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Original Research

Evaluation of Anti-Inflammatory and Analgesic Effects of Pioglitazone and Rosiglitazone in Wistar Rats

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ABSTRACT

The challenges faced in the treatment of various inflammatory disorders are immense. Inflammation is emerging as the pathological basis for many disorders like rheumatoid arthritis, uveitis etc. The purpose of the study is to evaluate analgesic effect of the test drugs Pioglitazone, Rosiglitazone and the standard drug Diclofenac sodium using Analgesiometer, in Wistar rats. NSAIDs and steroidal anti-inflammatory drugs have yielded good results in the treatment of inflammatory conditions, except for their potential to produce serious adverse effects, have resulted in widespread limitation of their use. In this study three models were selected for evaluation of acute and sub-acute anti- inflammatory activity, as well as analgesic activity of test drugs, Pioglitazone and Rosigliitazone. For comparison Diclofenac was used as a standard drug and vehicles of each test drug were used as control. Wistar rats were used as experimental animals.

KEY WORDS: *Anti-Inflammatory, Analgesic, Wistar rat, Pioglitazone, PPAR-gamma*

INTRODUCTION

It is well understood, accepted and scientifically proved that the inflammation is the basis for the pathogenesis of many disorders like rheumatoid arthritis, ankylosing spondylitis, uveitis, bronchial asthma, atherosclerosis, autoimmune diseases, and lung fibrosis, as well as life threatening hypersensitivity reactions to insect bites, drugs, and toxins. Such conditions are most commonly associated with pain and are very debilitating disorders. There are many drugs now available for treatment of different inflammatory conditions. Some of them like Non-steroidal anti-inflammatory drugs (NSAIDs) and steroidal anti-inflammatory drugs have yielded good results till now, except for their potential to produce serious adverse effects, sometimes even life threatening events have resulted in widespread limitation of their use.

Also, some other drugs like penicillamine, gold salts, methotrexate and allopurinol etc [1] have been in clinical use to treat inflammatory conditions like rheumatoid arthritis, gout etc. Some adrenergic agonists, calcium channel blockers and macrolides have also been reported to possess anti-inflammatory activity in experimental studies. Some other drugs unrelated to NSAIDs, like statins which inhibit HMG-CoA reductase enzyme and sulfonamides like sulfamethiazole have also been reported to posses anti-inflammatory activity in experimental models. (5-8) As these drugs have been reported to produce serious adverse effects, the search for safer and better anti-inflammatory agents is ongoing. The role of PPAR-gamma agonists as anti-inflammatory agents is now evident from few earlier studies [9]. There are very few studies till today which have evaluated the potential role of PPAR-gamma agonist like thiazolidinediones as anti-inflammatory agents [10]. Further studies to substantiate the role of PPAR-gamma agonists as anti-inflammatory agents are very scarce. Hence, this study was undertaken to evaluate anti-inflammatory effect of PPAR-gamma agonists like thiazolidinediones which include Rosiglitazone and Pioglitazone. Considering the potential role for PPAR-gamma agonists as anti-inflammatory agents as well as good anti-diabetic agents can open up new avenues in the research for potent anti-inflammatory agents, proving them to be useful in treating many inflammatory disorders as well as preventing complications of diabetes. It is for the first time that both the drugs of thiazolidinedione class Pioglitazone and Rosiglitazone, at three different doses have been evaluated in this study, till now. Not only anti-inflammatory activity but also the analgesic activity of Pioglitazone and

Rosiglitazone has been evaluated in this study, which was never done before.

AIMS & OBJECTIVES

AIM

To evaluate, anti-inflammatory and analgesic effects of Pioglitazone and Rosiglitazone in Wistar rats.

OBJECTIVES

To evaluate anti- inflammatory effect of the test drugs Pioglitazone, Rosiglitazone and standard drug Diclofenac sodium using acute and sub-acute models of inflammation in Wistar rats. To evaluate analgesic effect of the test drugs Pioglitazone, Rosiglitazone and the standard drug Diclofenac sodium using Analgesiometer, in Wistar rats. To compare the results of anti- inflammatory and analgesic effects of test drugs Pioglitazone, Rosiglitazone with the standard drug Diclofenac sodium. To compare the results of both anti- inflammatory and analgesic effects between two test drugs Pioglitazone and Rosiglitazone.

REVIEW OF LITERATURE

Acute inflammation is characterized by a sudden onset and has a short course (a few minutes to several hours). The signs of inflammation are present along with constitutional symptoms. The microscopic picture comprises of exudation of fluid, plasma and leukocytic emigration. Alteration in the microvasculature (arterioles, capillaries and venules) is the earliest response to tissue injury. These alterations include hemodynamic changes and changes in vascular permeability. In the initial stage, the escape of fluid is due to vasodilatation and consequent elevation in hydrostatic pressure. This fluid is transudate (does not contain protein) in nature. But subsequently, the characteristic inflammatory oedema, exudate (rich in proteins and inflammatory cells), appears by increased vascular permeability of microcirculation [11]. The early stage of an acute inflammatory process is reproduced in the guinea pig with the study of cutaneous erythema produced by UV rays (Ultraviolet rays). The technique was first described by Adams in the year 1960 [12]. Pedal inflammation (edema) is produced by injection of various phlogistic (irritating) agents into the hind paw of the rat. Various irritants used are carrageenan, mustard, dextran, egg-white, compound 48/80, yeast, serotonin, histamine, formalin, kaolin, substance-P, nystatin, glass powder, cobra venom, naphthoylheparamine etc. Carrageenan which was first used by Winter C.A, E.A Risley and G.W.Nuss, is still the most widely used irritant.It is injected as 0.05 ml to 0.1 ml volume of 1 % solution in normal saline, into one of the hind paws. The test drugs are administered appropriately prior to injecting carrageenan and paw edema is measured by plethysmograph at regular intervals of time.

Lee et al, 2007, induced ear edema in mice by topical application of 12-O- tetradecanoyphorbol 13-acetate. Arachidonic acid induced ear edema model in mice has also been described which is suitable for screening of lipoxygenase inhibitors.[13] During inflammation, vascular permeability increases to allow plasma constituents such as antibodies and complement to access injured or infected tissues. As a consequence, fluid and plasma proteins extravasation takes place and edema is formed. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan‘s blue. Albino Wistar rats are injected with 0.25 ml of 0.6 % v/v solution of acetic acid intraperitoneally. Immediately, 10 ml/kg of 10 % w/v Evans blue is injected intravenously via tail vein. After 30 minutes, the animals are sacrificed and the abdomen is cut open. The peritoneal fluid (exudates) collected, filtered and made up to the volume of 10 ml using normal saline solution and centrifuged at 3000 rpm for 15 minutes. The absorbance of the supernatant is measured at 590 nm using spectrophotometer. Increased concentration of dye with respect to the absorbance indicates increase in permeability.[14,15]

Weisinger D., in 1964, employed rats with Mycoplasma L4 induced arthritis and measured the grip function of the inflamed articulation [12,16] Phenylbutazone, aminopyrine, indomethacin and gold preparations were found to improve joint motility. Wiesenger, 1964 described a different type of arthritis which can be produced in the rat by means of intravenous injection of Mycoplasma arthritis isolated from Murphy Sturm lymphosarcoma of rats [16]. Granuloma formation method was first described by D‘Arcy et al. 1960, wherein, sterilized cotton pellets weighing 7-10 mg are implanted subcutaneously, in male albino rats, under anaesthesia [12]. Treatment is given daily throughout the study. The granulomas are dissected out on the fifth day for quantification. The cotton pellets are weighed after overnight drying at 60 °C.

However the technique has been suitably modified by using other suitable form of foreign bodies like grass piths, plastic rods, etc. and prolonging the study for 10 days [17]. The grass piths can be immersed in 10 % formalin for subsequent microscopic studies.

Pain can occur without tissue injury or evident disease and can persist after injury has healed. Serious tissue injury can occur without pain. Emotion (anxiety, fear, depression) is an inseparable concomitant of pain and can modify both its intensity and the victim‘s behavioral response. There is important processing of afferent nociceptive and other impulses in the spinal cord and brain. Pain is both a sensory and an e motional (affective) experience. Pain perception is a result of nociceptive input plus a pattern of impulses of different frequency and intensity from other peripheral receptors, e.g. heat, and mechanoreceptors whose threshold of response is reduced by the chemical mediators. These are processed in the brain whence modulating inhibitory impulses pass down to regulate the continuing afferent

input. But pain can occur without nociception [some neuralgia (Neuralgia is pain felt in the distribution of a peripheral nerve.)] and nociception does not invariably cause pain; pain is a psychological state, though most pain has an immediately antecedent physical cause.

