analyzed by the method of Arnao *et al.* [17] with some modifications. The working solution was prepared by mixing the stock solutions - 7 mM ABTS and 2.45 mM potassium persulfate solution in equal quantities, and allowing them to react for 14 h at room temperature in the dark. The solution was then diluted with ethanol to make the absorbance in the range 0.70 ± 0.01 units at 734 nm. Different concentrations of fractions were allowed to react with 1 mL of the ABTS solution for 7 min, and the absorbance was taken at 734 nm. The ABTS scavenging capacity of each fraction was compared with that of ascorbic acid, and percentage inhibition was calculated as:

$$\text{ABTS radical scavenging activity}\,(\%) = \left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

HPLC - Diode Array Detector (DAD) Analysis of Phenolic Compounds in 70% ME Fractions

The identification and quantification of phenolic compounds present in most active fractions of three variants of *S. cumini* were performed with a Shimadzu HPLC system containing two LC-8A preparative liquid chromatography pump units, a C18 reverse phase column (Phenomenex, 5 μm, 250×4.6 mm^2 dia.), and a (DAD; SPD-M10A VP) with a wavelength range of 200-450 nm. The fractions and 13 reference standards, namely, GA, ClA, caffeic acid, syringic acid, coumaric acid, FA, ellagic acid, cinnamic acid, catechol, myricetin, quercetin, kaempferol, and apigenin were prepared in HPLC grade ME at a concentration of 1 mg/mL and filtered through a 0.45 μm filter. Each sample (20 μL) were injected, and the HPLC analysis was done according to the standard method [18] with slight modifications. The mobile phase used was water: acetic acid (98:2, v/v) as solvent A and methanol:acetic acid (98:2, v/v) as solvent B with a time program of 0-15 min 15% B, 16-20 min 50% B, 21-35 min 70% B, 36-50 min 100% B. The flow rate was 1 mL/min and the column temperature was set at 30°C. Identification and quantification of the phenolic compounds were done by comparing the retention time and characteristic absorption spectra from the DAD with those of the authentic standards. To minimize variation in quantification, samples were taken in triplicates. Data acquisition and analysis were carried out using Shimadzu Class-VP version 6.14 SP1 software.

RESULTS

Yield of Extracts

Plants are rich in medicinally active and economically important compounds. Solvent extraction helps in segregating and

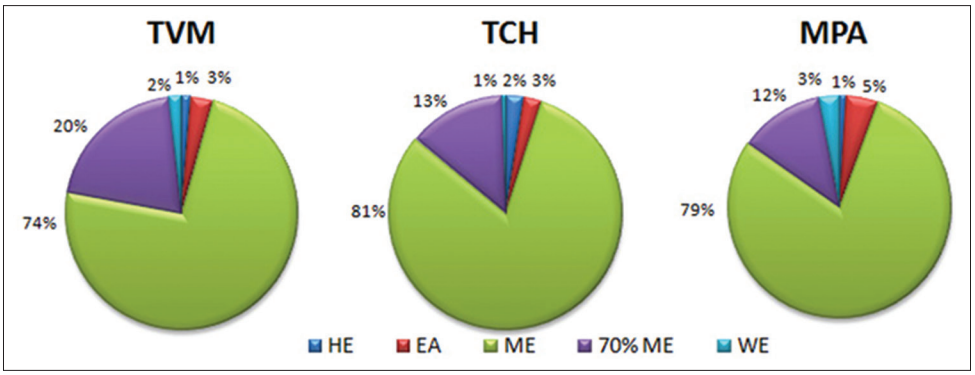

Figure 1: Graph showing percentage yield in different fractions of three different variants of *Syzygium cumini* seeds. TVM: Trivandrum variant, TCH: Trichy variant, MPA: Malampuzha variant

concentrating the active compounds. Initial extraction with hexane defatted the extract. EA, ME, 70% ME and water were sequentially used for the extraction. The yield of more than 70% was found for the methanolic fraction followed by 10-20% by 70% methanolic fraction of all the variants [Figure 1]. EA, hexane, and water fraction together constitute for only 6-8% yield in all the variants.

TPC

All the variants were initially analyzed for their total phenolic and flavonoid contents. The TPC of all fractions of *S. cumini* was expressed as milligram of GAE/g dry weight of fractions as represented in Figure 2a. All the fractions of *S. cumini* contained a significant amount of phenolic compounds. The highest phenolic content was exhibited by 70% methanolic fraction for all the three variants (TVM - 906 ± 7.2, TCH - 808.5 ± 3.9, MPA - 864.4 ± 5.6 mg GAE/g dry weight of fractions) and ME fraction of TVM (757.3 ± 6.2 mg GAE/g dry weight of each fraction). TPC increased in the following order for all the three variants: Hexane fraction <aqueous fraction <EA fraction <ME fraction <70% ME fraction [Figure 2a].

Total Flavonoid Content

Total flavonoid content for all the three variants of *S. cumini* was evaluated and had been represented in Figure 2b. Results revealed that 70% methanolic fraction of TVM variant possessed highest flavonoid content (233.8 ± 5.5 mg QE/g DW) compared to other variants. The total flavonoid content increased in the following order for all the three variants: Hexane fraction <aqueous fraction <EA fraction <ME fraction <70% ME fraction.

DPPH Radical Scavenging Activity

DPPH radical scavenging activity is one of the best methods to evaluate the antioxidant properties of natural products. DPPH• (1,1-Diphenyl-2-picrylhydrazyl radical) can accept an electron to become a stable diamagnetic molecule. The radical scavenging power of the sample was measured by the decrease in absorbance due to DPPH• at 517 nm, showing the formation of its reduced form, DPPH, which was yellow in color. The purple colored methanolic solution shows a strong absorption band at 517 nm due to the presence of odd electron.

In the present study, the DPPH radical scavenging activity increased in the following order- aqueous fraction <EA fraction <ME fraction <70% ME fraction. 70% ME fraction exhibited highest DPPH scavenging activity [Figure 3a]. The IC_{50} value of 70% ME fraction was found to be 5.1 μg/mL, 5.5 μg/mL, 6.2 μg/mL respectively for TVM, TCH, and MPA variants. However, the activity of each fraction was found to be less when compared to the standard, GA (1.8 ± 0.77 μg/mL). Methanol fraction also exhibited potential DPPH scavenging activity. The IC_{50} values for DPPH scavenging activity of EA, ME and water fractions of *S. cumini* seeds are represented in Figure 3a.

NO Scavenging Activity

Due to the presence of unpaired electron, NO is classified as a free radical and is a potent pleiotropic inhibitor of biological processes such as relaxation of smooth muscle, neuronal signaling and regulation of cell-mediated toxicity [19]. In addition to ROS, NO is also involved in inflammation, cancer, and other pathological conditions [20,21] and this free radical reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxynitrite. Peroxynitrite causes nitration or hydroxylation of aromatic compounds especially tyrosine and also triggers adduct formation with dissolved carbon dioxide in body fluids and damages various proteins [22]. SNP generates NO at physiological pH which reacts with oxygen to produce nitrite ions. A pink chromosphere was formed when nitrite ions react with Griess reagent, whose absorbance was measured at 540 nm.

The results from the assay illustrated that the 70% ME fraction of all variants demonstrated higher NO scavenging potential. The IC_{50} value of 70% ME fractions were 4.23 ± 0.34 μg/mL, 5.23 ± 0.24 μg/mL, 6.24 ± 0.31 μg/mL respectively for TVM, TCH, and MPA variants [Figure 3b]. The standard, curcumin, demonstrated NO radical scavenging potential with an IC_{50} value of 17.35 ± 2.3 μg/mL which was comparable to that of 70% ME fractions and ME fraction of TVM variant (IC_{50} value of 16.92 ± 2.3 μg/mL). The IC_{50} values for NO scavenging activity of EA, ME, 70% ME and aqueous fractions are represented in Figure 3b.

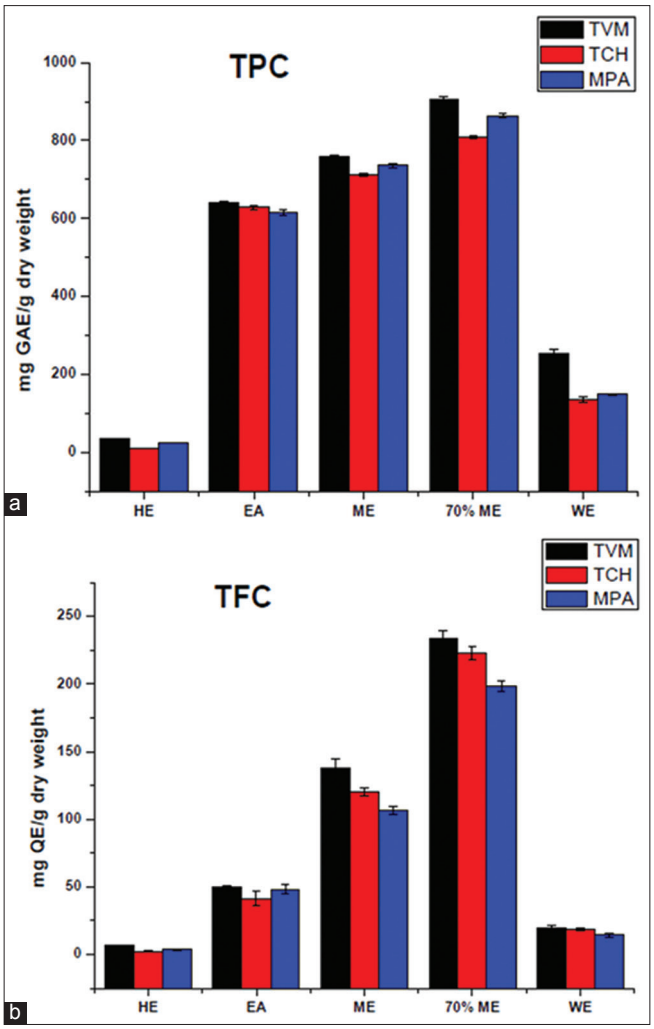

Figure 2: (a) Total phenolic content (mg GA equivalents/g dry wt.) (b) total flavonoid content (mg QE/g dry wt.) in different fractions of three different variants of *Syzygium cumini* seeds. Values are the means ± standard deviation of three replicated samples. Duncan's multiple range test was conducted and data in the same column with different letters/symbols indicate statistically significant differences among groups at $P < 0.05$. TVM: Trivandrum variant, TCH: Trichy variant, MPA: Malampuzha variant

Superoxide Radical Scavenging Activity

Superoxide anion radical is biologically quite toxic and is one of the strongest ROS among the free radicals and get converted to other harmful ROS such as hydrogen peroxide and hydroxyl radical, damaging biomolecules which results in chronic diseases [23]. The biological toxicity of superoxide is due to its capacity to inactivate iron-sulfur cluster containing enzymes, generate the highly reactive hydroxyl radical PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT [24]. The consumption of superoxide anion in the reaction mixture is indicated by the decrease in absorbance at 560 nm.

The results of the assay showed that 70% ME and ME fractions of all variants exhibited significant superoxide radical scavenging activity [Figure 3c] which was highly comparable with the standard, catechin, with an IC_{50} value 83.99 ± 2.34 μg/mL. The IC_{50} value of 70% ME fractions were 28.83 ± 2.14 μg/mL, 34.72 ± 1.24 μg/mL, 39.46 ± 2.64 μg/mL, respectively, for TVM, TCH and MPA variants. The IC_{50} value for superoxide radical scavenging activity of all fractions of all variants is represented in Figure 3c.

ABTS Scavenging Potential

ABTS is a protonated radical which has a characteristic maximum at 734 nm and the interaction with the fraction, or standard Trolox suppresses the absorbance of ABTS radical, and the results were expressed as Trolox equivalent antioxidant capacity value [Figure 3d].

The results from the study indicated that both ME and 70% ME fractions of all variants effectively scavenge ABTS radicals and the scavenging potential increased in a dose- dependent manner. The standard, trolox, exhibited an IC_{50} - 2.96 ± 0.87 μg/mL. Among the fractions 70% ME fraction of TVM variant exhibited highest ABTS scavenging ability (IC_{50} - 1.43 ± 0.05 μg/mL).

HPLC-DAD Analysis of Phenolic Compounds

The phenolic compounds of 70% ME fractions of three different variants were identified [Figure 4]. All fractions were individually spiked with each standard and recorded an increased peak height at almost same retention time, indicating the

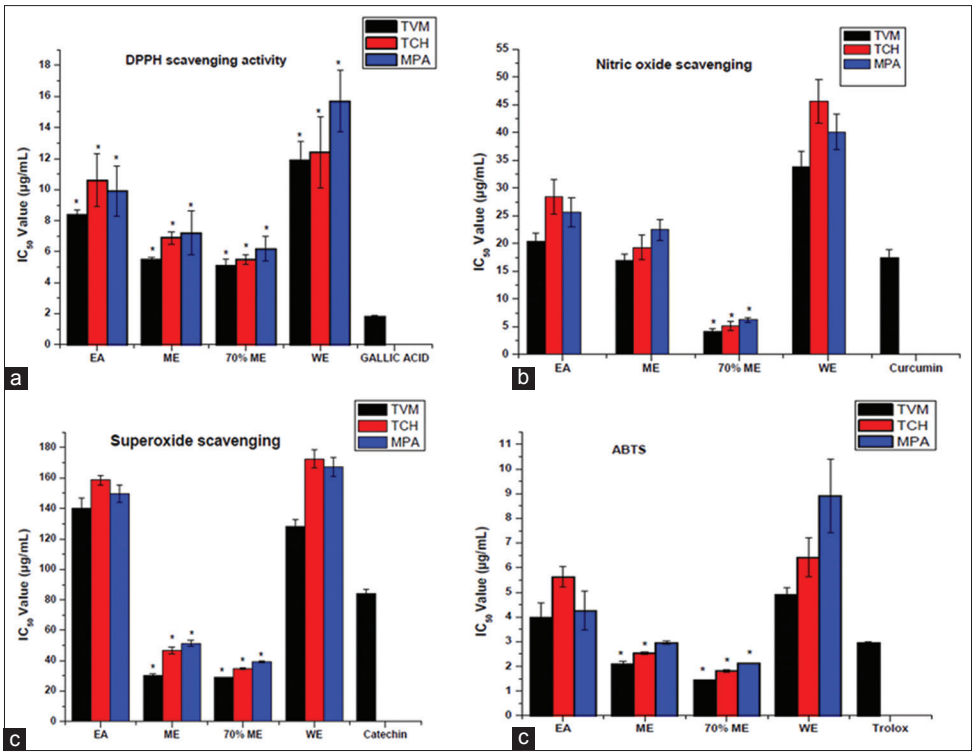

Figure 3: (a) 2,2-diphenyl-1-picrylhydrazyl (b) nitric oxide (c) super oxide (d) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activities of EA, ME, 70% ME and WEs of different variants of *Syzygium cumini*. EA: EA fraction, ME: ME fraction, 70% ME: 70% methanol fraction, WE: Water fraction, TVM; Trivandrum variant, TCH: Trichy variant, MPA: Malampuzha variant. Each value represents mean ± standard deviation (SD) from triplicate measurements. Values are means ± SD; $n = 3$; *Represents groups differ significantly from control group ($P \leq 0.05$)

presence of those compounds. The results showed that 70% ME fraction possessed the major quantity of phenolic acids and flavonoid compounds. Among the fractions, 70% ME fraction of TVM and MPA variants showed the highest concentration of ellagic acid (222.2 mg/g DW, 350.2 mg/g DW) and GA (272.2 mg/g DW, 341.24 mg/g DW, respectively). TCH variant (70% ME fraction) showed the presence of GA (21.6 mg/g DW), quercetin (13.2 mg/g DW) and other phenolic acids such as cinnamic acid, ClA, ellagic acid, and FA. The major phenolic compounds quantified from the active fraction of all the variants have been depicted in Figure 5.

DISCUSSION

In the present study, the antioxidant potential of *S. cumini* fractions of three geographical variants was assessed in terms of their phenolic and flavonoid content in addition to their free radical scavenging efficacies. Bajpai *et al*., [25] reported that 50% methanolic extract of seeds possessed 108.7 mg/g of TPC. An independent study using methanolic fraction of leaves had reported the presence of 610.32 mg/g of TPC and 451.5 mg/g of total flavonoid content [26]. In contrast, our results suggested higher TPC in 70% methanolic fraction of *S. cumini* seeds that were reported to contain in leaves, fruit, and pulp. This may be because sequential extraction of seeds using solvents would have fractionated phenolic and flavonoid compounds in 70% ME.