MATERIALS & METHODS

Protocol for this dissertation study was submitted to the Institutional Ethical Committee (IEC) and Institutional Animal Ethical Committee (IAEC) before starting the study. The study was approved by both IEC and IAEC constituted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals). Adult healthy Wistar rats of either sex weighing 150-250gm were obtained from the Central animal house, under Department of Pharmacology, Krishna Institute of Medical Sciences, Karad, maintained under suitable conditions of housing, temperature, ventilation and nutrition. Total 162 Wistar rats were included in the study over the period of 1 year.

OBSERVATIONS AND RESULTS

Carrageenan Induced Rat Hind Paw Edema

After inducing edema, the observations were made by measuring height of mercury displacement in Plethysmograph. The height of mercury displaced was measured first in small divisions, as marked on Plethysmograph. One small division is equal to volume of 0.25 ml. Number of small divisions measured multiplied by 0.25 ml denoted total volume displaced. The volume displaced after dipping the paw, was measured at different time intervals, starting from 0 hr, 30 min, 1 hr, 3 hrs, 4 hrs, and 5 hrs after injecting carrageenan in one of the hind paws. Immediate reading after the injection was taken as 0 hour reading.

The difference in volume displaced at different time intervals from 0 hour reading was taken as actual paw edema. Hence 0 hr readings was not considered as actual paw edema and not mentioned in the tables. Paw edema was measured in different groups, with six rats in each group (n=6). The observations were then tabulated as mean ± SEM as enumerated below.

Table 1: Comparison of mean paw edema volume (ml) between Diclofenac treated group (IX) and its Control group (V) at different time intervals

Groups	Paw edema volume in ml (MEAN ± SEM)					P value (Repeated ANOVA)
	30 min	1 hr	3 hrs	4 hrs	5 hrs	
V. CONTROL D.W 2 ml/kg	0.667 ± 0.0527	1.167 ± 0.0527 ***	1.125 ± 0.0559 **	0.916 ± 0.0527 *	0.708 ± 0.0768 ###	< 0.0001
IX. DICLOFENAC 5 mg/kg	0.333 ± 0.0527	0.458 ± 0.0768	0.25 ± 0.0645 #	0.167 ± 0.08333 ###	0.083 ± 0.0527 ** ###	< 0.0001
P value (Unpaired Student 't' Test)	0.0012 $$	< 0.0001 $$$	< 0.0001 $$$	< 0.0001 $$$	< 0.0001 $$$	

[***/###/$$$ Extremely significant P<0.001; **/##/$$ Very significant P<0.01;*/#/$ Significant difference P<0.05; n=6]

According to table no. 1, Observations at 1 hr, 3hrs, 4 hrs and 5 hrs compared with the observations at 30 min within the same group (Intra group comparison). Observations at 3hrs, 4 hrs and 5 hrs compared with the observations at 1 hr within the same group (Intra group comparison). Inter group comparison between group V and IX. For control group V, intra group comparison after Repeated measure ANOVA and Post hoc Tukey Kramer multiple comparison test revealed extremely significant (P<0.001), very significant(P<0.01) and significant difference (P<0.05) in paw edema at 1hr, 3 hrs, and 4hrs respectively as compared to its 30 minutes reading. This suggested significant increase in paw edema at 1 hr, 3 hrs, and 4 hrs from 30 minutes in control group V. Though extremely significant difference was noted at 5 hrs as compared to 1 hr with reduction in paw edema at 5 hrs, it was especially after significant increase in paw edema at 1hr in control group.

For Diclofenac treated group IX, intra group comparison after Repeated measure ANOVA and Post hoc Tukey Kramer multiple comparison test revealed no significant difference (P>0.05) in paw edema at 1 hr and 3 hrs from 30 min reading. This suggested no significant increase in paw edema at 1 hr and 3 hrs from 30 min. It also revealed extremely significant difference (P<0.001) at 4 hrs and 5 hrs from 1 hr, and significant difference (P<0.05) at 3 hrs from 1 hr. Also very significant difference (P<0.01) was noted at 5 hrs compared to 30 min reading. This suggested significant reduction in paw edema at 3 hrs, 4 hrs and 5 hrs. Inter group comparison between Diclofenac treated group IX and control group V using unpaired student 't' test revealed very significant difference (P<0.01) in paw edema

at 30 min and extremely significant difference (P<0.001) at 1 hr, 3 hrs, 4 hrs and 5 hrs.

Table 2: Comparison of paw edema volume (ml) within Pioglitazone treated groups (II, III, IV) and their Control group (I) at different time intervals. (Intra group comparison)

Groups	Paw edema volume in ml (Mean ± SEM)					P value (Repeated ANOVA)
	30 min	1 hr	3 hrs	4 hrs	5 hrs	
I	0.625	1.125	1.00	0.833	0.667	0.0001
CONTROL	±	±	±	±	±	
D.M.F	0.1070	0.08539	0.1118	0.1054	0.08333	
0.3 mg/kg		***	**		###	
II.	0.50	0.583	0.417	0.333	0.208	0.0003
PIOGLITAZONE	±	±	±	±	±	
5 mg/kg	0.1291	0.08333	0.0527	0.0527	0.07683	
				#	** ###	
III.	0.417	0.541	0.375	0.25	0.167	0.001
PIOGLITAZONE	±	±	±	±	±	
10 mg/kg	0.08333	0.07683	0.1070	0.06455	0.08333	
				##	* ###	
IV.	0.375	0.50	0.333	0.167	0.125	< 0.0001
PIOGLITAZONE	±	±	±	±	±	
15 mg/kg	0.05590	0.06455	0.10540	0.08333	0.05590	
				* ###	** ###	

[***/### Extremely significant; **/## Very significant; */# Significant difference; n=6.

According to table no. 2, Observations at1 hr, 3hrs, 4 hrs and 5 hrs compared with the observations at 30 min within the same group. Observations at 3hrs, 4 hrs and 5 hrs compared with the observations at 1 hr within the same group. Intra group comparison after Repeated measure ANOVA and Post hoc Tukey Kramer multiple comparison test in Control group I revealed extremely significant difference (P<0.001) and very significant difference (P<0.01) with increase in paw edema at 1 hr and 3 hrs respectively from 30 min. While no such significant difference (P>0.05) was noted in Pioglitazone treated groups II, III, IV at 1 hr and 3 hrs from 30 mins. This suggested there was no significant increase in paw edema at 1 hr and 3 hrs from 30 min in Pioglitazone treated groups.

Pioglitazone treated groups II and IV showed very significant difference (P<0.001) at 5hrs from 30 min. Group III showed just significant difference (P<0.01) at 5 hrs from 30 min. This suggested significant reduction in paw edema at 5 hrs from 30 min, noted with all pioglitazone treated groups. No such significant difference was noted with control group I.

DISCUSSION

In this experimental study on Wistar rats, the Thiazolidinedione class of drugs, Pioglitazone and Rosiglitazone, a PPAR-γ agonists used in the treatment of type 2 diabetes have demonstrated good acute and sub-acute anti- inflammatory activity. They have also demonstrated analgesic activity at two higher doses of each. The anti-inflammatory and analgesic activity is comparable with the standard anti- inflammatory drug Diclofenac sodium. This is in accordance with the few earlier studies like Snehalata VG et al.[18] who demonstrated acute as well as chronic anti- inflammatory effects of Rosiglitazone without ulcerogenic potential. There is hardly any study which has evaluated analgesic activity of PPAR- gamma agonists, and has been attempted this study. The probable mechanism of analgesic activity of PPAR- gamma still needs to be elucidated. Further studies in this aspect are essential.

Though serious concern were raised with the safety of Rosiglitazone regarding increased risk of cardiovascular events in last decade,[19-21] and the drug was withdrawn from the market, the FDA has recently revoked it in 2013 under black box warning,[22] stating the earlier reports showed inconclusive evidence relating to cardiovascular events. Pioglitazone was also withdrawn from the Indian market after raising concerns over its potential risk of causing bladder cancer. It was also revoked into Indian market in August 2013 with box warnings in bold red letters with respect to bladder cancer.[23-25] Attempts to develop novel glitazones (thiazolodindiones) a PPAR-γ agonists with potent glucose lowering and hypolipidemic activity, with improved cardiovascular and other safety profile of these class of drugs are ongoing.[26] Thus thiazolidinedione class of drugs, a PPAR- γ agonists can open up new avenues in the search for potent anti- inflammatory and analgesic agents with better safety profile, proving them to be useful in treating many inflammatory disorders as well as preventing complications of diabetes.

CONCLUSION

This study revealed that both Pioglitazone and Rosiglitazone, a PPAR-gamma agonists, possessed good anti- inflammatory (acute and sub-acute) as well as

analgesic properties. The anti- inflammatory and analgesic activity was prominent especially at two higher doses i.e Pioglitazone 10 mg/kg and 15 mg/kg dose and Rosiglitazone 5 mg/kg and 10 mg/kg dose. Effect of both the drugs was comparable with standard drug Diclofenac. Thus PPAR-gamma agonists could be useful in managing inflammatory conditions which are sometimes debilitating and are not responding to conventional anti- inflammatory agents. Also they can be used as add on therapy with conventional anti- inflammatory and analgesic drugs. Study of PPAR- gamma agonists can open up new avenues in search of potent, safe anti- inflammatory and analgesic agents. Further studies using different models of evaluation in animals, as well as clinical trials in humans are essential.

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Original Research

Assessing the Effect of Mode of Delivery and Birth Weight on Thyroid Stimulating Hormone and Glycemic Status in Neonates

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ABSTRACT

A negative link between TSH and period after birth has been discovered. In vacuum-assisted infants and neonates, the amount of TSH was higher than that of normal vaginal deliveries. Babies then got more TSH than babies later. The neonatal TSH is not linked to birth weight, gestatory duration, maternal age and period from membrane breakage to delivery. The degree TSH according to gender, labour induction, maternal disorder and maternal medicines was not substantially distinguished. The TSH and CH rates were acceptable but not the TSH and T4 levels. Statistical evaluation found that neonatal TSH was affected by sampling time, delivery mode, birth weight including BSL levels. Additionally, glycemic state were observed to be statistically significant in newborns at 24 hrs that suggests that blood sugar levels in newborns are influenced by mode of deliveries.

KEY WORDS: *Premature newborns, Thyroid stimulating hormone, Congenital hypothyroidism, Neonates screening, Hypothalamic-pituitary-thyroid*

1. INTRODUCTION

1.1 Background

The Congenital hypothyroidism (CH) is a typical congenial endocrinal condition with an estimated occurrence of 1 in 3000 to 1 in 4000 newborns [1-3]. CH is irreversible and occurs in anomaly (dysgenesis or organesis) in the growth of the pituitary gland or in deficiencies in thyroid hormone production. Moderately often there is intermittent hypothyroidism that may arise through transplacental motherhood, maternal antibody obstruction or iodine deficiencies. CH can be attributed to hypophysis or hypothalamic irregularity in extreme cases [4-7].

During the first half of pregnancy, the prenatal production of thyroid hormone (TH) is small. The fetus relies absolutely on maternal TH throughout this period. The infant hypothalamic-hypophysiology-thyroid axis begins functioning in the center of the feeding cycle and becomes established during puberty. Placenta forms a significant obstacle to thyroid hormone, rendering the prenatal hypothalamic-pituitary-thyroid network completely separate from the maternal framework [8]. The Placenta is impervious to TSH. Thyroid hormones have long been recognised as highly essential in the formation of CNS and mainly influence neuronal differentiation as well as synapse

formation [9].

Various study shows that Caesarean newborns are slightly higher in TSH than in the case of the vaginally delivered neonates although others have recorded higher rates in the blood of TSH in vaginally born babies. The impact of these variables is not obvious [10]. Several research have also demonstrated that the neonatal activity of the thyroid is not impaired by transmission [11].

Similarly there is no consensus on effect of birth weight on TSH values in neonates. High incidence of raised TSH has been documented in lower birth weight infants whereas some studies document that birth weight has no effect on neonatal TSH levels.

1.2 Research Objectives

1. To study the effect of mode of delivery and birth weight on thyroid stimulating hormone and glycemic status in neonates
2. To assess the effect of mode of delivery (Caesarean Section or Vaginal delivery) on measured TSH Levels and glycemic status in term neonates.
3. To determine the effect of birth weight on TSH levels and glycemic statusin term neonates.

2. LITERATURE REVIEW

Lakshminarayana et al. [12] performed an assessment of the presence of TSH and TH in the umbilical cord of the blood with perinatal causes. We observed that in caesarial neonates as well as those born with regular vaginal childbirth there was also no substantial variation in the mean TSH value.

A survey has been undertaken by Caverzere et al. [13] to evaluate the relationship seen between delivery method in full term healthy pregnancies of cord Fluid T4 and TSH. Concentration levels of umbilical cord plasma T4 and TSH with radio-immunoassay have been assessed. Infants born vaginally showed the umbilical cord plasma TSH to be slightly higher than babies delivered by caesarean process. The writers suggested that when analyzing the TSH findings of umbilical cord plasma the transmission mechanism would be taken into account.

3. METHODOLOGY

3.1 Data Collection
Subjects

The source of data was the neonates born at Krishna Hospital and Medical Research Center, Karad for two years duration. A total of 462 neonates were selected for this study.

Sample Size

Considering mean TSH (Normal delivery 3.4 & mean TSH (LSCS) =2-8 And SD = 2.2 WITH 95% confidence & 80% power minimum 212 babies from Normal delivery & 212 babies from (SCS) were included out of the total subjects & then applied statistical methods.

Inclusion criteria

 Apparently healthy neonates with gestational age more than 37 weeks.

Exclusion criteria

 Preterm babies (with gestational age less than 37 weeks)
 Neonates whose blood samples were collected less than 24 hours of birth.
 Neonates who were suffering from any illness or were on any antibiotics.
 Neonates born to mother with known thyroid disorders.
 Neonates born to diabetic mother

3.2 Method

After delivery, neonate specimens were drawn from the umbilical cord as well as measurements from the peripheral veins obtained 24 hours a day. TSH was measured within four days of selection of samples. TSH was evaluated using GOD-POD package technique with Chemiluminescence Immunoassay (CLIA).

3.3 Test for TSH
Application

The qualitative determination of TSH concentration in

human serum by a microplate. Chemiluminescence immunoassay (CLIA)

Reagents

1) Thyrotropin calibrators - 1
2) TSH tracers reagent
3) Light reaction wells
4) Signal reagent A– containing luminal in buffer store at 2-8°C
5) Wash solution concentrate –contain surfactant in buffered saline
6) Signal reagent B-H2O2, store at 2-8°C

Expected Ranges of values

Low normal range 0.42 µIU/ml
High normal range 5.45 µIU/ml
70% confidence interval to 2.5 percentile
Low range 0.33 to 0.5
High range 5.05 to 6.02

Test procedure

1) Formal the micro plate wells for each serum reference control & patient specimen to be assayed in duplicate.
2) Pipette 0.50 ml (50 µl) of appropriate serum reference, control or specimen into assigned well.
3) Add 0.100ml of TSH tracer request to each cell
4) Swirl the micro plate gently for20-30 second to mix and cover
5) Incubate 45 min at room temp.

3.4 Test for Sugar
Reagent composition in test

Active Ingredients	→	**concentration**
Peroxidase	→	≥800U/L
Glucose oxidase	→	≥20000 U/L
Mutarotase	→	≥1000U/L
Tris-phosphate buffer	→	50 mmol/L
Phenol	→	10mmo1/1

Expected Ranges of values

Fasting BSL 60-110 mg/dl

Sectional Criteria
1) Inclusion

a. Primi gravida.
b. Full term pregnancy.
c. Single-ton pregnancy
d. Normal pregnancy
e. K/C/O H/O Radiation.
f. Mother having diabetes mellitus.

2) Exclusion

a. Mother having thyroid dysfunction
b. Preterm baby.
c. Baby with Asphyxia.
d. Chronic diseases in neonate & Mother.