The radical scavenging activities such as DPPH, ABTS, NO, superoxide radical scavenging potential of the fractions were analyzed. The electron donating ability of *S. cumini* seed fractions were measured by the bleaching action of 1,1-Diphenyl-2-picrylhydrazyl radical purple colored solution. A study by Banerjee *et al*. [27] reported that ethanolic and methanolic fractions of *S. cumini* seeds showed a DPPH radical scavenging activity of 140 mg/GAE/g dry weight and 19.1 mg/g dry weight, respectively. From the results of the present study, the exhibition of higher DPPH radical scavenging activity demonstrated by 70% ME and ME fractions may be due to the presence of phenolic constituents that are more capable of donating hydrogen to a free radical and scavenge the radicals. In addition, 70% ME and ME fractions of all variants showed significant ABTS and NO radical scavenging potential which was significantly higher than the results reported by Lekha *et al*. (2013) [28] using EA fraction of *S. cumini* seeds. Although superoxide is a weak oxidant, powerful and dangerous hydroxyl radicals that can contribute to oxidative stress are generated from superoxides. Earlier reports on *S. cumini* fruit skin demonstrated IC_{50} value of 260 μg/mL for scavenging superoxide radicals [29]. The results from the present study demonstrated significant superoxide radical protection by 70% ME and ME fractions of all variants. The demonstration of significant radical scavenging activities (DPPH, ABTS, NO, and superoxide radicals) especially by the 70% methanolic fractions of all the variants of *S. cumini* seeds may be due to the presence of phenolic compounds in the fraction.

The phenolic profiling of active fractions of all the variants indicated the presence of prominent phenolic compounds which

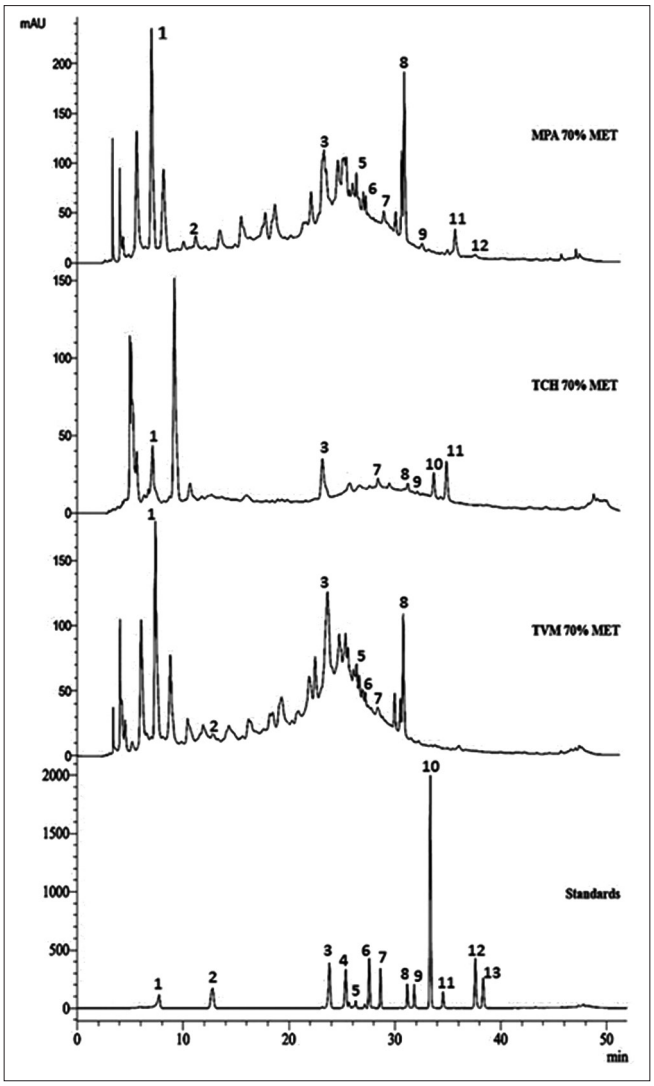

Figure 4: Representative high-performance liquid chromatography-diode array detector chromatograms of mixed standards and 70% methanol fractions of Trivandrum variant, Trichy variant and Malampuzha variant recorded at 280 nm. Standards are (1) gallic acid, (2) catechol, (3) chlorogenic acid, (4) caffeic acid, (5) syringic acid, (6) coumaric acid, (7) ferulic acid, (8) ellagic acid, (9) myricetin, (10) cinnamic acid, (11) quercetin, (12) kaempferol, (13) apigenin

were known for their antioxidant potential. GA, ellagic acid, ClA, and FA were found predominantly in 70% ME fraction of TVM variant. These phenolic acids have been very well known for the antioxidant activity [30-32], and these compounds were found in 70% methanolic fraction of all variants of *S. cumini* seeds. This difference in the composition of phenolics in 70% methanolic fractions of TVM variant may be responsible for its higher antioxidant efficacy when compared with the other variants. A positive correlation was reportedly observed between TPC and antioxidant activity [33,34]. A similar correlation between TPC and antioxidant activity was also reflected among the fractions in the present study.

In summary, 70% ME fraction of three variants of *S. cumini* seeds demonstrated significant radical scavenging activities which may be attributed to the higher levels of total phenolic compounds. TVM variant showed the best activity which can be attributed to the presence of highest phenolic content and relative composition of phenolics and flavonoids among all the variants.

CONCLUSION

The present study made an attempt to evaluate and compare the antioxidant potential of *S. cumini* fractions from different geographical locations of India and to correlate the activity with their phenolic content. The study demonstrated the fractionation of higher levels of phenolic compounds in 70% methanolic fractions of all variants resulting in increased radical scavenging potential. The active principle or individual polyphenols that may vary in their levels among the variants due to differences in geographical locations imparts significant contribution to their efficacy.

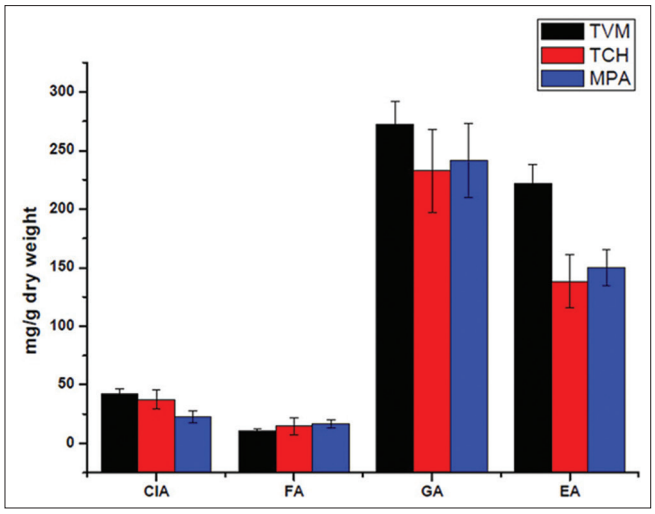

Figure 5: Prominent phenolic compounds (mg/g dry wt.) 70% methanol fractions of *Syzygium cumini* seeds quantified by high-performance liquid chromatography-diode array detector. Values are the means ± standard deviation of three replicated samples. GA: Gallic acid, ClA: Chlorogenic acid, FA: Ferulic acid, EA: Elagic acid. TVM: Trivandrum variant, TCH: Trichy variant, MPA: Malampuzha variant

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Original Research

Assessment of *in-vitro* cholinesterase inhibitory and thrombolytic potential of bark and seed extracts of *Tamarindus indica* (L.) relevant to the treatment of Alzheimer's disease and clotting disorders

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ABSTRACT

Background: Low level of acetylcholine (ACh) is an important hallmark of Alzheimer's disease (AD), a common type of progressive neurodegenerative disorder. Effective treatment strategies rely mostly on either enhancing the cholinergic function of the brain by improving the level of ACh from being a breakdown by cholinesterase enzymes. Again atherothrombosis is major life-threatening cerebral diseases. Traditionally *Tamarindus indica* (L.) has widely known for its medicinal values. Our aim is to investigate the cholinesterase inhibitory activities as well as thrombolytic activities of the bark and seeds crude methanolic extracts (CMEs) in the treatment of AD and clotting disorder. **Materials and Methods:** The crude methanol extract was prepared by cold extraction method and was assessed for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities by the Ellman's method. For thrombolytic activity clot lysis method was applied. **Results:** To compare both the fractions, extracts from the bark got more AChE inhibitory activity than the seed with the inhibitory concentration 50% IC_{50} values of 268.09 and 287.15 μg/ml, respectively. The inhibitory activity of BuChE was quiet similar to that of AChE as IC_{50} values of both the fractions were 201.25 and 254.71 μg/ml. Again *in-vitro* thrombolytic activity of bark was 30.17% and of seed it was 22.53%. **Conclusion:** The results revealed that the CME of bark and seed both have moderate cholinesterases inhibitory activities as well as thrombolytic activities, worth of further investigations to identify the promising molecule(s) potentially useful in the treatment of AD as well as in clotting disorders.

KEY WORDS: Alzheimer's disease, cholinesterase inhibition, clotting disorder, *Tamarindus indica*, thrombolysis

INTRODUCTION

With increasing medical advancements, reduction of birth rate and increase of life expectancy reflect age associated diseases such as neurodegeneration, cardiovascular (CVS) diseases, and other nervous system related disorders [1]. Among the neuron degrading disease, Alzheimer's disease (AD) is responsible for 60% of all dementia in the people having age more than 65 [2-4]. AD is a progressive neurodegenerative disorder characterize by impairment of learning, cognitive deficits, and behavioral disturbance [5-7].

Pathology of AD is complex and several pathogenic pathways are involved in the progression of this disease. Cholinergic deficit, oxidative stress, formation of neurofibrillary tangles, and deposition of senile plaque hypothesize are the main hallmark of AD [8,9]. Based on these information, several lines of pharmacological treatment have been developed such as cholinergic inhibitors, antioxidant therapy, neurotransmitter replacement, anti-inflammatory therapy, stimulation of muscarinic receptors, prevention of tau aggregation, prevention of tau hyperphosphorylation, increase of alpha-secretase activity, inhibition of beta and gamma secretase inhibitors, and others [10,11].

Among all the pathologic conditions of AD cholinergic deficits remarks as one of the main reason of AD. Loss of acetylcholine (ACh) is considered to play a pivotal role in the learning and memory deterioration of AD patients [12-14].

Several strategies are available to improve cholinergic neurotransmission including stimulation of cholinergic receptors or increasing the ability of ACh released in the synaptic cleft by inhibiting acetylcholinesterase (AChE) enzyme [15-17].

Similar to AChE, butyrylcholinesterase (BuChE) enzyme singly or synergistically act on AChE to hydrolyze ACh as well as butyrylcholine (BuCh) in the nerve endings [18]. In the AD brains increased levels of BuChE correlate with the progression of AD. In healthy human brain, the function of BuChE is highly dominated by AChE. In low concentration, BuChE is less effective in hydrolyzing ACh, but the concentration of BuChE increases enormously in Alzheimer's patient brain which is more capable of hydrolyzing ACh [19-21]. Therefore, inhibition of cholinesterase enzymes and preventing oxidative stress were found as a suitable strategy for AD treatment as well as for other types of neurodegenerative disorders including dementia.

Thrombosis is the process of blood clotting that obstructs the flow of blood through circulatory system. It is the first step of repairing process after injury [22]. However, in several cases, this clotted blood causes harm in the human body by blocking blood flow through an organ or part of the organ, which may lead to stroke, deep brain thrombosis, heart problems, and occlusion of peripheral artery. Beside this thrombosis is a critical stage for arterial disease associated with myocardial infarction and stroke which may be responsible for considerable morbidity and mortality of the patients. Moreover, for cancer patients, venous thrombosis can be the second leading cause of death [23]. To treat these this kind of diseased situation, thrombolytic agents such as tissue plasminogen activator, streptokinase (SK) or urokinase are frequently used [24]. However, these drugs display a huge side effect profile, including high risk of internal hemorrhage and severe anaphylactic reactions. Moreover, various treatments with SK are restricted due to immunogenicity [25].

Plants are the wide source of bioactive principles and medicine and traditional medicine is one of the primary health-care system in many developing countries. *Tamarindus indica* is a rich source of bioactive molecules with numerous uses. Phytochemical investigation carried out on this plant revealed the presence of many active constituents, such as phenolic compounds, glycosides, malic acid, tartaric acid, mucilage, pectin, arabinose, xylose, galactose, glucose, and uronic acid [26-28]. The ethanolic extract of *T. indica* contains fatty acids and various essential electrolytes [28].

The seed and pericarp of *T. indica* are mainly composed of phenolic antioxidant compounds [29]. All extracts of *T. indica* exhibited good antioxidant activity [30]. Fruits of *T. indica* were well known in virtue of their effects on the lipid profile, systolic and diastolic blood pressure and other CVS effects, and the body weight of humans [31]. *T. indica* is often used for the treatment of various skin problems such as cuts, wounds, and abscesses. Bark and leaves are most commonly used either as a decoction or as a powder form in the external surface of the skin [32].

Although *T. indica* has important medicinal value, no studies have yet examined for its cholinesterase inhibitory activities as well as thrombolytic activities. This study discusses the investigation of dried bark and seed extracts of *T. indica* in AChE and BuChE inhibitory potential as well as thrombolytic point of view, relevant to the treatment of AD and blood clotting disorders.

MATERIALS AND METHODS

Chemicals that were used in this study are analytical grade. Acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), donepezil, galantamine, 5'dithio-bis-(2-nitro) benzoic acid, and SK were purchased from Sigma-Aldrich (Japan). Tris-HCl buffer, sodium chloride, magnesium chloride, and Triton X-100 were collected from Wako Pure Chemical Company Ltd. (Japan). Solvents (methanol, chloroform, etc.) were used in this experiment were analytical grade also.

Plants Materials and Experiment

Bark and seed of *T. indica* were collected from Mymensingh, Tangail and Dhaka, Bangladesh in January and February 2016. All plant parts were authenticated by an expert taxonomist. All tests are done in the Laboratory of Pharmacy Department, East West University.

Preparation of the Sample

After collecting barks and ripe fruits, these were dried into sun under shadow. Seed was peeled out from the ripe fruit. The dried bark was cut into small pieces and ground into finer powder using a powerful grinder. The grinded sample was sieved to get uniform particle size and kept it into air-tight container to prevent it from light and moisture. Seeds were also ground by applying same method.

Extraction

Powdered bark (500 g) and grinded seed (200 g) were placed into an amber coated bottle and soaked into 1500 and 1000 ml of methanol, respectively. The contents were sealed into bottle for 10 days with occasionally stirred and shaken. After 10 days, the whole mixtures were filtered by Whitman No. 1 filter papers, and the filtrated solutions were concentrated under reduced pressure, heating below 50°C. Finally, 24.298 and 10.412 g of crude methanolic extracts (CMEs) of bark and of seed was obtained, respectively.

Determination of AChE Inhibitory Activity [33-36]

Modified Ellman's colorimetric method was applied to run *in-vitro* AChE inhibitory assay and ATCI used as a substrate. For enzyme source, bovine brain was used. To run this method, bovine brain was homogenized in a homogenizer with 10 times of a homogenation buffer (10 Mm Tris-HCl buffer, pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100 and centrifuged for 30 min at 10,000 rpm. The supernatant that was form after centrifugation was treated then with super-saturated ammonium sulfate solution which results floating

precipitation. Collected precipitation was then solubilize into extraction buffer. Solution of this precipitation was used as an enzyme source. Cool temperatures were maintained (4°C) throughout the enzyme excretion procedure. AChE hydrolysis rate was monitored spectrophotometrically. Each extract or standard (various concentrations) was mixed with an enzyme solution (200 μL) (collected from bovine brain) and incubated at 37°C for 30 min. After that Ellman's reaction mixture (400 μl, 0.35 mM ATCI, 200 μl, 0.7 mM 5, 5´-dithiobis (2-nitro benzoic acid)) (DTNB) in an extraction buffer (50 mM Tris.HCl buffer, 50 mM $MgCl_2$, 50mM NaCl, 1% Triton X-100, pH 8.0) to adjust it 3 ml of final volume. Absorbance at 412 nm was taken after 30 min incubated this mixture at 37°C. The blank reaction was measured by substituting buffer saline for the enzyme. Donepezil was used as a positive control. AChE inhibitory activity by the extract was calculated using following formula:

$$\%\ \text{of Inhibition of AChE} = \frac{A_{\text{Absorbance of control}} - A_{\text{Absorbance of sample}}}{A_{\text{Absorbance of control}}} \times 100\%$$

Determination of BuChE Inhibitory Activity [33-36]

BuChE inhibitory assay was also performed by modified Ellman's colorimetric method, where BTCI acts as a substrate. Human blood plasma was used as source of BuCh enzyme. BuChE hydrolysis rate was examined spectrophotometrically. Each extract or standard (various concentrations) was mixed with an enzyme solution (50 μL) and incubated at 37°C for 30 min. After adding Ellman's reaction mixture (400 μl, 0.35 mM butyrylthiocholine, 200 μl, 0.7 mM DTNB in a extraction buffer (50 mM Tris.HCl buffer, 50 mM $MgCl_2$, 50 mM NaCl, 1% Triton X-100, pH 8.0) to the above reaction mixture to adjust final volume 3 ml. To verify the result, all reading were repeated 3 times. The blank reaction was measured by substituting extraction buffer for the enzyme. Galantamine was used as a reference standard. BuChE inhibitory activity was calculated using the following formula:

$$\%\ \text{of Inhibition of BuChE} = \frac{A_{\text{Absorbance of control}} - A_{\text{Absorbance of sample}}}{A_{\text{Absorbance of control}}} \times 100\%$$

Thrombolytic Activity Test [37]

For thrombolytic activity test human blood was used. Blood was withdrawn from healthy human volunteers having no history of taking oral contraceptive pills or undergoing anticoagulant therapy. 1.0 ml of venous blood from each volunteer was transferred to the previously weighed eppendorf tubes (volume 1.5 ml) and incubated for 45 min at 37°C and was allowed to form clot. Bark and seed extracts (100 mg) were suspended into 10 ml of distilled water. After clot formation, the serum was completely removed from eppendorf tube. Clot was again weighed to determine the clot weight. For each eppendorf tube with the preweighed clot, 100 μl aqueous solution of the crude extract was added separately. 100 μl of SK (30,000 IU) were added to the positive and 100 μl distilled water were added to negative control tubes, respectively. All tubes were then again incubated for 90 min at 37°C to observe clot lysis. After 90 min, the released fluid was removed again and tubes were again weighed to observe the difference in weight. Difference obtained in weight taken before and after clot lysis by the extract, positive control and negative control, was expressed as percentage of clot lysis and the equation is shown below:

$$\%\ \text{of Clot lysis} = \frac{\text{Weight of clot after release of fluid}}{\text{Weight of clot before release of fluid}} \times 100\%$$

RESULTS

AChE Inhibitory Activity

Ellman's colorimetric method was applied to determine the AChE inhibitory activity of the plant extract. This method is based on determining the amount of thiocholine releases when ACh is hydrolyzed by AChE enzyme. DTNB is a coloring agent which binds with the ACh and forms color. In this test, the color indicates the presence of free ACh which was not hydrolyzed by AChE in the reaction mixture. As reduction of AChE in hippocampus and cortex is a remarkable hallmark of AD, inhibition of AChE ensures more ACh in the brain. The inhibitory activities of the different fractions are shown in Table 1. It can be observed that AChE inhibitory activity *T. indica* was concentration-dependent, because with the increase of the concentration, activity increased. To compare both the fractions, extract from the bark got more enzyme inhibitory activity than the seed sharing inhibitory concentration 50% (IC_{50}) values of 268.09 and 287.15 μg/ml, respectively. Donepezil was used as reference standard.