3.5 Instrument Used
Lumax based on CLIA technique

1) EM360 of TRANSASIA fully automated analyzer God – Pod method

Blood samples were analyzed for TSH and BSL by using Lumax based on CLIA technique TSH screening assay and GOD-POD method for BSL estimation respectively. A maximum of 462 subjects have been obtained and all have been used. The TSH values and sex, birth weight including delivery system have been analyzed for concise metrics. Depending on their birth weight and method of delivery, neonates, etc. were grouped into categories. The birth weight and distribution strategies of TSH and BSL values were statistically analyzed.

3.6 Statistical Methods

This work has implemented descriptive and inferential statistical assessment. Mean ± SD (Min-Max) is reported as a product of continuous measurements and findings are displayed by amount (percent) for propositional measures. Value was measured at 5% value point. The significance was

measured at the 5 percent point of value by the study of variation (ANOVA). The value of research parameters for 3 or more patients' groups were evaluated using the ANOVA (Variance Analysis). The definition of cumulative scale sample parameters among two classes (Inter-group Analysis) on metric variables had been used for student t research (two tailed, independent). The association between birth weight, TSH and BSL were evaluated by using Pearson method.

4. OBSERVATIONS AND RESULTS

Around 462 neonates depending on eligibility and rejection considerations were included in this analysis. At birth, birth weight and TSH samples have been obtained (Table 1 and Table 2). The mean was 234 and 228 being delivered via Caesarean from full term healthy vaginal deliveries.

Table 1: Mode of delivery and birth weight

Mode of Delivery		Birth Weight (in Kg)
Full Term Normal Delivery (FTND)	N	234
	Minimum	1.10
	Maximum	3.73
	Mean	2.7775
	Std. Deviation	0.46155
Lower Segment Cesserian Section (LSCS)	N	228
	Minimum	1.10
	Maximum	3.75
	Mean	2.7970
	Std. Deviation	0.45781
STATISTICAL ANALYSIS	Unpaired 't' test value	0.456
	p value	0.648

Table 2: Birth weight and the levels of TSH

Birth Weight		TSH (in μIU/ml)
LBW	N	97
	Minimum	0.40
	Maximum	10.90
	Mean	5.3598
	Std. Deviation	1.82355
NBW	N	365
	Minimum	0.40
	Maximum	19.50
	Mean	5.6077
	Std. Deviation	2.45942
Statistical Analysis	Unpaired 't' test value	0.927
	p value	0.354

In our study 365 neonates had birth weight more than 2500 g and 97 neonates had low birth weight of less than 2500g with TSH level 5.60±2.45 and 5.35±1.82 respectively which was statistically not significant (Table 2). Moreover, negative correlation was found between birth weight and BSL values however there was no correlation found between birth weight TSH values and BSL.

Table 3: levels of blood sugar at 24 hours after birth

Mode of Delivery		BSL (in mg/dl)
Full Term Normal Delivery (FTND)	N	234
	Minimum	32.00
	Maximum	100.00
	Mean	65.1880
	Std. Deviation	14.06266
Lower Segment Caesarean Section (LSCS)	N	228
	Minimum	38.00
	Maximum	120.00
	Mean	62.2368
	Std. Deviation	11.84243
Statistical Analysis	Unpaired 't' test value	2.437
	p value	0.015

In our study mean BSL Value in (mg/dl) born by FTND was 65.18 14.06 and mean BSL in LSCS born babies was 62.23 11.84 with *p* value 0.015 which is statistically significant (Table 3).

Due to the neonatal TSH surge, screen for CH using TSH as the primary method should be delayed until after 24-48 hours of life, otherwise there will be more number of false positive tests. In premature newborns the postnatal peaks of TSH and of T4 occur at the same time as term infants but their amplitude is somewhat lower (blunted response) than that observed in term newborns due to hypothalamo-pituitary-thyroid axis immaturity. Transient hypothyroxinemia is common in preterm infants.

5. CONCLUSION

In our study there was no statistical significance in the neonates having low birth weight and BSL at 24 hours. However statistical significance was found in mode of delivery and BSL values in term neonates. Also there was no significant correlation found amongst birth weight as well as TSH values in term neonates. Hence it can be concluded from this study that mode of delivery and birth weight do not affect TSH values of term neonates. Thus mode of delivery and birth weight need not be taken into account when TSH values, in samples collected at birth in term neonates.

This research also was carried out to determine a statistically important influence of delivery mode on the glycemic condition of newborns at 24hrs, and suggest that delivery mode influences neonatal blood sugar rates during 24hrs duration.

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Original Research

Antinociceptive Activity of Paroxetine with Morphine in Acute Pain in Albino Mice

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ABSTRACT

Antidepressants, mainly tricyclic antidepressants have been found useful for the treatment of chronic pain. Because of numerous undesirable side effects of traditional tricyclic antidepressants, SSRIs with favorable side effects profile are preferred. The purpose of the study is to evaluate antinociceptive activity of paroxetine in albino mice and to compare antinociceptive activity of paroxetine with morphine in albino mice. To delineate the possible mechanism of antinociceptive activity of paroxetine, paroxetine is producing its antinociceptive activity by acting through both mechanisms (opioid receptor pathway and 5-HT3 pathway) in this study. Study revealed that paroxetine has promising antinociceptive activity which needs to be explored. This drug can be used as add on therapy for treatment of pain with lower doses of conventional analgesic drugs.

KEY WORDS: *Paroxetine, Albino mice, Antinociceptive, Morphine, ANOVA test*

INTRODUCTION

"Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage."[1]

Pain is a protective mechanism. Pain occurs whenever any tissues are being damaged, and it causes the individual to react and to remove the pain stimulus [2]. Over the course of human history, pain has been treated by psychological techniques, physical methods (surgical intervention, electrical stimulant, pressure, cold, heat, counter-irritation, acupuncture) and by drugs [3]. Pain is a symptom of many diseases requiring treatment with analgesics [4]. Paroxetine provided analgesic effect for patients with refractory chronic pain. However, this effect was remarkable with 20 mg or more and accompanied by antidepressive effect [5]. A study has shown that paroxetine improves pain symptoms in physical illness and it also revealed that there is evidence linking the analgesic properties of paroxetine with its serotonergic, opioidergic and noradrenergic activity [6-8]. From this, we can assume if paroxetine produce the antinociceptive activity; it may be producing by acting on these serotonergic or opioidergic pathway. So, in this study, to delineate the possible mechanism of antinociceptive activity of paroxetine, we studied the interaction of paroxetine with naltrexone and paroxetine with ondansetron.

AIM & OBJECTIVE

AIM

To evaluate and compare antinociceptive activity of paroxetine with morphine and probe into the possible mechanism.

OBJECTIVES

To evaluate antinociceptive activity of paroxetine in albino mice. To compare antinociceptive activity of paroxetine with morphine in albino mice. To probe into the possible mechanism of action of paroxetine. To study the interaction between paroxetine and naltrexone. To study the interaction between paroxetine and ondansetron.

REVIEW OF LITERATURES

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Pain has been an important factor in human experience throughout history. It may be difficult to realize however that the concept of pain has not always been held as we do today. Management of any except acute pain. Primitive man had no difficulty in appreciating the association of pain with obvious injury but one was mystified by pain of internal disease or pain that persisted with no apparent cause. It is probable that many amulets, nose rings, animal talismans or other unearthed artifacts represented magical techniques to ward off pain. Little has changed in pain management until Renaissance (14^{th}-16th centuries). Pain management continued to be centered on the same narcotic and analgesic agents that had been described centuries before. Management of pain in the 19th century saw many advances in the science of pharmacology, several of which concerned narcotic, analgesic and topical and general anaesthesia. Morphine was isolated from opium in 1806 by Serturner and named after Morpheus, the Greek god of dreams. The synthetic manufacture of specific compounds led to the introduction of salicylic acid and related compounds including acetyl salicylic acid, marketed as aspirin, the most commonly prescribed analgesic throughout the last century. Concepts of pain changed as medicine itself took on a more scientific cloak. Management of pain made significant advances during the 1st half of 20th century, especially in the development of analgesics and related drugs for pain and variety of non-narcotic analgesics appeared including acetaminophen and phenacetin. Semisynthetic and synthetic narcotic analgesic agents were developed. Surgical management of pain continued to focus on interruption of the neospinothalamic tract at various levels or interruption of sympathetic innervation. The most significant contribution to the concepts of pain perception occurred in 1965, when Melzack and Wall proposed the gate theory of pain. The anatomical concepts incorporated into the gate control theory paved way for the use of stimulation of nerve structure for the modulation of pain. This was the decade that saw the introduction of stimulation techniques for the management of chronic pain, based primarily on the gate theory, which indicates that stimulation of large fibers peripherally inhibits transmission of pain.

MATERIAL & METHODS

Protocol for this dissertation study was submitted to the Institutional Ethical Committee (IEC) and Institutional Animal Ethical Committee (IAEC) before starting the study. The study was conducted after the IAEC permission was obtained strictly in accordance with the protocol. Swiss albino mice of either sex, weighing 20-40g, were used for the study. The mice were inbred in the Central animal house, Department of Pharmacology, Krishna Institute of Medical Sciences, Karad, under suitable conditions of housing, temperature, ventilation and nutrition. Total numbers of 108 animals were used in our study.

OBSERVATION & RESULTS

For analgesiometer method, reaction time was noted for tail flick before and 15 min, 30 min, 60 min, 120 min after injection of respective drugs.

Table 1: Comparison between control and morphine groups in analgesiometer method

GROUPS / DRUG-TIME INTERVAL		BEFORE	AFTER				REPEATED MEASURES ANOVA	
			15 MIN	30 MIN	60 MIN	120 MIN	F	P
CONTROL (DW)		3.77 ± 0.761	3.75 ± 0.543	4.33 ± 0.634	4.55 ± 0.538	4.5 ± 1.021	0.6105	0.6599
MORPHINE 0.5		4.27 ± 1.058	7.83 ± 0.75***	9.93 ± 0.067***	9.43 ± 0.442***	9.25 ± 0.546***	20.600	<0.0001
UNPAIRED 't' TEST	t	0.384	4.392	8.779	7.012	4.101		
	P	0.7092	0.0014	<0.0001	<0.0001	0.0021		

[n=6 in each group; latency in seconds for tail flick expressed as mean ± SEM;P<0.05 considered as significant] *** P<0.001.

In table no. 1, there was no significant difference in mean duration of latency in control group using repeated measures ANOVA test (P=0.6599). There was a significant difference in mean duration of latency in morphine group using repeated measures ANOVA test (P<0.0001). Post hoc Tukey-Kramer multiple comparison test revealed that the mean duration of latency in morphine group was significantly increased at all the time intervals i.e. 15, 30, 60 and 120 min (P<0.001) as compared to before reading. The mean duration of latency in morphine group was significantly more at all the time intervals i.e. 15 min (P=0.0014), 30 min (P<0.0001), 60 min (P<0.0001) and 120 min (P=0.0021) as compared to control using unpaired 't' test. Thus, morphine produced a significant increase in the latency of tail flick at all time intervals with onset and maximum antinociceptive activity within 15 min, maintaining the activity till 120 minutes.

Table 2: Comparison of intragroup readings in paroxetine 2.5, paroxetine 5 and paroxetine 10 groups in analgesiometer method (by repeated measures ANOVA followed by post hoc Tukey-Kramer multiple comparison test)

GROUPS / DRUG-TIME INTERVAL	BEFORE	AFTER				REPEATED MEASURES ANOVA	
		15 MIN	30 MIN	60 MIN	120 MIN	F	P
PAROXETINE 2.5	3.92 ± 0.886	5.17 ± 1.094	5.77 ± 1.168	6.78 ± 0.703	6.48 ± 0.741	1.994	0.1343
PAROXETINE 5	2.3 ± 0.384	7.37 ± 0.773 ***	8.72 ± 0.812 ***	8.95 ± 0.627 ***	9.15 ± 0.602 ***	37.212	<0.0001
PAROXETINE 10	3.42 ± 0.723	7.78 ± 1.112 ***	9.35 ± 0.650 ***	9.42 ± 0.583 ***	9.55 ± 0.450 ***	36.788	<0.0001

[n=6 in each group; latency in seconds for tail flick expressed as mean ± SEM;P<0.05 considered as significant] *** P<0.001.

As in table no. 2, there was no significant difference in mean duration of latency in paroxetine 2.5 group using repeated measures ANOVA test (P=0.1343). There was significant difference in mean duration of latency in both paroxetine 5 (P<0.0001) and paroxetine 10 (P<0.0001) groups using repeated measures ANOVA test. Post hoc Tukey-Kramer multiple comparison test revealed that the mean duration of latency was significantly increased at all the time intervals i.e. 15, 30, 60 and 120 min (P<0.001) as compared to before reading in both paroxetine 5 and paroxetine 10 groups.

Table 3: Comparison of intergroup readings in control, paroxetine 2.5, paroxetine 5 and paroxetine 10 groups in analgesiometer method (by one way ordinary ANOVA followed by post hoc Tukey-Kramer multiple comparison test)

GROUPS / DRUG-TIME INTERVAL		BEFORE	AFTER			
			15 MIN	30 MIN	60 MIN	120 MIN
CONTROL (DW)		3.77 ± 0.761	3.75 ± 0.543	4.33 ± 0.634	4.55 ± 0.538	4.5 ± 1.021
PAROXETINE 2.5		3.92 ± 0.886	5.17 ± 1.094	5.77 ± 1.168	6.78 ± 0.703	6.48 ± 0.741
PAROXETINE 5		2.3 ± 0.384	7.37 ± 0.773 *	8.72 ± 0.812 **	8.95 ± 0.627 ***	9.15 ± 0.602 **
PAROXETINE 10		3.42 ± 0.723	7.78 ± 1.112 *	9.35 ± 0.650 **	9.42 ± 0.583 ***	9.55 ± 0.450 ***
ONE WAY ORDINARY ANOVA	F	1.05	4.322	8.006	13.152	10.466
	P	0.3921	0.0166	0.0011	<0.0001	0.0002

[n=6 in each group; latency in seconds for tail flick expressed as mean ± SEM;P<0.05 considered as significant] *P<0.05, **P<0.01, *** P<0.001.

In table no. 3, there was significant difference in mean duration of latency in control, paroxetine 2.5, paroxetine 5 and paroxetine 10 groups using one way ordinary ANOVA test at all the time intervals i.e. 15 min (P=0.0166), 30 min (P=0.0011), 60 min (P<0.0001) and 120 min (P=0.0002). Post hoc Tukey-Kramer multiple comparison test revealed that the mean duration of latency was not significantly different for paroxetine 2.5 as compared to control at all the time intervals. But, the mean duration of latency was significantly more in both paroxetine 5 and paroxetine 10 groups as compared to control at all the time intervals i.e. 15 min (P<0.05), 30 min (P<0.01), 60 min (P<0.001) and 120 min (P<0.01 for paroxetine 5 and P<0.001 for paroxetine 10). There was no significant difference in mean duration of latency between paroxetine 5 and paroxetine 10 groups at all the time intervals (P<0.05). Thus, paroxetine 2.5 didn't produce the antinociceptive activity while paroxetine 5 and paroxetine 10 produced the antinociceptive activity with no dose dependency. Paroxetine 5 and paroxetine 10 produced the antinociceptive activity with onset and maximum activity within 15 min, maintaining the activity till 120 minutes.

Table 4: Comparison among morphine 0.5, paroxetine 5 and paroxetine 10 groups in analgesiometer method

GROUPS / DRUG-TIME INTERVAL		BEFORE	AFTER			
			15 MIN	30 MIN	60 MIN	120 MIN
MORPHINE 0.5		4.27 ± 1.058	7.83 ± 0.754	9.93 ± 0.067	9.43 ± 0.442	9.25 ± 0.546
PAROXETINE 5		2.3 ± 0.384	7.37 ± 0.773	8.72 ± 0.812	8.95 ± 0.627	9.15 ± 0.602
PAROXETINE 10		3.42 ± 0.723	7.78 ± 1.112	9.35 ± 0.650	9.42 ± 0.583	9.55 ± 0.450
ONE WAY ORDINARY ANOVA	F	1.631	0.082	1.023	0.243	0.1506
	P	0.2286	0.9217	0.3833	0.7871	0.8615

[n=6 in each group; latency in seconds for tail flick expressed as mean ± SEM; P<0.05 considered as significant]

As shown in table no. 4, there was no significant difference in mean duration of latency in morphine 0.5, paroxetine 5 and paroxetine 10 groups at all the time intervals using one way ordinary ANOVA test. Thus, both paroxetine 5 and paroxetine 10 produced the antinociceptive activity statistically similar to morphine at all time intervals.