BuChE Inhibitory Activity

BuChE possess the capability of hydrolyzing ACh, as it can act both itself and synergistically with AChE. Modified Ellman's method was also applied to quantify the BuChE activity of the plants extract. BuChE inhibitory activity of different fractions of *T. indica* is reported in Table 2. The result truly indicates that both bark and the seed extracts have the ability to inhibit

Table 1: AChE and BuChE inhibitory activity of CME fractions of *T. indica*

Concentration (μg/mL)	% AChE inhibition		
	Bark	Seed	Donepezil (Standard)
25	6.32±0.79	5.13±0.64	84.02±1.19
50	10.48±1.18	9.37±1.17	91.29±1.72
100	19.52±1.97	16.43±1.85	92.57±1.56
200	38.71±0.89	32.82±1.92	93.73±1.82
300	55.02±1.55	51.77±1.32	94.62±0.79

AChE: Acetylcholinesterase, BuChE: Butyrylcholinesterase, CME: Crude methanolic extracts, *T. indica*: *Tamarindus indica*

BuChE. IC_{50} values of both the fractions were 201.25 and 254.71 μg/ml, respectively. Galantamine was used as reference standard.

Thrombolytic Activity Test

100 μl SK as a positive control (30,000 IU) was added to the clots along with 90 min of incubation at 37°C, showed 72.95% clot lysis. Clots when treated with 100 μl distilled water (negative control) showed only negligible clot lysis (3.60%). The *in-vitro* thrombolytic activity study with *T. indica* revealed 30.17% clot lysis with bark and 22.53% with the seed. The percentage of weight loss of clot after application of extract solution was the functional indication of thrombolytic activity of the extract. % of clot lysis obtained after treating clots with different concentration of the sample is given in Table 3.

DISCUSSION

Due to medical advancement reduction of birth rate is increasing life expectancy which results an increase of problem in this century [38]. AD is one of the most common among them having no cure at all. However, there are several hypotheses that have been established to suppress the severity of this disease. A number of factors have been identified; among them oxidative stress and cholinergic dysfunctions were found as the major contributor factors of AD [38,39]. In AD patients, the number of degrades time to time, so a neuroprotective approach also might be beneficial to this condition [40-42].

T. indica was used from the very past for various disease condition. It has been renowned for its several medicinal importance. It is a rich source of antioxidant that was previous established. Extract of the *T. indica* possesses lipid peroxidation, antioxidant enzyme activities, H_2O_2-induced ROS production, and gene expression patterns in liver HepG2 cells [43,44]. In this study, we evaluate its anti-Alzheimer's activity along with its thrombolytic activity. Our finding suggested that *T. indica* not only possesses anti-Alzheimer's activity but also thrombolytic activity. It is well recognized that inhibition of AChE is one of the most effective strategy.

In this study, we found that inhibition of AChE by both CME was occurred by dose-dependent manner. From Table 1, it is found that AChE inhibitory activity *T. indica* was concentration-dependent. To compare both the fractions, extract from the bark got more enzyme inhibitory activity than the seed with the IC_{50} of 268.09 and 287.15 μg/ml, respectively. Donepezil was used as positive control, and at the same concentration, it inhibits near about 94% of the enzyme. All the tests were run for at least three times to get linear and reliable results.

Like AChE, BuChE is also important in the breakdown of ACh. It can cleave ACh alone with synergistically with AChE. So preventing this BuChE is also important strategy in the treatment of AD [45]. Our study revealed that *T. indica* has anti-BuChE activity [Table 2]. CME of both bark and seed indicates that these are capable of inhibiting this enzyme too. IC_{50} values of bark and seed the fractions were 201.25 and 254.71 μg/ml, where at the same concentration galantamine inhibits almost 95% of the enzyme.

Plants are always been an excellent source for treating clotting disorder and stroke [46,47]. There are several thrombolytic drugs obtained from various plant sources. In our study, two different extracts from *T. indica* showed the thrombolytic activity in compare to the standard drug. The maximum clot lysis activity was mostly observed in CME of the bark with 30.17% than the seed 22.53%, which means bark can lysis more blood clot than the seeds. At the given concentration SK solubilize 72.95% clot.

Table 2: BuChE inhibitory activity of CME fractions of *T. indica*

Concentration (μg/mL)	% BuChE inhibition		
	Bark	Seed	Galantamine (standard)
25	15.64±1.09	10.33±1.81	76.12±2.19
50	28.03±1.21	22.19±1.10	87.29±1.77
100	37.66±1.57	30.07±1.75	92.57±2.56
200	49.54±1.74	41.52±1.68	93.73±1.23
300	65.64±1.07	57.41±1.55	94.62±1.79

BuChE: Butyrylcholinesterase, CME: Crude methanolic extracts, *T. indica*: *Tamarindus indica*

Table 3: % lysis of clot by SK, methanolic extract of *T. indica* bark, seed and control

Sample	Concentration (μg/ml)	Blood clot weight before treatment (ava.)	Blood clot weight after treatment (ava.)	% of clot lysis
Control (distilled water)	100	0.648±0.038	0.611±0.046	3.60
Bark extract	100	0.748±0.027	0.447±0.039	30.17
Seed extract	100	0.792±0.035	0.569±0.022	22.53
Standard (SK)	100	0.825±0.0411	0.0955±0.0478	72.95

SK: Streptokinase, *T. indica*: *Tamarindus indica*

CONCLUSION

The result suggested that CMEs of both bark and seed from *T. indica* moderately inhibit both AChE and BuChE. These fractions also possess thrombolytic activity. Further study is needed to identify the specific molecule (or molecules) that are mainly responsible for those effects which might be a potential source for cholinesterase inhibitors in AD treatment and in neuroprotection, in general.

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Review Article

Garcinia subelliptica Merr. (Fukugi): A multipurpose coastal tree with promising medicinal properties

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ABSTRACT

In this short review, the current knowledge on the botany, ecology, uses, and medicinal properties of the multipurpose *Garcinia subelliptica* (Fukugi) is updated. As yet, there are no reviews on this indigenous and heritage coastal tree species of the Ryukyu Islands in Japan, which has ethnocultural, ecological, and pharmacological significance. Planted by the Okinawan people some 300 years ago, Fukugi trees serve as windbreaks and accord protection against the destructive typhoons. The species has become a popular ornamental tree, and its bark has been used for dyeing fabrics. It forms part of the food chain for mammals and insects and serves as nesting sites for birds. Endowed with bioactive compounds of benzophenones, xanthones, biflavonoids, and triterpenoids, *G. subelliptica* possesses anticancer, anti-inflammatory, anti-tyrosinase, trypanocidal, antibacterial, DNA topoisomerase inhibitory, DNA strand scission, choline acetyltransferase enhancing, hypoxia-inducible factor-1 inhibitory, and antiandrogenic activities. Fukugetin and fukugiside are two novel biflavonoids named after the species. The chemical constituents of Fukugi fruits when compared with those of mangosteen yielded interesting contrasts.

KEY WORDS: Anticancer, anti-inflammatory, chemical constituents, Fukugi, *Garcinia subelliptica*

INTRODUCTION

The genus *Garcinia* consists of ~450 species, distributed in Africa, Madagascar, Asia, Australia, Polynesia, and Central America [1]. China has 20 species, of which 13 are endemic and one is introduced [1] while 35 species occur in India [2]. The plant list of 2013 has listed a total of 396 species of *Garcinia* with accepted names [3]. Southeast Asia has about 30 species that produce edible fruits, of which most of them are sour because they contain citric acid [4]. *Garcinia mangostana* L. (mangosteen) is the most popular as it produces the sweetest fruits.

Garcinia species are known to contain a wide variety of chemical constituents, notably, benzophenones and xanthones. Benzophenones have a wide range of biological and pharmacological properties, for example, antioxidant, antimicrobial, anti-inflammatory, anti-HIV, cytotoxic, hepatoprotective, antiparasitic, and antidiabetic properties [5-7]. Antioxidant, anti-inflammatory, antimicrobial, cytotoxic, and antiplasmodial activities have been reported in xanthones [8,9].

In this short review, the current knowledge on the botany, ecology, uses, and medicinal properties of *Garcinia subelliptica* is updated. It is an indigenous and heritage coastal tree species of the Ryukyu Islands in Japan. To date, there are no reviews on *G. subelliptica*, and there is adequate information in literature on the ethnocultural, ecological, and pharmacological significance of the species to warrant this update.

BOTANY AND USES

G. subelliptica Merr. of the family Clusiaceae (previously Guttiferae) is native to the Ryukyu Islands of Japan, China, Taiwan, India, Sri Lanka, and the Philippines [1,2]. Locally known as Fukugi (Japanese mangosteen), the tree can grow up to 15-20 m tall [10]. Young trees have a compact conical crown with alternating pairs of erect branches sprouting from a main trunk. Older trees have broader crowns and a thick trunk with grayish bark. Characteristic features of the tree are the upward-pointing branches and leaves, cone-shaped crown, and yellowish latex.

Leaves of *G. subelliptica* are simple, spirally arranged in opposite pairs, ovate-oblong in shape, thickly leathery, and rounded at the apex [10]. Twigs are 4-6 angled. The undersurface is yellow-green, and the uppersurface glossy and dark green. The midrib is prominent while the side veins are not visible. Leaves are reddish-bronze when young, turning yellow-green, and dark green when mature. Sun leaves of *G. subelliptica* are smaller, thicker, and more elliptic than shade leaves [11]. Having more and larger stomata, sun leaves also have higher chlorophyll content than shade leaves.

The species is monoecious with male and female flowers occurring on the same trees [10]. Fruits are oval, green when young and yellowish-orange when mature, and very sour in taste. Photos of male flowers, young fruits, and mature fruits are shown in Figure 1. Fukugi trees begin to flower in early May and fruits mature in late August each year [12]. Fallen ripe fruits emit a strong and unpleasant odor [13].

Fukugi trees have been planted on the Ryukyu Islands by the Okinawan people some 300 years ago, embracing the concept of Feng Shui [14,15]. Dominating the rural landscape, the trees provide shade, serve as windbreaks, and accord protection against the destructive typhoons. The species is planted as forest belts or groves along the coast and the boundaries of villages, and as hedges in the gardens of houses. On the island of Ishigaki, a survey of 12 villages showed that 30% of the houses have Fukugi trees [14].

Fukugi trees lining the stone walls of traditional houses with red roof tiles and a pair of guardian lions are very much of the Okinawan cultural landscape. The walls of 1.2-1.5 m in height protect the lower parts of buildings while the crown foliage of Fukugi shelters the upper parts, particularly the roofs and eaves [16]. On the Ryukyu Islands, the esthetics of Fukugi trees forming the landscape of such villages embodies traditional wisdom and the Satoyama concept of man living in harmony with nature.

In Okinawa, *G. subelliptica* also forms the landscape of shrines and other sacred sites [15]. Out of 683 trees enumerated and measured, data on the average tree height, trunk diameter, and tree age were 10 m, 35 cm, and 140 years. Fukugi trees reaching almost 80 cm in trunk diameter and more than 300 years old have been recorded. These growth data suggest that Fukugi is a very slow growing tree.

In recent years, trees of *G. subelliptica* have become popular ornamentals along roadsides and in gardens. Ripe Fukugi fruits are a food source for the Ryukyu flying foxes (*Pteropus dasymallus*) during the summer although their main food items are the fruit crops cultivated by the farmers [17]. The larvae of a new species of moth named *Heleanna fukugi* were found feeding on young leaves of *G. subelliptica* [18]. Adult moths of this species were found emerging from nests of the bull-headed shrike built on branches of Fukugi trees [19]. In the Ryukyu Islands, the yellow pigment from the bark of Fukugi trees has been used to dye traditional fabrics including the Okinawan Bingata [13,20]. On the occasion of the Shichi festival on Iriomote Island, we observed that Miruku (God of Harvest) wore a traditional yellow kimono dyed using the bark of Fukugi.

Elsewhere, trees of *G. subelliptica* have gained popularity as an ornamental and landscape plant in East and Southeast Asia. Although *G. subelliptica* is not known for its uses in folk medicine, there is now convincing *in vitro* scientific evidence that the species is rich in bioactive compounds with pharmacological properties of medicinal values.

CHEMICAL CONSTITUENTS

Major classes and the number of compounds of *G. subelliptica* are benzophenones (58), xanthones (30), biflavonoids (15), and triterpenoids (7) [Table 1]. Benzophenones and xanthones have been isolated from the fruit, seed, wood, bark, and root. Garcinielliptones are the dominant benzophenones while garcinia xanthones and subelliptenones are the major xanthones. Biflavonoids are found primarily in the leaf. *Garcinia* flavones are the dominant biflavonoids. Triterpenoids are found in the leaf and fruit. Fukugetin and fukugiside [Figure 2] were the first two biflavonoids isolated from the stem bark of *G. subelliptica* [21,22]. Other compounds that are novel to *G. subelliptica* are shown in Table 1.

Benzophenones or phloroglucins are a diverse class of phenolic compounds consisting of more than 300 members and characterized by having a common phenol-carbonyl-phenol skeleton [4,6]. The A ring generally contains up to two substituents while the B ring can undergo prenylation and cyclization producing a wide variety of structurally unique compounds with bi-, tri-, and tetra-cyclic ring systems. Benzophenones are major intermediates in the biosynthetic pathway of xanthones and are typically found in *Garcinia* species. They are non-polar compounds that become increasingly hydrophobic with more prenyl groups attached.