DISCUSSION

In the present study to find out the possible mechanism of action of paroxetine, we studied the interaction of paroxetine with naltrexone and paroxetine with ondansetron. It was observed that morphine 0.5mg/kg produced the significant antinociceptive activity in both tail flick method and acetic acid induced writhing method. These findings coincide with findings of Barbara J et al.[9] where, morphine showed the antinociceptive activity in mice using hot plate method; and with the findings of M Kesim et al.[10] where, intraperitoneally injected morphine (0.5 mg/kg) inhibited the nociceptive effect of acetic acid in writhing method.

It was found that paroxetine 5 and paroxetine 10 produced the antinociceptive activity in both tail flick method and acetic acid induced writhing method with no dose dependency. It was observed that paroxetine 5 and paroxetine 10 produced the antinociceptive activity which was statistically similar to morphine at all time intervals in tail flick method. It was also found that only paroxetine 10 produced the antinociceptive activity which was statistically similar to morphine in acetic acid induced writhing method and paroxetine 5 was having less antinociceptive activity as compared to morphine. It was observed that pre-treatment with both naltrexone and ondansetron with paroxetine 5 blocked the antinociceptive activity of paroxetine. There was no significant difference found between both the combination groups. So, it is evident that paroxetine 5 was producing its antinociceptive activity by acting through both the pathways (opioid pathway and 5-HT3 pathway).

It was observed that pre-treatment with both naltrexone and ondansetron with paroxetine 10 blocked the antinociceptive activity of paroxetine. There was no significant difference found between both the combination groups. So, it was evident that paroxetine 10 was producing its antinociceptive activity by acting through both the pathways (opioid pathway and 5-HT3 pathway). So, antinociceptive activity of paroxetine at both doses (5mg/kg and 10 mg/kg) was antagonized by naltrexone 5mg/kg and also by ondansetron 0.1mg/kg.

There are studies available with naloxone (another opioid receptor antagonist) combination and few studies where, they have combined naltrexone with fluoxetine (another SSRI).[11,12]

CONCLUSION

Study revealed that paroxetine has promising antinociceptive activity which needs to be explored. This drug may become important for treatment of pain when patients can't tolerate the conventional analgesic drugs because of their adverse effects and also in cases where depression is coexisting with painful conditions. This drug can be used as add on therapy for treatment of pain with lower doses of conventional analgesic drugs. The limitations of the study being the study is very primitive in the method and parameters used to evaluate analgesia, and it has been carried out only in single species i.e. mice. Further studies need to be done in various other acute and chronic models using different species to establish the efficacy of paroxetine as an analgesic agent.

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Original Research

Animal Study of Effect of Lamotrigine Pretreatment in Bipolar Disorder

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ABSTRACT

In the present study treatment with high doses of dexamphetamine (5 and 10 mg/kg ip) as well as apomorphine (1.5 and 3 mg/kg ip) was found to induce sniffing and oral movement variety (OMV) of stereotyped behaviour (SB) in rats. Pretreatment with haloperidol antagonised the SB induced by lamotrigine and that induced by apomorphine and dexamphetamine. This indicates that DAergic mechanisms are involved in the induction of SB by lamotrigine in rats. Pretreatment with alpha-methyl-p-tyrosine results in depletion of both DA and noradrenaline from the DAergic and noradrenergic neurons respectively whereas pretreatment with small doses of apomorphine cause selective depletion of DA from the DAergic neurons. Further, the intensity of haloperidol catalepsy depends on the dose of haloperidol used, increasing with increase in dose. The effect of pretreatment with small doses of apomorphine was therefore studied on the SB induced by lamotrigine, apomorphine and dexamphetamine. The results were statistically analyzed based on student's t-test.

KEY WORDS: *Haloperidol, Lamotrigine, Phenyltriazine, Bipolar depression, Neurotransmitter, Cataleptic disorder, Antiepileptic stabilizer*

1. INTRODUCTION

1.1 Background

Lamotrigine a phenyltriazine related medication is an antiepileptic product that prevents the production of an excitatory neurotransmitter glutamate [1] and blocks voltage-dependent sodium (Na+) channels. Animal studies have demonstrated, but not prevented clonic engine seizures induced using pentylenetetrazole, that lamotrigine suppresses the tonically enlarged hind extremity of the main electroshock type, with partial and secondary generalized seizures of the enzyme models [2,3]. Nonetheless, Lennox-Gastaut syndrome in both children and adults and lack of seizures is therapeutic lamotrigine effective for selective and secondarily expanded add-on treatment and monotherapy with tonic-clonic seizures in children [4].

Recently, multiple clinical studies found that lamotrigine was successful in managing bipolar conditions, indicated effectiveness during severe bipolar depression, though not in acute medical systems, and was long-term therapy for bipolar depression. The anti-glutamatergic behaviour and improved dopaminergic Neuro-space Delivery are pathways hypothesized for its efficacy in managing bipolar disorders, respectively [5]. Frontal cortical lesions have been shown to improve behavioral reactions to amphetamines and decrease haloperidol catalepticism, contributing to reduced rates of striatal glutamate [6].

Lamotrigine, by blocking the voltage-dependent Na+ channels, will inhibit the release of glutamate from the corticostriatal and cortico-nigral glutamatergic neurons.

Consequently, it will decrease the concentration of glutamate in the striatum and SNc and thereby produce a functional lack of glutamate at the NMDA receptors in the striatum and SNc. The NMDA receptor antagonist MK-801 produces a functional lack of glutamate at the NMDA receptor sites in the striatum and SNc by blocking the NMDA receptors [7].

1.2 Research Objectives

The present study was undertaken with following aims and objectives in mind. Objectives for performing this part of the study are as follows:

1. To compare the intensity of lamotrigine-induced SB with the intensity of SB induced by high doses of apomorphine and dexamphetamine.
2. To study the effects of pretreatment with haloperidol, alpha-methyl-p-tyrosine and small doses of apomorphine on the SB induced by high doses of apomorphine and dexamphetamine.
3. To determine whether the cataleptic effect of the neuroleptic haloperidol is antagonized by lamotrigine treatment. This part of the study was undertaken as haloperidol induced catalepsy is reported to be antagonized by amphetamine and apomorphine treatment in rats.
4. To compare the effects produced by treatment with lamotrigine, high doses of apomorphine and dexamphetamine on the cataleptic effect of haloperidol.

2. LITERATURE REVIEW

The effects of lamotrigine on various seizure types have been demonstrated in animal models [7,8]. Although, the relationship between animal and human data is unclear, studies in animals give insight as to possible applications for a new antiepileptic drug.

Mitra-Ghosh et al. [9] have reported that lamotrigine does not affect the basal release of glutamate. However, Alghatani et al. [10] have reported that lamotrigine decreases the spontaneous release of glutamate but increases the spontaneous release of GABA in the rat entorhinal cortex in vitro.

A drug can dampen neuronal excitability either by preventing depolarization or by causing hyperpolarization. Lamotrigine, by blocking high threshold voltage-gated calcium (Ca2+) channels prevents depolarization [11], whereas, by enhancing transient outward potassium (K+) currents causes hyperpolarization. These additional actions of lamotrigine aid in limiting the pathological excitation of the brain neurons which occurs during seizures.

Alabi et al. [12] studied the effect of lamotrigine treatment on reserpine-induced catalepsy in rats. The authors have reported that lamotrigine treatment failed to antagonise reserpine-induced catalepsy.

Dalic et al [13], carried out a subsequent open-label, uncontrolled replication and extension study in a number of patients of BPD and cocaine dependence. They have reported that in this study too LTG treatment caused significant improvements in HAM-D, YMRS, BPRS and CCQ. In addition, there was significant reduction in the use of cocaine as judged by the dollars spent per week on cocaine.

Metcalf et al [14] performed the FST in mice 30 mins after single administration of LTG (8 and 16 mg/kg ip). At these doses though LTG impaired the spontaneous locomotor activity it however, decreased the immobility time in the FST.

3. MATERIALS AND METHODS

3.1 Subject

Albino rats of either gender (100-180 g) have been used in this study. The subjects have been housed in controlled situations and have had ample exposure before the trial period. They were held on a 12-hour light / dark process. At least one hour until the experimental acclimation tests, the creatures were carried into the clinic and placed in a noiseless, diffusely lit room. There were 10 animals in each party. Just once did any animal included. Both measurements have been made at 27 ° C-30 ° C within 10 and 17 hours.

3.2 Medications

Drugs used were lamotrigine (Kopalle Ltd.), dexamphetamine sulphate (Koch-Light), apomorphine hydrochloride (Sigma), alpha-methyl-p-tyrosine methyl ester hydrochloride (Sigma) and haloperidol (Senorm injection,

Sun Pharmaceuticals). Lamotrigine was dissolved in 2% solution of Tween 80 in distilled water.

All drug solutions were prepared immediately before use and were injected intraperitoneally. The volume of injection was 5 ml/kg body weight for 2.5 to 40 mg/kg dose range of lamotrigine and alpha-methyl-p-tyrosine and 10 ml/kg body weight for 80 and 160 mg/kg doses of lamotrigine, while for the remaining drugs it was 2 ml/kg body weight.

3.3 Observational Process

Haloperidol effects and lamotrigine, amorphine, dexamphetamine studies

The animals were housed in individual cages sized 30x20x20 cms, "30 min prior to drug therapy to enable tolerance to the test environment, to track stereotypical behaviors (SB).

1) Lamotrigine study

Control groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by vehicle (5 ml/kg ip of 2% solution of Tween 80 in distilled water). Experimental groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by lamotrigine (5, 10, 20 and 40 mg/kg ip).

2) Amorphine study

Control groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by distilled water (2 ml/kg ip). Experimental groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by apomorphine (1.5 and 3 mg/kg ip). Animals were tested and scored for catalepsy 20 min after distilled water or apomorphine injection in the same manner.

3) Dexamphetamine study

Control groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by distilled water (2 ml/kg ip). Experimental groups received haloperidol (1 and 1.5 mg/kg ip) followed 0.5 hr later by dexamphetamine (5 and 10 mg/kg ip).

3.4 Statistical Analysis

The findings were tested under statistical analysis through unpaid t-tests of the pupil with $p < 0.05$ significant differences.

4. OBSERVATIONS AND RESULTS

We had investigated the effects of Tween 80 (2 % solution, 5 and 10 ml/kg i.p) on the gross behaviour of the animals, on the behaviors induced by the DA agonists and on catalepsy induced by haloperidol. Its effects were compared with control groups receiving distilled water (5 and 10 ml/kg i.p). Tween 80 did not produce motor incoordination, ataxia or muscular hypotonia, neither did it stimulate locomotor activity or induced stereotyped behaviour or catalepsy in rats. It did not significantly influence the behaviors induced by the DA agonists. Further, Tween 80 had no significant effect on haloperidol induced catalepsy.

Rats treated with 2.5 to 40 mg/kg LTG appeared sedated between 10 to 30 min period after injection but did not exhibit catalepsy when tested at 30 min time interval after LTG injection. After 30 min interval animals receiving 2.5 mg/kg LTG returned to normal state and appeared the same as control animals treated with 5 ml/kg distilled water or Tween 80. After 30 min interval animals receiving 5 and 10 mg/kg LTG exhibited only stereotyped sniffing behaviour whereas animals treated with 20 and 40 mg/kg LTG also exhibited OMV of SB.

The stereotyped behaviors usually manifested about 30 min after administration of LTG, reached maximum intensity about 60 min after LTG injection, and based on doses used, lasted for about 90 to 110 min, after which the animals became quiet and appeared the same as distilled water or Tween 80 treated control animals. LTG, at 80 and 160 mg/kg doses, had produced muscular hypotonia, ataxia as well as motor incoordination which interfered with the proper expression of OMV of SB in rats. These doses were therefore not used for subsequent studies. Table 1 through Table 3 represent the experimental outputs of the study.

Table 1: Effect of haloperidol (HAL) pre-treatment on lamotrigine (LTG) in rats.

Study 1	Treatments (dose mg/kg ip)	Intensity Score (Mean ± S.E.M.)
A	1. DW + LTG 5	1.3 ± 0.15
	2. HAL 0.25 + LTG 5	0.0
B	1. DW + LTG 10	1.6 ± 0.16
	2. HAL 0.25 + LTG 10	0.4 ± 0.16*
	3. HAL 0.5 + LTG 10	0.0
C	1. DW + LTG 20	2.3 ± 0.15
	2. HAL 0.25 + LTG 20	1.1 ± 0.10*
	3. HAL 0.5+ LTG 20	0.0
D	1. DW+ LTG 40	2.7 ± 0.15
	2. HAL 0.25+ LTG 40	1.5 ± 0.16*
	3. HAL 0.5+ LTG 40	0.0

* P<0.001 in contrast with the distilled water (DW) treatment category of Student Unpaired t-testers, pretreated administration of lamotrigine.

Table 2: Effect of alpha-methyl-p-tyrosine (AMT) pre-treatments

Study 2	Treatments (dose mg/kg ip)	Intensity Score (Mean ± S.E.M.)
A	1. DW + LTG 5	1.2 ± 0.13
	2. AMT 100 + LTG 5	0.6 ± 0.16*
	3. AMT 200 + LTG 5	0.0
B	1. DW + LTG 10	1.8 ± 0.13
	2. AMT 100 + LTG 10	1.2 ± 0.13*
	3. AMT 200 + LTG 10	0.5 ± 0.16**
C	1. DW + LTG 20	2.4 ± 0.16
	2. AMT 100 + LTG 20	1.8 ± 0.13*
	3. AMT 200 + LTG 20	1.1 ± 0.10**
D	1. DW + LTG 40	2.9 ± 0.10
	2. AMT 100 + LTG 40	2.3 ± 0.15*
	3. AMT 200 + LTG 40	1.6 ± 0.16**

*p<0.05; **P<0.001 in contrast with the distilled water (DW) treatment (5ml/kg ip) category of Student Unpaired t-testers, pretreated administration of lamotrigine.

Table 3: Effects of pretreatments with Amorphine

Study 3	Treatment (doses mg/kg ip)	Intensity Score (Mean ± S.E.M.)
A	1. DW + LTG 5	1.1 ± 0.10
	2. APO 0.05 + LTG 5	0.5 ± 0.16*
	3. APO 0.1 + LTG 5	0.0
B	1. DW + LTG10	1.7 ± 0.15
	2. APO 0.05 + LTG 10	1.1 ± 0.10*
	3. APO 0.1 + LTG 10	0.3 ± 0.15**
C	1. DW + LTG20	2.2 ± 0.13
	2. APO 0.05 + LTG 20	1.6 ± 0.16*
	3. APO 0.1 + LTG 20	1.1 ± 0.10**
D	1. DW + LTG40	2.8 ± 0.13
	2. APO 0.05 + LTG 40	2.2 ± 0.13*
	3. APO 0.1 + LTG 40	1.6 ± 0.16**

*p<0.05; **P<0.001

Table 4: Effects of pretreatments with dexamphetamine

Study 4	Treatments (doses mg/kg ip)	Intensity Score (Mean ± S.E.M.)
A	1. DW + DAM 5	± 0.13
	2. HAL 0.25 + DAM 5	1.5 ± 0.16*
	3. HAL 0.5 + DAM 5	0.0
B	1. DW + DAM 10	3.6 ± 0.16
	2. HAL 0.25 + DAM 10	2.3 ± 0.15*
	3. HAL 0.5 + DAM 10	0.0

*P<0.001

5. CONCLUSION

Lamotrigine, by blocking the voltage-dependent Na+ channels, decreases the release of glutamate in the striatum and SNc. The cortico-glutamatergic neurons innervate the striatum and the substantia nigra pars compacta (SNc). High doses of apomorphine induce sniffing and OMV of SB in rats by directly stimulating the postsynaptic striatal D2 and D1 dopamine (DA) receptors. Treatment with apomorphine and dexamphetamine antagonize the cataleptic effect of haloperidol, we have therefore investigated whether lamotrigine treatment reduces the cataleptic effect of haloperidol.