Figure 1: A wasp visiting male flowers (left), young green fruits (middle), and mature orange fruits of *Garcinia subelliptica*

Table 1: Classes and names of compounds isolated from *Garcinia subelliptica*

Compound class and name	Plant part	Reference
Benzophenones		
Cycloxanthochymol*	Fruit	[23-25]
4′,6-Dihydroxy-2,3′4-trimethoxybenzophenone*	Wood	[26]
Garcinialiptones A–D*	Fruit	[25]
Garcinielliptones A–S*	Fruit, seed	[27-32]
Garcinielliptones FA–FE*	Fruit	[24,33-35]
Garcinielliptones HA–HF*	Fruit, wood	[34,36]
Garcinielliptin oxide	Seed	[28]
Garcinol	Fruit	[23]
Garsubellins A–E*	Seed, wood	[28,32,37,38]
Isogarcinol	Fruit	[23]
Isoxanthochymol	Fruit	[23-25]
Subellinone*	Wood	[39]
2′,3′,6-Trihydroxy-2,4-dimethoxybenzophenone*	Wood	[40]
Xanthochymol	Fruit, seed	[13,23-25]
Xanthones		
1,2-Dihydroxy-5,6-dimethoxyxanthone*	Wood	[26]
1,5-Dihydroxy-3-methoxylxanthone	Root bark	[41]
2,5-Dihydroxy-1-methoxylxanthone*	Wood	[42]
2,6-Dihydroxy-1,5-dimethoxyxanthone*	Wood	[26]
1,7-Dihydroxyxanthone	Fruit	[33]
1,6-*O*-Dimethylsymphoxanthone*	Wood	[40]
Garciniaxanthones A–E*	Wood, root, root bark, stem bark	[20,26,42-44]
Globuxanthone	Root bark	[45]
4-Hydroxybrasilixanthone B*	Stem bark	[20]
12β-Hydroxy-des-d-garcigerin	Root bark	[45]
Isogarciniaxanthone E*	Stem bark	[20]
1-*O*-Methylsymphoxanthone*	Wood	[42]
Subelliptenones A–I*	Wood, root bark, stem bark	[20,41,42,45-47]
Symphoxanthone	Wood	[42]
1,3,6,7-Tetrahydroxyxanthone	Fruit	[24]
1,4,5-Trihydroxy-2-(1,1-dimethyl-2-propenyl) xanthone	Stem bark	[20]
1,2,5-Trihydroxyxanthone*	Wood	[26]
1,4,5-Trihydroxyxanthone*	Root	[43]
Biflavonoids		
Amentoflavone	Leaf	[48]
I7, II4′-Dimethylamentoflavone	Fruit	[33]
Garciniaflavones A–F*	Leaf	[48]
3‴-*O*-Methylfukugetin	Fruit	[13]
4‴-*O*-Methylfukugetin*	Seed	[13]
4‴-*O*-Methylmorelloflavone	Leaf	[48]
Morelloflavone (fukugetin)*	Leaf, fruit, seed, stem bark	[13,20,21,48]
Morelloflavone-7″-*O*-β-glucopyranoside (fukugiside)*	Leaf, stem bark	[22,48]
Podocarpusflavone A	Leaf	[48]
Volkensiflavone	Seed	[13]
Triterpenoids		
β-Amyrin	Leaf, fruit	[31]
Canophyllic acid	Fruit	[33]
Canophyllol	Fruit	[33]
Cycloart-25-ene-3β,24-diol	Leaf	[48]
Cycloartenol	Leaf	[48]
5-Hydroxymethylfurfural	Fruit	[33]
Oleanan-3-one	Leaf	[48]

Compounds with an asterisk are novel.

Xanthones are secondary metabolites that have a unique tricyclic C6-C3-C6 aromatic structure [49]. The substitution of isoprene, methoxyl, and hydroxyl groups at various locations of the A and B rings can result in a diverse array of xanthone compounds.

Biflavonoids are flavonoid-flavonoid dimers of varying structures due to the different dimer combinations such as flavanone-flavone, flavone-flavone, and flavone-flavonol [50]. Linking the flavonoids, there are two general types of bond connections: C-C and C-O-C. Structural diversity arises when the connecting bond and many hydroxyl and/or methoxyl substituents groups can occur in different positions.

An interesting study on the chemical constituents of green and ripe fruits and seeds of Fukugi in Okinawa reported that the yield of xanthochymol content of ripe fruits is about six times that of green fruits [13]. The content of fukugetin of ripe seeds is about 10 times more than that of green seeds. The isolation of volkensiflavone from ripe seeds of *G. subelliptica* was reported for the first time.

Among the 110 compounds isolated from *G. subelliptica* [Table 1], 85 compounds (77%) are novel, of which 53 are benzophenones, 23 are xanthones, and 9 are biflavonoids. Major novel compounds are garcinielliptones A-S, FA-FE, and HA-HF (29), subelliptenones A-I (9), *Garciniaflavones* A-F (6), garsubellins A-E (5), *Garciniaxanthones* A-E (5), and garcinialiptones A-D (4).

When the chemical constituents of fruits of *G. subelliptica* are compared with those of *G. mangostana* (mangosteen), some interesting contrasts become evident. Only two xanthones (1,3,6,7-tetrahydroxyxanthone and 1,7-dihydroxyxanthone) have been isolated from the fruit of *G. subelliptica* [Table 1]. In contrast, phytochemical and pharmacological reviews on mangosteen have listed 48-50 xanthones from the fruits, notably those of mangostins, garcinones, mangostenones, and garcimangosones [51,52]. Two reviews on the benzophenones of *Garcinia* species have included *G. subelliptica* but not *G. mangostana* [4,6]. The fruit of *G. mangostana* contains low contents of guttiferone A and xanthochymol [53]. In contrast, a total of 48 benzophenones have been isolated from *G. subelliptica* [Table 1].

PHARMACOLOGICAL ACTIVITIES

Anticancer

Xanthones isolated from the stem bark of *G. subelliptica* were the first compounds reported to be cytotoxic to cancer cell lines [20]. Against HeLa cells, the growth inhibition of 50% (GI_{50}) values of garciniaxanthone A, garciniaxanthone E, and 1,4,5-trihydroxy-2-(1,1-dimethyl-2-propenyl) xanthone were 17, 10, and 14 μM, respectively. 4-Hydroxybrasilixanthone B and isogarciniaxanthone E had GI_{50} values of 17 and 16 μM, respectively. Interestingly, fukugetin showed no inhibitory activity against HeLa cells.

Benzophenonoids of *G. subelliptica* also possess cytotoxic activity. Isolated from the fruits of *G. subelliptica*, garcinielliptone FB displayed marginal cytotoxicity against MCF-7, Hep 3B, and HT-29 human cancer cell lines [33]. An initial study on benzophenonoids isolated from the fruit of *G. subelliptica* reported that xanthochymol was moderately cytotoxic to MCF-7, Hep2, HeLa, and KB human cancer cells [24]. A follow-up study by the same group of scientists [25] reported that all benzophenonoids from the fruit of *G. subelliptica* displayed cytotoxicity against A549, DU145, KB, and vincristine-resistant KB human tumor cell lines with inhibitory concentration 50% (IC_{50}) values ranging from 3.3 to 7.3 μg/ml. They included garcinialiptones A-D, xanthochymol, isoxanthochymol, and cycloxanthochymol. Another compound (GP-1) from the fruit of *G. subelliptica*-induced apoptosis and autophagy of HT-29 human colorectal cancer cells through caspase- and mitochondria-related pathways [54].

From the seed of *G. subelliptica*, β-amyrin and garcinielliptone FC caused NTUB1 human bladder cancer cell death in a concentration-dependent manner after exposure for 24 and 72 h [31,32]. Cell death was by apoptosis through a ROS-dependent mechanism. Three benzophenones, garcinol, isogarcinol, and xanthochymol isolated from the fruit of *Garcinia purpurea* displayed potent cyctoxic activity against four leukemia cell lines in the order of isogarcinol > xanthochymol > garcinol [55]. As shown in Table 1, all these three compounds have also been isolated from the fruit of *G. subelliptica* [23]. A recent publication has reported that garcinielliptone FC from the fruit of *G. subelliptica*-induced apoptosis in HT-29 cells involving both caspase-dependent and caspase-independent pathways [35]. In addition, garcinielliptone FC also effectively suppressed the activity of nuclear factor-κB, a key inflammation-related molecule.

Anti-inflammatory

Fukugi has been reported to possess anti-inflammatory activity. The ethanol leaf extract of *G. subelliptica* reduced nitric oxide production in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages. Expression of cyclooxygenase-2 was notably reduced and production of proinflammatory cytokines was inhibited [56]. From the seed of *G. subelliptica*, garcinielliptones (garsubellin A, garcinielliptin oxide, and garcinielliptone F) had potent inhibitory effects on the release of β-glucuronidase and lysozyme, and on superoxide formation from neutrophils stimulated with fMLP/CB [28,29]. Garcinielliptones L and M showed potent inhibitory effects on the release of β-glucuronidase from peritoneal mast cells stimulated with *p*-methoxy-*N*-methyl phenethylamine and on nitric oxide production in RAW 264.7 cells in response to LPS [30].

Anti-tyrosinase

Using L-dihydroxyphenylalanine as substrate, leaves of 39 coastal plant species found in Iriomote, Japan, were screened for tyrosinase inhibition [57]. Leaves of *G. subelliptica* with 57% inhibition ranked third. Fukugetin was one of the two biflavonoids isolated from the leaves of *G. subelliptica*. The compound exhibited much stronger inhibition (IC_{50} of 2.5 μg/ml) than kojic acid (IC_{50} of 9.1 μg/ml) and arbutin (IC_{50} of 62 μg/ml) when tested using L-tyrosine as substrate. This suggested that biflavonoids, such as fukugetin, may be developed into agents for skin lightening and/or for prevention of food browning. However, the acetone fruit extract of *G. subelliptica* displayed very weak anti-tyrosinase activity [58].

Other Bioactivities

Other pharmacological properties of *G. subelliptica* include trypanocidal, antibacterial, DNA topoisomerase inhibitory, DNA strand scission, choline acetyltransferase (ChAT) enhancing, hypoxia-inducible factor-1 (HIF-1) inhibitory, and anti-androgenic activities. They are briefly described as follows:

From the stem bark of *G. subelliptica*, xanthones exhibited trypanocidal properties when tested against epimastigotes and trypomastigotes of *Trypanosoma cruzi* [20]. The parasitic

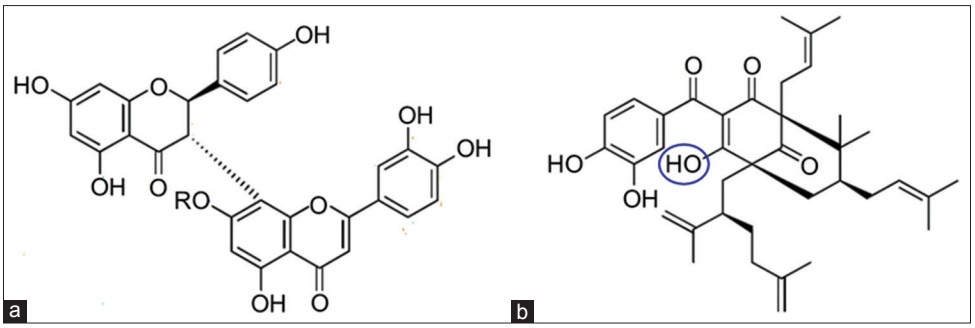

Figure 2: Molecular structures of fukugetin, fukugiside, and xanthochymol. (a) Biflavonoids: Fukugetin (R = H) and fukugiside (R = β-D-glc), (b) Benzophenone: (+)-xanthochymol

protozoan *T. cruzi* is the etiologic agent of Chagas' disease, which is transmitted to humans and animals by insect vectors. Among the nine xanthones isolated, subelliptenone B had the strongest activity against epimastigotes with IC_{100} value of 51 μM while garciniaxanthone B was most effective against trypomastigotes with IC_{100} value of 8 μM.

Xanthochymol from the fruit of *G. subelliptica* inhibited the growth of methicillin-resistant *Staphylococcus aureus* with minimum IC (MIC) of 3.1 μg/ml, which was superior to that of vancomycin (6.3 μg/ml) used as positive control [23]. Against methicillin-sensitive resistant *S. aureus*, the MIC of xanthochymol was 3.1 μg/ml similar to that of vancomycin (6.3 μg/ml). It was postulated that the chelated OH group at C-1 of xanthochymol [Figure 2b] may be involved in the inhibitory activity.

Subelliptenone F from *G. subelliptica* has been reported to inhibit DNA topoisomerases I and II with IC_{50} values of 30 and <1.0 μg/ml, respectively [59]. Topoisomerases are the key enzymes of cells that regulate the topological structure of DNA and cells die when these enzymes are inhibited [60]. Topoisomerase inhibitors are therefore among the most active anticancer agents.

Benzophenones (garcinielliptones FC, HC, and HF) from the heartwood and fruit of *G. subelliptica* exhibited DNA strand scission activity [34,36]. In the presence of Cu(II), all three garcinielliptones caused significant breakage of the supercoiled plasmid pBR322 DNA. Compounds with the ability to induce DNA breakage and degradation, for example, bleomycin, have been used as anticancer drugs [36].

Garsubellin A isolated from the wood of *G. subelliptica* was found to enhance the activity of ChAT at 10 μM in P10 rat septal neuron cultures [37]. Deficiency in ChAT is a key enzyme involved in the synthesis of neurotransmitter acetylcholine and its deficiency is believed to be implicated in the development of the dementia of Alzheimer's disease.

From the leaf of *G. subelliptica*, amentoflavone strongly inhibited HIF-1 in human embryonic kidney 293 cells under hypoxic conditions [48]. Overexpression of HIF-1 is associated with increased tumor growth and angiogenesis, and HIF-1 inhibitors are known to be anticancer agents [61].

Hydroxyxanthones from the root bark of *G. subelliptica*, notably subelliptenone F, displayed strong antiandrogenic activity in LNCaP prostate cancer cells [62]. Androgens are primary regulators of prostate cancer cell growth and proliferation in human males [63]. As activation of the androgen receptor is crucial for prostate cancer growth, current therapies are adopting androgen depletion and antiandrogen approaches [64].

CONCLUSION

As heritage trees of the Ryukyu Islands in Japan, the multipurpose *G. subelliptica* has ethnocultural, ecological, and pharmacological significance. Planted some 300 years ago, Fukugi trees dominate the rural landscape, providing shade, and protection of villages during the typhoons. The species has gained popularity as an ornamental tree for landscaping roadsides and gardens, and its bark is still being used as a natural dye for fabrics. It is an important food source for mammals and insects, and serves as nesting sites for birds. Endowed with bioactive compounds of benzophenones, xanthones, biflavonoids, and triterpenoids, Fukugi possesses anticancer, anti-inflammatory, anti-tyrosinase, trypanocidal, antibacterial, DNA topoisomerase inhibitory, DNA strand scission, ChAT enhancing, HIF-1 inhibitory, and antiandrogenic activities. Fukugetin and fukugiside are two novel biflavonoids named after the species. Not known for its uses in folk medicine, there is now convincing *in vitro* scientific evidence that *G. subelliptica* is rich in bioactive compounds with pharmacological properties of medicinal values. *In vivo* and toxicity studies would be the next phase of research, and the prospects for clinical trials are far from the horizon.

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Review Article

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Solving the puzzle: What is behind our forefathers' anti-inflammatory remedies?

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ABSTRACT

Inflammation is a ubiquitous host response in charge of restoring normal tissue structure and function but is a double-edged sword, as the uncontrolled or excessive process can lead to the injury of host cells, chronic inflammation, chronic diseases, and also neoplastic transformation. Throughout history, a wide range of species has been claimed to have anti-inflammatory effects worldwide. Among them, *Angelica sinensis, Tropaeolum majus, Castilleja tenuiflora, Biophytum umbraculum*, to name just a few, have attracted the scientific and general public attention in the last years. Efforts have been made to assess their relevance through a scientific method. However, inflammation is a complex interdependent process, and phytomedicines are complex mixtures of compounds with multiple mechanisms of biological actions, which restricts systematic explanation. For this purpose, the omics techniques could prove extremely useful. They provide tools for interpreting and integrating results from both the classical medical tradition and modern science. As a result, the concept of network pharmacology applied to phytomedicines emerged. All of this is a step toward personalized therapy.

KEY WORDS: Inflammation, omics applied to phytomedicine, rational phytotherapy, traditional medicinal system

INTRODUCTION

Inflammation, an important component of innate immunity, is a ubiquitous host response to foreign physical and/or chemical challenges, invading pathogens [1] or tissue injury, ultimately isolating the source of disturbance, eradicating infection, and restoring normal tissue structure and function (homeostasis) [2]. It is a complex process that is frequently associated with pain and involves several events such as vasodilatation, plasma extravasations, cellular migration (neutrophils) and in some cases, the activation of coagulation cascades [3]. Its characteristic signs include redness, heat, tumor, pain, and loss of function [4]. On the one hand, inflammation is a host defense mechanism meant to eliminate invading pathogens and initiate the healing process. On the other hand, inflammation is a double-edged sword, as the uncontrolled or excessive production of inflammatory products can lead to the injury of host cells, chronic inflammation, chronic diseases, and also neoplastic transformation [5]. Despite progress within medical research during the past decades, the treatment of many serious diseases remains problematic, and chronic inflammatory diseases remain one of the world's major health problems [6,7].

Nowadays, we know that inflammation is a global term that encompasses a multitude of processes that can vary widely in spite of the interdependency and the existence of overlapping signaling pathways. Not only is chronic inflammation different from acute, but inflammatory activity also differs depending on the disease, even if the classical signs and symptoms are similar. For example, the inflammation involved in dry eye disease is accompanied by a specific profile that is wholly separate from inflammation in Crohn's disease sustained, among others, on diverse defects on regulatory mechanisms [8,9]. For this reason, it makes sense that patients with different illnesses do not receive the same prescription for inflammation management. Targeted therapies could increase treatment success [10], reduce toxicity and adverse events including overdose, over the duration, tolerance, dependence/addiction, hypersensitivity, and mid- and long-term toxic effects [11].

Inflammation is not something new. For a long time, different cultures around the world have used herbs and spices - or their formulations - to treat inflammatory disorders and related diseases more or less gracefully [12]. In the course of history, an extraordinarily large number of plant species have been claimed worldwide to possess anti-inflammatory abilities. Some of them have demonstrated their ethnopharmacological relevance and are the main medical treatment for two-thirds of the world population, which is due to the limited availability and affordability of the standard pharmaceutical medicines used in developed countries [13]. The World Health Organization estimates that 80% of people in the developing countries use traditional medicine as primary health care [14].

In light of the above, when referring to the pharmacological anti-inflammatory activity of each species, there is a need for specificity. What is the principle role that each species plays in reducing inflammation? With regard to anti-aggregation, for example, a concrete compound or group of compounds have been described for the extract of *Ginkgo biloba*; a precise mechanism of action and a specific posology have been described to assess its therapeutic effect and reduce adverse events or achieve synergy [15], but the explanation is not always easy. In the case of the anti-inflammatory action, very diverse pathways could achieve it, and the molecular mechanism underlying it sometimes remains unclear. Anti-inflammatory natural products chemistry is complex and the structure diverse. It includes fatty acids, for example, γ-linolenic acid in *Oenothera biennis*, phenylpropenes such as cinnamaldehyde in *Cinnamomum zeylanicum*, or phenolic glycosides as salicin in *Salix purpurea*, to name just a few. In recent years, the concept of network pharmacology, in which low doses of multiple drugs are used to treat diseases, has been developed to understand complex diseases as networks, for which the most efficient treatment is multi-targeted [16]. This depth of knowledge would enable the development of potential new agents, particularly in the inflammation field.

Here, four different actions generally investigated when anti-inflammatory activity is assessed have been selected, and a number of popular species used to treat inflammatory ailments by people in totally different cultural backgrounds (China, America, Africa, Europe, India, and Australia) have also been chosen [Table 1]. Ethnopharmacological relevance (cultural meaning, bioprospective, and/or standardized use), state of science (e.g., scientific rigor, innovation, novelty, or preclinical *in vivo* studies), scientific and social impact of publications under them and use in differentiated geographical locations have been the main parameters chosen to design this research. In most cases, only earlier studies on ethnobotany, phytochemistry, and the pharmacological effects on these species have been reported to date, but there is enough information to form an initial idea of possible trends, therapeutic potentials, possibilities for future research and, no less important, to understand inflammation as an intricate process that involves a wide range of pathophysiologic disorders, cells and signaling pathways. This does not imply that rationality does not must guide all decisions when phytotherapy is selected as a health resource and phytomedicines designed and prescribed.

METHODOLOGY

Information about the anti-inflammatory medicinal plants presented here was obtained from published papers and texts on ethnobotanical studies. The selection of relevant data was made by systematically searching the electronic scientific databases including PubMed, Scopus, SciFinder, and the Web of Science and other web machineries such as Google and Google Scholar. Textbooks, theses, Government survey reports, unpublished materials as well as articles published in peer-reviewed journals were also used for the compilation of data. References were also searched to retrieve the related literature. Some plant name databases including "The Plant List" (www.theplantlist.org) and "Kew Royal Botanic Gardens" (mpns.kew.org) were used to validate the scientific name of the plant.

RESULTS

Regulation of Nuclear Factor-kappa B (NF-κB) Pathway [Figures 1 and 2]

The expression of various pro-inflammatory cytokines and chemokines such as tumor necrosis factor (TNF-α) and interleukin (IL-1) (the main cytokines responsible for neutrophil recruitment to the inflammatory site, activation, modulation, and the trafficking of AMPA receptors in models of inflammatory pain [17]) as well as IL-6, IL-12p35, macrophage (MPH) inflammatory protein-2 (MIP-2), the C-X-C motif chemokine 10 (CXCL-10 or IP-10), or the C-C motif chemokine ligand 3 (CCL-3 or MIP-1-α) [18] are induced by NF-κB activation [19]. NF-κB is an important transcription factor, and it is essential for inducing the expression of a wide variety of cellular genes that are related to the pathophysiological process of inflammation in mammalian cells. Five members constitute the NF-κB family: (1) p65 (or RelA), (2) RelB, (3) c-Rel, (4) the p50/p105 complex (or NF-κB 1), and (5) the p52/p100 complex (or NF-κB 2) [18]. NF-κB exists within the cytoplasm in an inactive form that is associated with a set of regulatory proteins called I-κBs, including I-κBα, and I-κBζ. Stimulation leads to the phosphorylation of I-κB

Table 1: Species used on inflammatory ailments. The origin, the principal mechanism involved and the ethnopharmacological use are collected

Species used on inflammatory ailments				
Native to	Mechanism involved	Species	Ethnopharmacological use	Bibliographical reference
China	1. Nuclear factor kappa-β	*Angelica sinensis*	Blood stasis, analgesia, rheumatism and	[27]
	2. MPH and mononuclears	*Magnolia* spp.	bruises	[52]
	3. COX and LOX inhibition	*Tripterygium wilfordii*	Allergic rhinitis and sinusitis	[120]
	4. Toll-like receptors and inflammasomes	*Zanthoxylum myriacanthum var. pubescens*	Neuralgia, dropsy, and gout Gastrointestinal disorders, parasite invasion, and centipede bites	[71]
America	1. Nuclear factor kappa-β	*Polygala molluginifolia*	Pain and inflammation	[31]
	2. MPH and mononuclears	*Critonia aromatisans*	Inflammation and arthritis	[4]
	3. COX and LOX inhibition	*Castilleja tenuiflora*	Coughs, dysentery, nerves, nausea, vomiting	[96]
	4. Toll-like receptors and inflammasomes	*Polygala sabulosa*	Tonic remedy, topic an esthetic and expectorant	[72]
Africa	1. Nuclear factor kappa-β	*Harpagophytum procumbens*	Pain and osteoarthritis	[37]
	2. MPH and mononuclears	*Biophytum umbraculum*	Dysregulated inflammation, conjunctivitis,	[59]
	3. COX and LOX inhibition	*Kigelia africana*	vaginitis	[104]
	4. Toll-like receptors and inflammasomes	*Xanthium strumarium*	Articular rheumatism, waist pain, wounds Rheumatism, constipation, diarrhea	[23]
Europe and India	1. Nuclear factor kappa-β	*Calea urticifolia*	Gastric ulcers, diabetes, and inflammation	[40]
	2. MPH and mononuclears	*Zataria bracteata*	Antiseptic, analgesic, carminative,	[61]
	3. COX and LOX inhibition	*Tropaeolum majus*	anthelmintic	[107]
	4. Toll-like receptors and inflammasomes	*Origanum vulgare*	Cardiovascular disorders (antihypertensive and diuretic) Convulsive coughs, colds, skin diseases	[81]
Australia	1. Nuclear factor kappa-β	*Salvia plebeia*	Hepatitis, cold, tumors, and inflammation	[48]
	2. MPH and mononuclears	*Carpobrotus rossii*	Upper respiratory tract and throat infections	[66]
	3. COX and LOX inhibition	*Clematis pickeringii*	Headaches, colds, and muscular pain	[112]
	4. Toll-like receptors and inflammasomes	*Vitex trifolia*	Fever, pain, rheumatism and sprained joints	[86]

A. sinensis: Angelica sinensis, T. wilfordii: Tripterygium wilfordii, Z. myriacanthum: Zanthoxylum myriacanthum, P. molluginifolia: Polygala molluginifolia, C. aromatisans: Critonia aromatisans, C. tenuiflora: Castilleja tenuiflora, P. sabulosa: Polygala sabulosa, H. procumbens: Harpagophytum procumbens, B. umbraculum: Biophytum umbraculum, K. Africana: Kigelia Africana, X. strumarium: Xanthium strumarium, C. urticifolia: Calea urticifolia, Z. bracteata: Zataria bracteata, T. majus: Tropaeolum majus, O. vulgare: Origanum vulgare, S. plebeian: Salvia plebeian, C. rossii: Carpobrotus rossii, C. pickeringii: Clematis pickeringii, V. trifolia: Vitex trifolia, MPH: Macrophage, COX: Cyclooxygenases

by I-κB kinase (IKK), I-κBα proteasomal degradation [20] and the nuclear release and accumulation of p65, which allows the NF-κB dimers translocate to the nucleus [21]. Microtubule-associated protein (MAP) and AGC kinases are also important factors in the signal transduction [22]. The phosphorylation of ERK 1/2, JNK, and p38 MAPK activates a series of transcription factors such as AP-1, CREB, c-Jun, and signal transducer and activator of transcription 1 (STAT1), which result in NF-κB activation [23]. The overexpression of the NF-κB pathway leads to the formation, among others, of IL-1β and prostaglandin E (PGE_2) [24] and the induction of cyclooxygenases-2 (COX-2) and inducible nitric oxide synthase (i-NOS) [25].

A. China

The prescreening of 22 commonly used Chinese herbs by NF-κB-dependent activity showed that the ethyl acetate (EtOAc) fraction of *Angelica sinensis* ("Dang Gui" in China, Apiaceae) root, *Morus alba* (Moraceae) root and bark and *Andrographis paniculata* (Acanthaceae) aerial part extract (both 10 g of drug in 300 mL of 95% ethanol at 50°C for 3 h, twice) all suppressed NF-κB luciferase activity and decreased NO and PGE_2 production in lipopolysaccharide (LPS)/interferon (IFN)-γ-stimulated peritoneal MPHs [26]. *A. sinensis* root extract achieved the highest NF-κB luciferase activity inhibition. This root herb is commonly used in traditional Chinese medicine (TCM) and as a dietary supplement in women's care in Europe [27]. It has been called "female ginseng" for its use in the treatment of various gynecological conditions that are generally not easily treated with conventional therapies [27]. The Pharmacopoeia of the People's Republic of China [28] records that it can be applied to treat blood stasis, analgesia, rheumatism, and bruises, and it can also be used as an anti-inflammatory herb. The most commonly used prescriptions containing *A. sinensis* are formulated in decoctions, pastes, vine extracts, and tablets [29]. The EtOAc extract from *A. sinensis* root has been widely evaluated for its ability to modulate NF-κB transactivation activity in an NF-κB-promoted luciferase reporter gene assay and for its *in vivo* anti-inflammatory effects in a murine model of LPS-induced endotoxic shock [19]. Two major compounds of *A. sinensis* - ferulic acid and Z-ligustilide - do possess anti-inflammatory properties. This last, a highly lipophilic compound, has been shown to have a significant anti-inflammatory effect related to the inhibition of the TNF-α-activated NF-κB signaling pathway. Metabolomics-based on gas chromatography and mass spectrometry have been employed to investigate, discriminate and classify the anti-inflammatory effects of *A. sinensis* root and its different

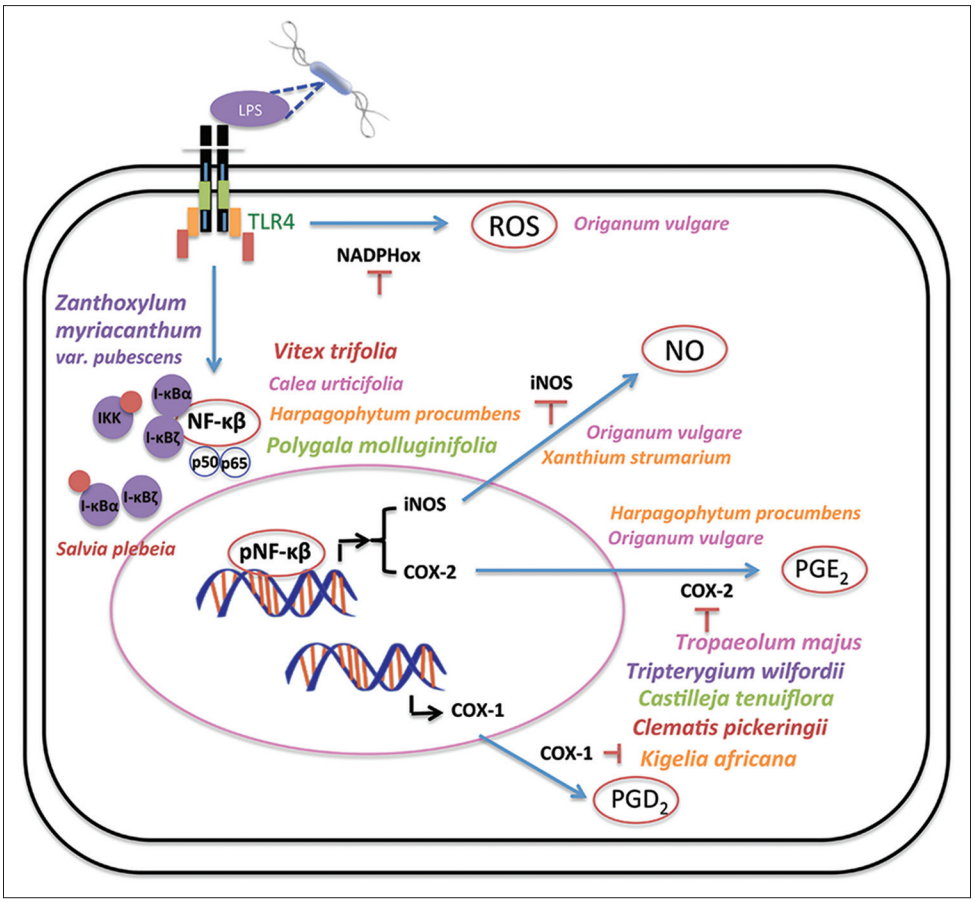

Figure 1: Regulation of the NF-κB pathway. *Polygala molluginifolia, Harpagophytum procumbens, Vitex trifolia* and *Calea urticifolia* inhibit p50 and/or p65 phosphorylation and NF-κB translocation into the nucleus; *Salvia plebeia* suppresses IκBα degradation, which hinders nuclear translocation of NF-κB; and *Zanthoxylum myriacanthum* var. *pubescens* blocks the downstream IKK and I-κB phosphorylation. *Xanthium strumarium* diminishes NO and PGE_2 levels. *Tripterygium wilfordii* and *Clematis pickeringii* inhibit Cyclooxygenases-2. *Tropaeolum majus*, *Castilleja tenuiflora* and *Kigelia africana* act downregulating Cyclooxygenases-2 expression. *Origanum vulgare* possesses pleiotropic effects, as it is able to reduce NO and ROS species. Adapted from Leyva-López *et al*., 2016 and Torres-Rodríguez *et al*., 2016

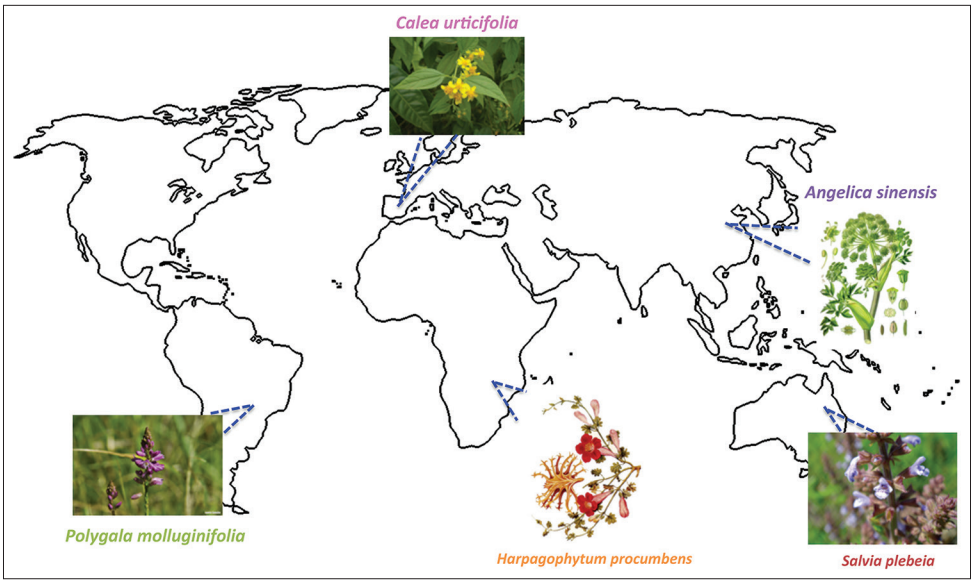

Figure 2: Species with anti-inflammatory effect mainly based on the regulation of the NF-κB pathway

processed products obtained according to the dialectical rule in TCM (parched with alcohol) and based on local Chinese prescriptions (stir-fried, parched with sesame oil or soil) [30]. The metabolite-based profile obtained on male Wistar rats differs for each treatment with regard to inhibiting the secretion of inflammatory mediators, readjusting the level of potential biomarkers and regulating multiple perturbed pathways. The treatment effects of the drug parched with alcohol and stir-fried are superior to those of other processed products of A. *sinensis* root.

B. South America

The genus *Polygala* has been used for a long time to treat several diseases, and especially for pain and inflammation [31]. Aqueous preparations and decoctions of the roots of *Polygala tenuifolia* ("Yuanzhi" in Chinese or "Onji" in Japanese) have been demonstrated to affect TNF-α, IL-1α, and IL-1β [32,33]. Modern pharmacological studies on another species from the genus *Polygala*, *Polygala molluginifolia* - which is native to Rio Grande do Sul (southern Brazil) and popularly known as "canfora," one of the ingredients of the local Brazilian tea - showed that a crude hydroalcoholic extract from dried whole plant material, extracted three times for 7 days each, achieved significant anti-inflammatory efficacy in an *in vivo* inflammation model by inhibiting leukocyte migration, exudate concentrations, the activities of some pro-inflammatory enzymes and the production of important mediators in the carrageenan-induced pleurisy murine model [34]. This effect can be attributed, at least in part, to the large amounts of its two main compounds, rutin and 5,3′,4′-trihydroxy-6″,6″-dimethylpyrano [2″,3″:7,6] isoflavone, which affect the NF-κB pathway by inhibiting p65 phosphorylation and its translocation into the nucleus, similar to the effect observed with dexamethasone. The potential for the extract to inhibit mechanical and thermal hyperalgesia (heat and cold) in postoperative pain in mice without causing sedation or locomotor dysfunction has been evidenced recently [35]. However, the precise mechanism is not completely known. These authors suggest that this may be mediated, at least in part, by the activation of endogenous opioid receptors and/or by the inhibition of TRPV1 and TRPA1 channels, peripherally, and centrally.

C. Southern Africa

The famous species *Harpagophytum procumbens* ("Devil's claw," Pedaliaceae), which grows in southern Africa, can still surprise scientists. Although the preparations of the secondary roots of this perennial herbaceous plant are used in every part of the world successfully to treat pain and osteoarthritis and to decrease the need for non-steroidal anti-inflammatory drugs [36,37], the molecular mechanism by which the principal active compound, harpagoside, inhibits the synthesis of inflammatory proteins remains partially unknown. Huang *et al*. [25] investigated the effect of harpagoside on the activity of the nuclear transcription of the NF-κB system. In previous studies of rat mesangial cells, harpagoside failed to attenuate NF-κB translocation to the nucleus, while the whole extracts of *Harpagophytum* succeed. However, Huang *et al*. demonstrated that harpagoside (200 μM) inhibited the translocation of NF-κB into the nuclear fraction in HepG2 cells. This discrepancy suggests that the ability of harpagoside to attenuate NF-κB activity is specific to the cell and/or stimulus and that there are other active components in the extract involved in the inhibition of the NF-κB translocation to the nucleus. The real effect could be defined as modulatory. The structural features required for active principles acting against the NF-κB target have yet to be elucidated. The harpagoside inhibition of COX-2 *in vitro* [38] and the suppression of c-FOS, one of the main components of AP-1 transcription factors, in osteoarthritis chondrocytes [39] have also been reported.

D. Europe

Calea urticifolia ("Juanislama" in Colombia) is a medicinal plant introduced in Europe from South America in the mid-fifteenth century. It is commonly known in Spain as "Jaral de Castilla" and "Hierba del negro." This species grows in semi-warm and warm climates and is located predominantly in oak forests. It is a shrub, 1-3 m high, with yellow flowers that belong to the Asteraceae (Compositae) family. Local people from Castilla (Spain) use the tea prepared with its leaves as a remedy for gastric ulcers, diabetes and inflammation [40] and as a topical bactericide [41]. The main active compounds from leaves are germacranolide, sesquiterpene lactones with marked cytotoxicity *in vitro* against human tumor cell lines HL60 and SW480 [42]. *C. urticifolia* extract prepared in boiling water (10 g/100 mL) and freeze-dried (yield 14.8%) was able to modulate anti-inflammatory activity through the suppression of the NF-κB signaling pathway by suppressing the nuclear translocation of p50 and p65 subunits of NF-κB that resulted in reduced iNOS expression, inhibited NO/ROS production and the decreased production of pro-inflammatory markers of chronic low-grade inflammation [43]. Phenolic compounds such as caffeoylquinic acids and flavonoids glycosides could be responsible for the anti-inflammatory and antioxidant properties of *C. urticifolia* tea since germacrolides did not inactivate NF-κB as expected and shown in parthenolide [42]. Their pivotal role seems to be involved on Nrf2/ARE pathway activation and the induction of phase II detoxification/antioxidant enzymes [44].

E. Australia

In traditional medicine, *Salvia plebeia* ("Sage weed," Labiatae) is used in the treatment of hepatitis [45] and a variety of inflammatory diseases, colds, and tumors [46]. It is an annual or biennial hairy herb that grows in mountainous regions of many countries such as Australia, Korea, China, India, and Iran. Phytochemical studies on *S. plebeia* have demonstrated that it contains eudesmane-type sesquiterpenoid lactones [47], hispidulin, royleanonic acid, nepetin, eupatorin [46], lignans, diterpenoids, homoplantaginin, luteolin, luteolin-7-glucoside, aliphatic compounds, and caffeic acid [45]. Its active compounds include rosmarinic acid and flavones [48]. The dried extract (yield 16.8%) prepared from a previous aqueous extract (dried and ground whole parts of *S. plebeia* in 70% ethanol extract, 1 week) has demonstrated significant anti-nociceptive activity and *in vitro* and *in vivo* anti-inflammatory activity in a dose-dependent manner [46].

An oral dose of 200 mg/kg in male ICR mice showed an inhibition of 66.4% in the writhing number compared to the control group (indomethacin 10 mg/kg). When the extract was prepared in 95% ethanol (10 kg of air-dried and well pulverized aerial parts at 70°C, 5 h and yield 12.35%) and administered orally to 8-week-old male BALB/c mice at a dose of 2 mg/kg, the expression of inflammatory cytokines and chemokines - IL-4, IL-17, MMP-1, and MMP-3 - were inhibited after 54 days [45]. The mechanism of action is partially related to the suppressed IκBα degradation and the nuclear translocation of NF-κB. Nrf2/HO-1 signaling cannot be ruled out as a mechanism underlying the biological effects of *S. plebeia* and its active ingredients [49]. The extract has also demonstrated the inhibition of the Akt and MAPKs pathways, thereby reducing Akt and ERK p38 expression [45].

Anti-inflammatory Role on the Activation of MPHs and other Mononuclear Cells [Figures 3 and 4]

MPHs play an important role in acute and chronic inflammatory response, both locally and systemically [4], and they serve as an essential interface between innate and adaptive immunity [20]. The activation of MPHs by various stimuli such as bacterial endotoxins, LPS, and viruses massively increases the production of numerous inflammatory mediators, including NO, PGE_2, and various cytokines such as TNF-α, TNF-β, IFN-γ, IL-4, IL-6, IL-10, IL-12, interleukin-1β (IL-1β), granulocyte/MPH colony stimulating factor, MIP-2, monocyte chemotactic protein 1 (MCP-1), and other inflammatory mediators (e.g., 5-HT and L-tryptophan). An increment of some of these mediators (e.g., IL-4) consequently stimulates the activity of B-cells toward the production of IgE, which can exacerbate allergic reactions and stimulate pro-inflammatory responses that are mediated by others mononuclear cells (mast cells, basophils and/or eosinophils). This is particularly the case in allergic diseases such as airway inflammation associated with asthma [50]. These cells also contribute to tissue remodeling, wound healing, phagocytosis and the clearing of apoptotic and necrotic cells [51].

A. China

IL-4 is not the only allergic and inflammatory mediator related to asthma. Histamine, PGE_2, NO, IL-1α, TNF-α,

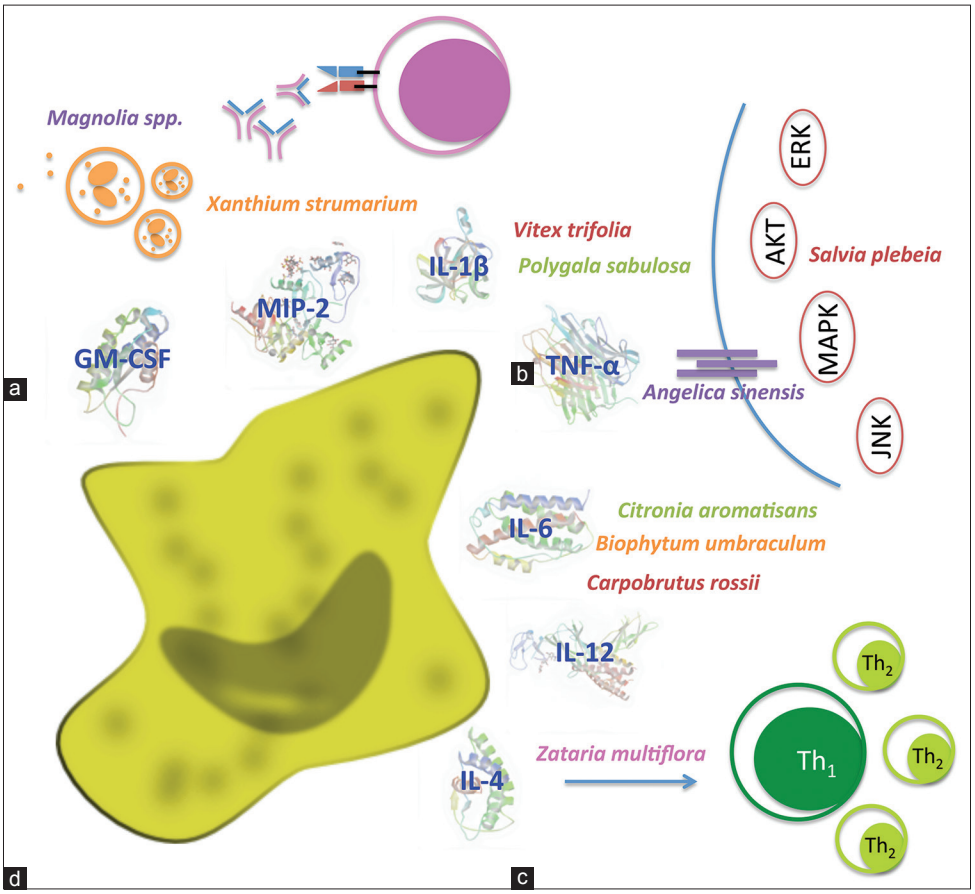

Figure 3: Activity on macrophages and other mononuclear cells. a) *Magnolia* spp. inhibit histamine release in mast cells, and *Xanthium strumarium* has the ability to downregulate IgE levels. b) *Polygala sabulosa* and *Vitex trifolia* reduce pro-inflammatory IL-1β, *Citronia aromatisans*, *Biophytum umbraculum* IL-6 and *Carpobrutus rossii* TNF-α, among others. *Salvia plebeia* inhibits Akt and MAPKs pathways, whereas *Angelica sinensis* does so in the TNF-α-activated NF-κB signaling pathway. c) *Zataria multiflora* inhibits total IL-4 and enhances IFN-γ gene expression and IFN-γ/IL-4 ratio favoring Th_1 activities and suppressing Th_2 functions. C) Salvia plebeia inhibits Akt and MAPKs pathways, whereas Angelica sinensis does so in the TNF-α-activated NF-κB signaling pathway. D) Zataria multiflora inhibits total IL-4 and enhances IFN-γ gene expression and IFN-γ/IL-4 ratio favoring Th1 activities and suppressing Th2 functions.

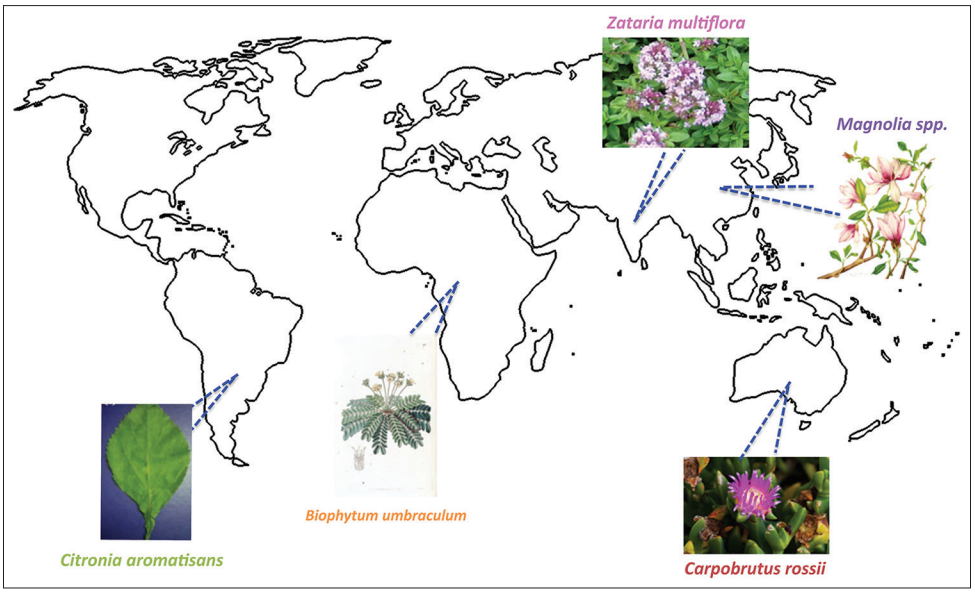

Figure 4: Species with anti-inflammatory effect mainly based on acting upon macrophages and other mononuclear cells

and platelet active factor (PAF) but also play an important role. *Flos magnoliae* (Chinese name "Xin-Yi" or "Shin-I") is one of the most commonly used Chinese medicinal herbs with a long history of clinical application for managing the symptoms of allergic rhinitis and sinusitis [52]. In patients with mild to moderate asthma, a purified extract improved both the asthma-related symptoms and the pulmonary function after 8 weeks [53]. The drug consists in the dried flower buds of *Magnolia* spp. The Pharmacopoeia of the People's Republic of China [28] includes three magnolia species: *Magnolia biondii*, *Magnolia Denudate*, and *Magnolia sprengeri*. Among these, *M. biondii* is the most commonly used form of *F. magnoliae*, which accounted for more than 80% of the market in 2003 [54]. A dry extract of the first two species (from a previous absolute ethanol extract, 1 g fine powder of dried flower buds in 60 mL ethanol, three static cycles at 100°C) 05 mg/mL of absolute ethanol or above in a concentration- and inhibition-dependent manner [55]. Lignans from *F. magnoliae*, including magnolin, magnoshinin, magnosalin [56], lirioresinol-b-dimethyl ether, pinoresinol dimethyl ether, fargesin, demethoxyaschantin, and aschantin may contribute to the pharmacological activities of *M. biondii*. These bioactive lignans have been shown to inhibit various inflammatory mediators such as the PAF, cytosolic phospholipase A2 (PLA), hexosaminidase, lipoxygenase (LO), the complement system and the production of leukotriene (LT) C_4 [53]. Although magnolin, adopted by the Chinese pharmacopeia as a quality control marker, and fargesin have been linked to beneficial therapeutic effects, the assay demonstrated that there were no correlations between the contents of magnolin and fargesin and the inhibition of *F. magnoliae* on induced histamine release.

B. South America

The ointment prepared by placing crushed leaves of *Critonia aromatisans* (synonym of *Eupatorium hemipteropodum*, Compositae, in Spain commonly called "Trebol Oloroso") in a fire along with melted petrolatum is used for anti-inflammatory and anti-arthritis remedies. It can be apply directly to the painful area as a semi-solid preparation. It allows the lipophilic compounds that are responsible for the pharmacological activity and captured by the petrolatum to be absorbed. Leaves in decoction can also be used as a poultice. Despite this, the Composite family is one of the most frequent causes of allergic plant contact dermatitis and bullous allergic contact dermatitis [57]. Strong anti-inflammatory activity has been demonstrated in preclinical *in vivo* assays conducted in BALB/c mice and in NIH mice only for the n-hexane extract [4]. Their results suggest that part of the mechanism of action of the n-hexane extract of *C. aromatisans* is due to its inhibitory effect on MPHs. Some of the classical pro-inflammatory mediators produced by MPHs, including TNF-α, IL-1β, IL-6, and NO, and their release is reduced significantly in cell culture from a concentration of 50 μg/mL. The compounds involved and the mechanism of action are not clear. The probable presence of the species of cyclocolorenone, a compound with an α, β unsaturated cyclopentenone ring (a reactive structural element similar to sesquiterpene lactones), and stigmasterol, a phytosterol that inhibits some pro-inflammatory and pro-degradative mediators involved in inflammation, may contribute to or produce the anti-inflammatory effect observed.

C. Africa

The annual herb *Biophytum umbraculum* (Oxalidaceae), which is commonly found in tropical and subtropical Africa and Asia, is a highly valued medicinal plant used to treat hemorrhoids, wounds, stomach aches, fevers [58], dysregulated inflammation, conjunctivitis, vaginitis and colon illnesses, among others [59]. In Mali, the aerial parts are used to treat both children and adults, and no toxicity has been reported. The most common way to prepare and administer the medicine is to mix the powder of the flowering aerial parts with water; it can be

applied topically, massaging to facilitate the transdermal bioavailability of the compounds, or it can be taken orally [59]. Cassiaoccidentalin A, isovitexin, and isoorientin are flavone-C-glycosides that are isolated from the EtOAc-soluble fraction of the methanol crude extract [58]. They revealed strong antioxidant activity but were inactive when the inhibition of LPS-induced MPHs was tested, and this was in spite of the high dose-dependent inhibition showed by the EtOAc extract. The activity appears to be due to unidentified compounds [59].

D. India

The inflammatory condition, particularly in allergic diseases such as airway inflammation associated with asthma, is regulated by the balance of Th_1/Th_2 cells. An inappropriate response or change in Th_1/Th_2 balance, as well as an uncontrolled activation of Th_2, can cause hyper-reactivity, resulting in asthma disease. *Zataria multiflora* (or *Zataria bracteata*), also called "Avishan-e-Shirazi" or "Shirazi thyme," is a perennial plant belonging to the Lamiaceae family. It is commonly prescribed in Iranian traditional medicine for its antiseptic [60], analgesic, carminative, anthelmintic, and antidiarrheal properties [61]. An aqueous-ethanolic extract (ethanol 50°/water 50:50) was administered to guinea pigs sensitized to ovalbumin. It inhibited the total IL-4 and enhanced IFN-γ gene expression and the IFN-γ/IL-4 ratio, which is an index for Th_1/Th_2 balance, at doses of 30 mg of extract per day [62]. It, therefore, potentiated Th_1 activities and suppressed Th_2 functions. Sensitized animals treated with the extract showed significant improvement in all histological changes of the lung including interstitial inflammation, interstitial expansion, atelectasis, and epithelial damage. There were no adverse effects on treated animals. The anti-inflammatory effect of *Z. multiflora* has been associated, at least in part, with its flavonoids and essential oils, but other active substances of the plant might also be involved [61,63]. Further investigations are needed to unravel the anti-inflammatory properties of this plant, which is employed not only for its thymol and carvacrol content (higher than 65% in both fresh and dried plant), but also for its potential to treat coughs, bronchitis, pneumonia and laryngitis, among others, when multidrug-resistant strains are involved [64].

E. Australia

Carpobrotus rossii (commonly "pigface"), a haplophyte from the Aizoaceae family, is a salt-tolerant coastal succulent ground cover that is native to southern regions of Australia, including the island state of Tasmania. There are many anecdotal (verbal) accounts of *C. rossii* juice being used by local Aboriginals and early European settlers as a traditional remedy for a number of ailments, including upper respiratory tract and throat infections, gastrointestinal upset and itching caused by spider and tick bites, and as an astringent used

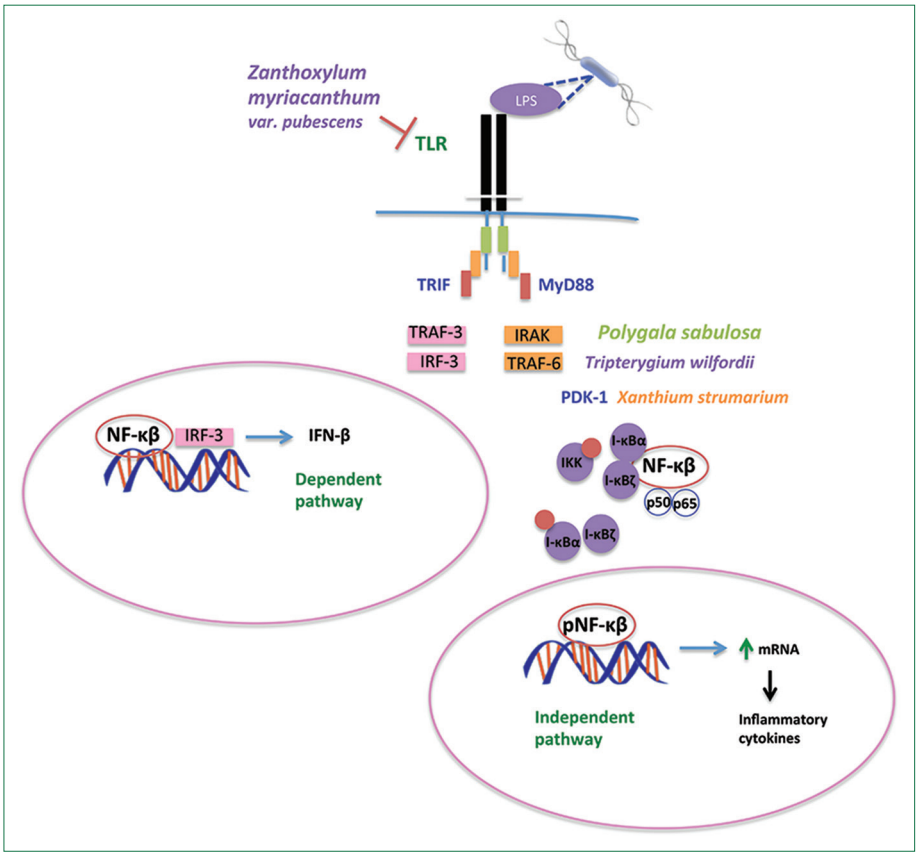

Figure 5: Toll-like receptor-mediated actions. *Zanthoxylum myriacanthum* var. *pubescens* demonstrates suppresses the expression of TLR4 in LPS-activated cells. *Polygala sabulosa* inhibits the activation of TLR4 by LPS. *Tripterygium wilfordii* affects the expression of TRAF-6, which is downstream to the MyD88 signaling pathway, and *Xanthium strumarium* blocks PDK1 and Akt kinase activity, inhibiting the formation of the PDK1/Akt complex. Adapted from Liao *et al.*, 2013 and Hossen *et al.*, 2016

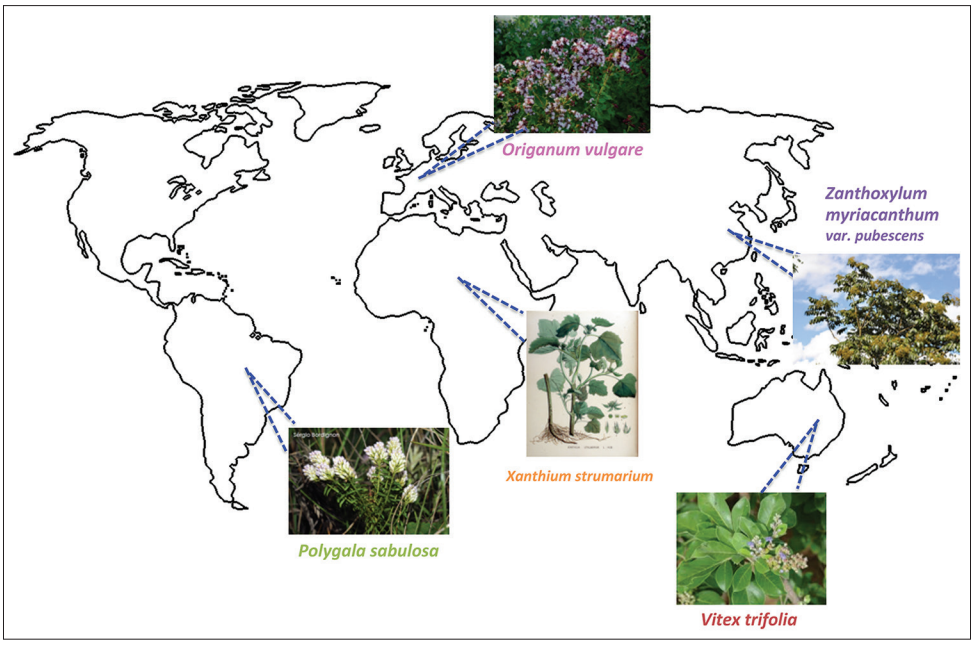

Figure 6: Species with anti-inflammatory effect mainly based on a toll-like receptor-mediated action

externally on wounds, burns, eczema and bluebottle and jellyfish stings [65,66]. Geraghty *et al.* (2011) demonstrated that pretreatment with a dry extract (100 μg/mL of RPMI 1640 media formulation) of fresh juice from succulent leaves reduced IL-10, TNF-α and MCP-1 in peripheral blood mononuclear cells by 57, 72 and 81%, respectively, when stimulated with 5 μg/mL of LPS. It also reduced MCP-1 by 74%, at a dose of 100 μg/mL of RPMI 1640 media, when 5 μg/mL of phytohaemagglutinin A (PHA) was added. The *C. rossii* extract was a more effective inhibitor of cytokine release in the presence of LPS than PHA, indicating the inhibition of cytokine release from a wide variety of leukocytes, including B-lymphocytes, monocytes, and MPHs. No *in vivo* studies were carried out. Fractionation of the extract revealed an important polyphenolic fraction consisting of flavonoids (epicatechin, among others) and tannins, which is consistent with previous assays where epicatechin at concentrations around 200 μg/mL suppressed both RNA expression and the secretion of TNF-α and MCP-1 by NR8383 MPHs [67].

Toll-like Receptors Mediated Actions [Figures 5 and 6]

Toll-like receptors, a subtype of the pattern recognition receptors, are able to identify structural components present on the surface of pathogens (e.g., lipids, carbohydrates, peptides and nucleic acids) that are unique to bacteria, fungi and viruses. These structural components, which are called pathogen-associated molecular patterns, are present on the surface of immune-competent cells such as monocytes/MPHs, dendritic cells, neutrophils and endothelial cells, and signal to activate a host's inflammatory response [68]. TLR4 has been regarded as the main sensor for the recognition of Gram-negative bacteria and as critical upstream mediators that trigger excessive inflammatory cascades [69].

A. China
Recently, the essential oil from the fruits of *Zanthoxylum myriacanthum* var. *Pubescens* (or "Maqian") have been demonstrated to possess healing activity against dextran sulfate sodium- induced intestinal inflammation in mice [70]. Z. *myriacanthum* is a TCM herb native to the Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China, distinguished by its soft hairy rachis, leaves, and petiolules. The fruits are commonly consumed as a spice and used for the treatment of gastrointestinal disorders (e.g., abnormal pain), parasite invasion and centipede bites [71]. When they were extracted using the hydrodistillation method and the resulting essential oil was administered to TPH-1 cells (v/v 0.01%, 0.02%, 0.04%, and 0.05%), the expression of TLR4 in LPS-stimulated THP-1 cells was effectively suppressed in a dose-independent manner. The essential oil effectively prevented the LPS-stimulated increase in TLR4 expression and markedly blocked the downstream IKK and I-κB phosphorylation. Moreover, the pro-inflammatory cytokines, including TNF-α and IL-1 β, were also suppressed.

B. South America
Polygala sabulosa, popularly known as "Timutu-pinheirinho" in Brazil, is a small herb with an abundant number of secondary metabolites, including coumarins, saponins, lignans, flavonoids, steroids, and many xanthones [72]. They are used in folk medicine for the treatment of disorders of the bowel and kidney, as a tonic remedy and as a topical anesthetic and expectorant. In male Swiss mice, the peritonitis model was induced by LPS injection, and the pretreatment with hydroalcoholic extract (500 g air-dried whole plant ground to a powder and extracted at room temperature with 96% ethanol for 14 days) in doses from 3 mg/kg demonstrated an ability to reduce pro-inflammatory (IL-1 β, TNF-α, and IL-6) and increase

anti-inflammatory (IL-10) cytokines levels in the peritoneal leakage [3]. These authors suggest that the extract may be acting directly or indirectly to inhibit the activation of TLR4 by LPS. As is well-known, LPS, a component of the outer membrane of gram-negative bacteria, is a potent activator of MPHs and an exogenous ligand of TLR2, which is expressed in lymphoid tissues, T cells, and MPHs, and TLR4, which is expressed in monocytes, MPHs, dendritic cells and Th1 and Th2 cells. LPS may regulate TLR-2 expression directly and/or through TLR4 [73]. TLR4 signaling involves two pathways: One is dependent on the activation of myeloid differentiation primary response gene 88 (MyD88), and the other is MyD88-independent and unique to TLR3 and 4 [69]. Notably, the MyD88-dependent pathway leads to the transcription of pro-inflammatory genes, resulting in increases in the production of pro-inflammatory cytokines [68,74,75]. Through MyD88-dependent pathways, the IKK complex is activated and induces the expression of inflammatory cytokines through the nuclear translocation of NF-κB. The MyD88-independent pathway utilizes the adapter TRIF to mediate the activation of IRF-3 and the production of type-1 interferons. The suppression of NF-κB activation, direct or indirectly by *P. sabulosa* extract - for example, through the MyD88-dependent pathway - cannot be ruled out.

C. South Africa

Among the plants in the genus *Xanthium* (Asteraceae), *Xanthium strumarium*, commonly referred to as "cocklebur," has traditionally been used as an herbal medicine in South Africa [76]. When mature, this robust, annual weed bears numerous oval, brownish, and spiny burs. The entire plant has been used as a medicine to cure inflammatory ailments such as rheumatism and to relieve constipation, diarrhea and vomiting, chronic bronchitis and rhinitis [23,77]. The essential oil is highly appreciated [78]. Its bioactive compounds include glycosides, phytosterols, phenolic acids, and xanthiazones. Only the seeds in the burs and young seedlings (cotyledonary leaves) contain the toxic principle, carboxyatractyloside [76]. The methanolic extract from the whole plant demonstrated the suppression of LPS-induced phosphorylation of AKT and IKKα/β through TLR2 and 4 by blocking PDK1. It also suppressed the Akt kinase activity and inhibited the formation of the PDK1/Akt complex in cells treated with RAW 264.7 [22]. It leads to the reduced production of IL-6 without affecting IL-1β or TNF-α [23]. The main component that may be involved in PDK1 regulation is resveratrol [22]. The extract has no effect on COX-2 expression at either the mRNA or protein level [23]. NO and PGE_2 levels were also diminished. Upregulation of 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which produces biologically inactive 15-ketoprostaglandins from active PGE_2, has been hypothesized as a possible explanation. The reduced production of IL-6 has been confirmed by Wang *et al*. (2015) for caffeoylxanthiazonoside isolated from *Z. stramarium* fruits and administrated by intraperitoneal injection on a sepsis mice model at doses of 10 mg/kg or higher, but in this case, it was TNF-α production that decreased. The same compounds demonstrated favorable anti-allergic effects and the ability to ameliorate the nasal symptoms and to downregulate IgE levels in an allergic rhinitis rat model [79].

D. Europe

Oregano is the common name for a herb primarily derived from a wide group of plant genera and species used throughout the world in cooking. At least, 61 species from 17 genera belonging to six families are given the name oregano, though the Verbenaceae and Lamiaceae families are the most important [12]. In Europe, *Origanum vulgare* L. is the species most often commercialized as oregano. It is a widespread species native throughout the Mediterranean region, in most parts of the Euro-Siberian region and the Irano-Turanian region [80]. *O. vulgare* has been collected since ancient times to flavor traditional dishes and to relieve various complaints such as convulsive coughs, colds, skin diseases, digestive disorders, headaches, and inflammation-related disorders [81]. The leaves and flowering branches are used in fresh infusions and decoctions and as hydroalcoholic extracts. The bioactivity of the active compounds depends on the preparations consumed [82]. The phenolic acids, flavonoids, and monoterpenes deriving from the "cymyl"-pathway (mainly carvacrol and/or thymol and their biosynthetic precursors c-terpinene and p-cymene) present in the essential oil are hypothesized to possess anti-inflammatory properties [80]. When the essential oils of lavender, salvia, and oregano were dilluted in unscented cream at 3% concentration (in a 2:1:1 ratio) and used daily to massage the lower abdomen of primary dysmenorrhea patients, the blended essential oils demonstrated analgesic properties [83]. With the growing number of reports on the biological activity and nutritional benefits derived from oregano consumption, the identification of the compounds responsible for its activity and the study of its anti-inflammatory potential are relevant and important [12,84]. Both polyphenols, monoterpenes, and sesquiterpenes are active compounds of oregano for which an anti-inflammatory mechanism model based on the amelioration of pro-inflammatory mediators (NO and ROS) and mitochondrial and COX activity produced by LPS-activated MPHs through TLR4 has been proposed [12].

E. Australia

Vitex trifolia L. (simpleleave chastetree, Labiatae) is a tropical shrub or tree (up to 5 m tall) that grows mainly in the coastal areas and is widely employed by Pacific islanders to cure numerous illnesses [85]. The leaves are used in maceration or decoction, both internally and externally, as a remedy to cure illnesses and especially to abrogate diseases that may involve inflammatory processes [18]. For example, it can be heated and rubbed on the forehead to treat headaches, and it can be applied topically as an infusion to relieve fever or alleviate pain derived from rheumatism and sprained joints [85,86]. The traditional aqueous decoction of *V. trifolia* leaves (after lyophilization, 1 mg/mL) has shown the ability to decrease the expression of numerous inflammatory mediators (e.g. cytokines, chemokines, COX-2) through the diminution of the nuclear translocation of the transcription factor NF-κB, which is related to the inhibition of the expression of the NF-κB p50 subunit (but interestingly, not the p65 subunit) after TLR4 activation [18]. It also inhibits IL-1 β and

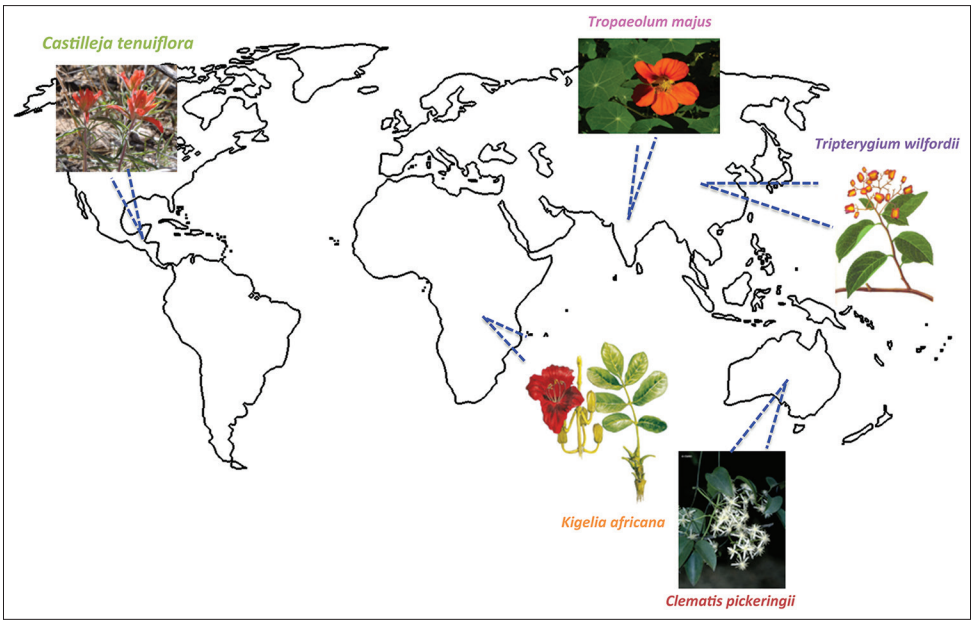

Figure 7: Species with anti-inflammatory effect mainly based on cyclooxygenases and LOXs inhibition

caspase-3 in RAW 264.7 cells, since it is able to downregulate apoptosis due to non-activation of caspase 8, which allows it to avoid the apoptosis of cells. A flavonoid, vitexicarpin (also named casticin), has been reported as the molecule responsible for significant anti-inflammatory effects on acute inflammation and on cell proliferation [86,87]. Among others, it was shown to exert immune-modulatory effects by inhibiting monocyte oxidative burst, chemotactic action on stimulated neutrophils, T- and B-lymphocyte proliferation, the inflammatory activity of isolated human neutrophils and interference with the activity of the STAT1. It also played a vascular protective role through the suppression of NF-κB nuclear translocation.

Inhibition of COX and LO [Figure 7]

When the efficacy of plant extracts on inflammatory conditions needs to be investigated and/or compared [6], researchers fully study the inhibitory effect on the biosynthesis of important inflammatory mediators and PG, for example, PGE_2 and PGD_2, which are potent dilators of vascular smooth muscle that account for the characteristic vasodilatation and erythema (redness) in acute inflammation and symptoms such as fever and pain—from free cell membrane arachidonic acid (AA) liberated by PLA_2 and catalyzed by the molecular targets COX-1, COX-2, 5-LO, and 12-LO. It was initially suggested that the isozyme COX-1 was constitutively expressed in most tissues and involved in the regulation of physiological "housekeeping" (e.g., in the stomach, COX- 1 is important for mucosal fusion, bicarbonate production, and mucus production [5]) and that COX-2 was an isoform that could be induced in pathological conditions by inflammatory stimulation. Selective COX- 2 inhibitors were developed to prevent gastrointestinal side effects caused by the inhibition of COX-1 [88]. However, recent studies have demonstrated that COX-1 and COX-2 have overlapping actions and that are both involved in homoeostasis processes. COX-1 has a significant role in the synthesis of pro-inflammatory PGs and may be induced at the site of inflammation, and COX-2 is constitutively expressed in some tissues, including the brain and kidneys [89]. Furthermore, inhibiting the COX pathway shunts the metabolism of AA toward the 5-LOX pathway [90]. The 5-LOX pathway generates an important class of inflammatory mediators, including LTs, which play a major role in asthma and vascular changes during the inflammatory process. Therefore, in recent years, dual inhibitors of both the COX and LOX pathways are seen as a promising new approach to inhibiting inflammation with no or low toxicity, and they could be an important treatment for many inflammatory disease states [88,91].

A. China

Of 46 Chinese materia medica vine plants, 44 have been used to treat rheumatism and related inflammatory diseases [92], alleviate pain in joints and muscles, relieve fever and stiffness in the limbs and expel "wind-dampness" [93], a term related to rheumatic fever, rheumatoid arthritis, and osteoarthropathy [91]. Of these plants, *Tripterygium wilfordii* ("léi gong téng" in China, *Celastraceae*) roots and stems and *Trachelospermum jasminoides* ("confederate-jasmine", *Apocynaceae*) stems have been used in decoctions for centuries. COX-1, COX-2, and 5-LOX inhibition assays were carried out to evaluate the anti-inflammatory effects of these traditionally used vine plants. The dry extracts (from previous one in absolute ethanol under 6º for 8 h, reconstituted in ethanol before each experiment) of both *T. wilfordii* roots and stems (DER 1.08:30) and *T. jasminoides* stems (DER 0,85:30) demonstrated significant inhibition of COX-1 (inhibitory concentration (IC_{50}) 27±1 μg/mL and 35±2 μg/mL, respectively) and COX-2 (IC_{50} 125±8 μg/mL and 138±7 μg/mL, respectively). Only *T. wilfordii* extract showed linear regression (IC_{50} 22±2 μg/mL) for 5-LOX [91]. Although *T. wilfordii* extract showed a significantly higher

IC_{50} value on COX-2 than indomethacin under the same experimental conditions ($P < 0.01$), there is ample evidence that *T. wilfordii* is toxic and that its use has resulted in cases of leukopenia and thrombocytopenia. Therefore, complete cytotoxicity studies should be undertaken and reported before further clinical practice [94]. Qin *et al*. [95] reported the potential therapeutic effect of *T. wilfordii* on inflammation through the inhibition of the expression of TLR4 affecting the expression of TRAF-6, which is downstream of the MyD88 signaling pathway, thereby suppressing the activation of NF-κB and reducing the release of inflammatory factors such as TNF-α and IL-1β.

B. South America

Castilleja tenuiflora (also known as *C. angustifolia* and *C. canescens*, popularly "cola borrego" in Spain) is an annual small perennial shrub belonging to the *Orobanchaceae* family that is distributed in disturbed areas of pine-oak temperate forests of the Southern United States and Mexico [96]. In The History of the Plants of New Spain (16th Century), this species was recorded under the Náhuatl name "Atzoyatl." It is described as having a "hot nature" and root preparations that heal colics caused by fecal mass retention [97]. It is commonly known in Mexico as "garaiiona," "cola de borrego" (lamb tail) or "hierba del cancer" and in English as "Indian paintbrush" [98]. It is harvested wild for traditional use. The decoction of its leaves has been used in Mexican traditional medicine to treat coughs, dysentery, nerves, nausea, and vomiting as well as hepatic and gastrointestinal diseases [96]. *C. tenuiflora* accumulates iridoid glycosides, such as aucubin and geniposidic acid, in its aerial parts and roots. This glycosides have *in vitro* antitumor and cytotoxic effects [99] as well as neuroprotective, immunomodulatory, hepatoprotective, cardioprotective, and anti-inflammatory (COX-2 and COX-1 related) effects [100]. In addition to iridoid glycosides, the phenylethanoid glycosides verbascoside and isoverbascoside have been detected in the genus *Castilleja* and isolated from the roots and aerial parts [96,98]. PhGs are natural compounds that can be absorbed by human intestinal cells, and they are potent anti-inflammatory agents because they inhibit the accumulation of pro-inflammatory molecules such as NO and cytokines along with the expression of the COX-1 and COX-2 [101]. The presence of these compounds in *C. tenuiflora* could be related to the significant effect of 20% (20.01±3.45%) inflammation inhibition produced by 1 mg/ear of a dry extract from a previous methanolic one obtained from the aerial parts of *C. tenuiflora* (255 g of whole plants extracted twice with 0.4 L absolute ethanol) in a topical model of inflammation [TPA-induced ear edema in male ICR mice (1 mg/ear and n=5)] [96]. In contrast, the control, indomethacin, showed 40% inhibition.

C. Africa

Among the African medicinal plants, *Kigelia africana* (also *K. pinnata* or "sausage tree") of the family Bignoniaceae happens to be one of the most recognized [102]. Locally known as the cucumber or sausage tree because of its huge fruits (which average 0.6 m in length and 4 kg in weight), it can grow to more than 20 m tall [103]. People in different parts of the world have claimed that parts of this plant serve various purposes, but there is not enough scientific research to back up their claims. In Ivory coast, eating the bark powder daily and bathing in its maceration are folk and traditional treatments for articular rheumatism [104]. In Sudan, Senegal, Tanzania, Benin, and Kenya, the decoction of the stem bark mixed with soda or cow milk for oral use or flour for dressing inflamed body parts is used for waist pain, wounds [105], ulcers or sores. For toothaches, the powdered bark is applied around the inflamed teeth [106]. The mature fruits and roots are also used to treat abdominal pain and fever. Researchers have evaluated the anti-inflammatory activity of several parts of the plant, including the stem bark and roots. The ethanolic stem bark extract (500 g powdered bark with 2.5 L of absolute ethanol, 72 h, yield 3.78% w/w) has been researched for its activity against carrageenan-induced inflammation on the hind paws of rats (a classic and highly reproducible method for assessing the acute inflammatory responses in antigenic challenges and irritants) and for its analgesic properties in the hot plate test and mouse-writhing assays [103]. The dry extract in doses of 200 mg/kg produced a higher inhibition of the synthesis and release of PG than aspirin (100 mg/kg), which resulted in a dose-dependent anti-inflammatory effect. This is most likely the mechanism by which the analgesic effect occurs.

D. India

Nasturtium ("Indian cress," *Tropaeolum majus*), an herbal plant from the order *Brassicales*, is traditionally employed in the treatment of cardiovascular disorders for its antihypertensive and diuretic effects [107] Its effects on inflammation and microbes meet the 1978 German Commission E standards for herbal medicines [108]. This species contains high amounts of benzyl glucosinolate. Some authors [109,110] attribute its biological effects, at least the anti-bacterial and the anti-inflammatory ones, to the products of its enzyme-mediated hydrolysis. Tran *et al*. (2016) demonstrated that aqueous nasturtium plant extract (1 g of whole plant powder in 10 ml of distilled water [pH 6.32] or DMSO at 37°C for 30 min at 100 rpm) at a concentration of 111 μg/mL not only selectively inhibits COX-2 protein expression and PGE 2 release but also has no impact on COX-2 enzyme activity in primary human peripheral mononuclear cells (PBMCs). They found that 5-LOX protein expression was not regulated at any of the tested plant extract concentrations, even though when PBMCs were pretreated with nasturtium extract (333 μg/mL) followed by 1 μg/mL LPS and 1 μg/mL fMLP, the release of the LT B4 was inhibited (by half) compared with the negative control. The authors suggest that the reduced COX-2 protein levels that follow pretreatment with the compounds could be related to a temporary inhibitory effect on ERK1/2 downstream signaling and blocking of the c-Jun activation involved in forming the early response transcription factor AP-1, which starts the transcription of pro-inflammatory cytokines (e.g., TNF-α). A 90% ethanolic extract of *T. majus* leaves a lyophilized yield of 14% and showed no sub-chronic toxicity after a 28-day long oral treatment in Wistar rats at a dose of 750 mg/kg [111]. These results suggest that this species is safe. However, other toxicological studies are necessary to evaluate the overall safety of this plant.

E. Australia

The species *Clematis pickeringii*, *Clematis glycinoides* ("traveler's joy" and "headache vine"), and *Clematis microphylla* have been traditionally used in Australia to treat inflammatory conditions [6]. Both indigenous Australians and bushmen have used the crushed foliage to cure headaches and colds by inhaling the strong and sharp aroma [112,113] and applying the juice, freshly ground leaves or bark to the painful area [114]. The dry extract of *C. pickeringii* stems (mixed with PVP 5% to remove the tannins that have been reported to produce antiphlogistic activity during the preparation of extracts) from a previously ethanolic plant (absolute ethanol is 500mL x3, 48h each at room temperature with a final yield of 4.3%) showed COX-1 (IC_{50} 73.5±1.2 μg/mL), COX-2 (IC_{50} 101.2±1.2 μg/mL), and 5-LOX (IC_{50} 29.3±2.3 μg/mL) inhibition. *C. pickeringii* stems activated the protein expression of PPARα and PPARγ, which then cause cell replication, differentiation, and inflammatory responses in HepG2 cells (60 μg/mL; [89]. Experiments performed this way showed better results than previously published studies in which the extraction was carried out with absolute ethanol for 12 h at 70°C and produced a 4.7% yield [6]. These authors speculate that *C. pickeringii* contains active ingredients that act as a natural peroxisome proliferator-activated receptor (PPARα) and PPARγ ligands.

AN OVERVIEW

There are many plants used to treat inflammatory diseases worldwide. Here, interesting examples of early approaches to deciphering the enigma of the pharmacological relevance and mechanisms from plants used in different cultures have been provided. As has been shown, "traditional medicine" around the world has directly or indirectly discovered, without accurate scientific knowledge, the same anti-inflammatory actions. Nonetheless, the ethnopharmacological investigation of these species is still relatively limited, and the state of science to date on the selected species is still poor and inconclusive. The cellular and molecular mechanisms underlying the anti-inflammatory actions of these plants or their chemical ingredients remain poorly characterized. Observation, description, and investigation of the ingredients, their effect and these indigenous drugs would allow the development of an "ethnopharmacy" process that encompasses relevant disciplines, such as pharmacognosy, pharmacology, toxicology, and drug delivery, to apply knowledge from orally transmitted medical systems to drug development [115] in an evidence-based and scientifically rational approach.

However, inflammation is a complex combination of processes, some of which are not fully understood, involving several interdependent events that vary in duration, intensity, and consequences over time and between people. Thus, the therapeutic strategy for treating inflammation should be streamlined. If the ailment is different, then why should the remedy be the same? Researchers have paid close attention to this issue in recent decades. Using a model analysis, Kell [116] demonstrated how targeting a particular step in the signaling pathway of the transcription factor NF-κB can have qualitatively (directionally) different effects depending on the actual state of the system and whether systems are diverse. In the case of phytomedicines, combinations of ingredients are subject to considerable batch-to-batch variability since this will depend on where plants go, harvesting techniques, storage and the preparation of decoctions [16] where the interactions between the components of the medicine and potential molecular targets (required for optimal effect) are more complex than those associated with a single chemical entity in no particular step. This is far for simple. The same approach could be applied to TCM, a complex theoretical system that encourages interactions and synergies between the various phytomedicines comprising any given therapy.

A characterized compound or group of compounds with a perfectly defined mechanism of action at a particular dose fulfills the negative requirements (such as toxicity) inherited from classical pharmacology. The new trends suggest an integrative approach in which a wide variety of compounds act together on multiple targets to produce a final action through a balance resulting from minor changes. The researchers have made suggestions wherever possible about "the most active principal compounds" involved in the events. However, in this way, an assumption is made. The plant has one or a few ingredients that determine its therapeutic effect, what's called "the silver bullet method" [117]. In this method, the described effects would not be explained completely. In recent years, "the shotgun approach," which has been understood as a therapeutic strategy that targets multiple parts of an organism, has gained increasing acceptance [118].

Omics technologies and methods and systems biology approaches will become principal tools in current and future endeavors to integrate traditional and modern medical systems. Through them, classical concepts will fit into new strategies of coping with chronic and emerging diseases. An integrated approach using genomics, transcriptomics, proteomics, and metabolomics as well as phytomedicine for the assessment of the mode of action of multidrug treatments will likely yield the most reliable results, but it remains the great challenge for the future [119]. Promising results can be expected in the inflammation field. Urlich-Merzenich *et al*. (2007) use microarrays to obtain the gene expression profile (mRNA) of willow bark extract STW-33-1, quercetin, diclofenac, and acetylsalicylic acid in human chondrocyte cultures and willow bark extract STW-33-1 in the blood cells of rats. Gene expression rates were up- or down-regulated differently for each substance, which clearly demonstrated that they had specific expression profiles. The modulation of genes is not the only effect a compound/extract could promote. It is converted at the protein level, and the metabolomics is understood quantitatively and completely, which has led to patterns associated or not associated with anti-inflammatory results. Classical pharmacology can add onto this step each compound membrane disturbance, receptor agonism or antagonism and protein inhibition or stabilization, among other things. Even in systems biology, the protein networks, and regulatory feedback loops must be taken into account. If an extract is administered,

the complexity grows greatly. The combination of constituents could yield a new, fully differentiated state and the effect could or could not be (synergism or antagonism) dose-dependent. The interactions could be much more sophisticated.

Despite the high level of complexity and cost of the omic techniques, they are the only way the pharmacological action of many species can be fully understood. They are the key to a rational and fully accepted form of phytotherapy. The application of "omic" technology unfolds the possibility to investigate phytopreparations without prominent active principle(s) for their complex mechanisms of action and helps us to rationalize the therapeutic superiority of many plant extracts over single isolated constituents" [119]. Furthermore, through them, the prescription could be selected according to the patient's needs in individualized medicine or more generally in stratified medicine targeted at subgroups of patients. In terms of ailment, developing the appropriate drug for each health problem and physiological condition means assessing "personal safety," shortening the drug development process and prompting cost reduction. How this could be introduced into regulatory laws to comply with the legal conditions is another matter.

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