These findings further suggest that the SB is indirectly triggering lamotrigine by the release of DA from the DAergic neurons, with the subsequent activation of the D2 and D1 DA receptors by the activated DA after synaptic response. The released DA displaces haloperidol from the post-synaptic striatal D2 and D1 DA receptor sites and stimulates them. Consequently, there is reduction in or abolition of the cataleptic effect of haloperidol by lamotrigine treatment. To conclude our results with lamotrigine, indicate that there is a functional interaction between the cortico-glutamatergic neurons and the nigrostriatal DAergic neurons.

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Original Research

In vitro antimicrobial activity of five essential oils on multidrug resistant Gram-negative clinical isolates

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ABSTRACT

Aim/Background: The emergence of drug-resistant pathogens has drawn attention on medicinal plants for potential antimicrobial properties. The objective of the present study was the investigation of the antimicrobial activity of five plant essential oils on multidrug resistant Gram-negative bacteria. **Materials and Methods:** Basil, chamomile blue, origanum, thyme, and tea tree oil were tested against clinical isolates of *Acinetobacter baumannii* ($n = 6$), *Escherichia coli* ($n = 4$), *Klebsiella pneumoniae* ($n = 7$), and *Pseudomonas aeruginosa* ($n = 5$) using the broth macrodilution method. **Results:** The tested essential oils produced variable antibacterial effect, while Chamomile blue oil demonstrated no antibacterial activity. Origanum, Thyme, and Basil oils were ineffective on *P. aeruginosa* isolates. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration values ranged from 0.12% to 1.50% (v/v) for tea tree oil, 0.25-4% (v/v) for origanum and thyme oil, 0.50% to >4% for basil oil and >4% for chamomile blue oil. Compared to literature data on reference strains, the reported MIC values were different by 2SD, denoting less successful antimicrobial activity against multidrug resistant isolates. **Conclusions:** The antimicrobial activities of the essential oils are influenced by the strain origin (wild, reference, drug sensitive, or resistant) and it should be taken into consideration whenever investigating the plants' potential for developing new antimicrobials.

KEY WORDS: Acinetobacter, antimicrobial activity, *Escherichia coli*, essential oil, multidrug resistant, *Klebsiella, Pseudomonas*

INTRODUCTION

Medicinal plants have been used for centuries by traditional medicine, first in India and China. In the ancient Western world, the Greeks contributed significantly to the rational development of herbal drugs with Hippocrates (460-377 BC), Aristotle (384-322), and Theophratus (circa 300 BC) to have dealt with the medicinal properties of herbs. According to the World Health Organization, still about 80% of the world population depends on traditional medicine for primary healthcare, especially in the developing countries [1,2]. Plant essential oils have formed the basis of pharmaceuticals and natural therapies being used for a wide variety of purposes, from treating infectious, systematic and inflammatory diseases to food preservation, and perfume and cosmetics production [3,4].

Over the past years, the emergence of drug-resistant pathogens has drawn attention on medicinal plants and their metabolites for potential antimicrobial properties. Multidrug-resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, extended-spectrum-beta-lactamase (ESBL) producing *Escherichia coli*, and carbapenemase-producing *Klebsiella pneumoniae*

have become a worldwide major problem in the hospital environment and are the main causes of hospital-acquired infections or healthcare-associated infections, not excluding their potential of transmission in the community [5,6]. Such bacterial isolates can be resistant to all currently available antibiotics or may remain susceptible only to past agents such as the polymyxins [7].

One of the actions to mitigate the drug-resistance problem includes the development of new antimicrobials and in this sense essential oils are being investigated for potential antibacterial activities. Many plant oils or extracts have been reported to have antimicrobial properties and this is attributed to their ability to synthesize aromatic substances, most of which are phenols or oxygen-substituted derivatives [8,9]. However, most of the published studies deal with either non-pathogenic or reference bacterial strains and there is a scarcity of data about wild multidrug resistant isolates. The objective of this study was to determine the antimicrobial activity against multidrug resistant Gram-negative bacteria isolated from clinical samples, of plant essential oils, which are widely used in studies with non-pathogenic or reference strains but their actual effect against resistant pathogens is hardly addressed in the available literature.

MATERIALS AND METHODS

Microorganisms

The bacterial strains used in this study were *A. baumannii* ($n = 6$), *E. coli* ($n = 4$), *K. pneumoniae* ($n = 7$) and *P. aeruginosa* ($n = 5$), isolated from blood cultures ($n = 9$), urine ($n = 5$), vascular catheters ($n = 2$), and wound swabs ($n = 6$) collected from equal number of hospitalized patients entering the University Hospital of Ioannina, Greece. Based on the susceptibility tests [Table 1] all the *K. pneumoniae* isolates were carbapenemase-producing and the *E. coli* isolates were producing ESBLs. Among the *K. pneumoniae* strains producing carbapenemases, four were resistant to all tested antibiotics, while the rest were sensitive only to colistin. Resistant bacteria to colistin and tigecycline were further confirmed by the E-test (BioMerieux SA, France).

The isolation of the bacterial strains used in the present study was performed according to the following routine procedures employed by the Clinical Microbiology Laboratory of the University Hospital of Ioannina:

1. Blood specimens were inoculated directly into Bact/Alert® (BioMerieux SA, France) disposable culture bottles containing 30 ml of liquid substrate consisted of 22 ml of complex media and 8 ml of a charcoal suspension. The media component consists of soybean-casein digest (2.0% w/v), brain heart infusion solids (0.1% w/v), sodium polyanethol sulfonate (SPS) (0.05% w/v), pyridoxine HCl (0.001% w/v), menadione (0.0000725% w/v), hemin (0.000725% w/v), L-cysteine (0.03% w/v), and other complex amino acid and carbohydrate substrates in purified water. Moreover, the bottles contain an atmosphere of carbon dioxide (CO_2) in oxygen under vacuum. The Bact/Alert® disposable culture bottles are commercially available, ready-to-be-used with the Bact/Alert® Microbial Detection System (BioMerieux SA, France), which is a fully automated blood culture system for detecting bacteremia, utilizing a colorimetric sensor and reflected light to monitor the presence and production of CO_2 dissolved in the culture medium. When the growth of the microorganism produces CO_2, the color of the gas-permeable sensor installed in the bottom of each culture bottle changes from blue-green to yellow. The lighter color results in an increase of reflectance units monitored by the system. Bottle reflectance is monitored and recorded by the instrument every 10 min.
2. Urine specimens were inoculated on Uricult Plus media (Orion Diagnostica, Finland), which are intended for diagnosing urinary tract infections by demonstration and presumptive detection of total bacteria count, Gram-negative bacteria, Enterococci, and *E. coli* in urine samples. Uricult Plus is a dip-slide system based on three agar media: The Cystine-lactose electrolyte-deficient agar intended for the determination of the total bacterial count in urine samples, the selective Mac Conkey agar supporting the growth of Gram-negative bacteria and the selective Enterococcus medium intended specifically for the detection of enterococci. The Uricult Plus slide-plates were incubated at 37°C for 24-48 h.
3. Vascular catheters and wound swabs were cultured in blood agar, Mac Conkey agar (Oxoid, UK), Mannitol salt agar (Oxoid, UK), and Sabouraud Dextrose agar (Oxoid, UK) plates and were incubated at 37°C for 24-48 h.

Identification to species level was performed using the VITEK®2 automated system (BioMerieux SA, France). This system uses advanced colorimetry, an identification technology enabling identification of routine clinical isolates (bacteria, yeast), and antibiotic susceptibility testing and resistance mechanism detection.

The selected Gram-negative isolates were stored at –70°C in Microbank® beads (Prolab diagnostics, Canada), a ready-to-use system for storage and retrieval of bacterial isolates, which is comprised of cryovials incorporating treated beads and a special cryopreservative solution enhancing longer survival of the fastidious microorganisms and higher quantitative recoveries. Prior to any experimentation, the so cryopreserved isolates were revived by subculturing in appropriate culture media.

The susceptibility testing was performed using the VITEK®2 automated system and the E-test (BioMerieux SA, France). Susceptibility to the following antibiotics was tested: Aminoglycosides (amikacin, gentamicin, netilmicin, and tobramycin), carbapenems (doripenem, imipenem, and meropenem), cephalosporins (cefalotin, cefepime, cefixime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, cefuroxime, and cefuroxime axetil), monobactams (aztreonam), nitrofurans (nitrofurantoin), penicillins (ampicillin, piperacillin, and ticarcillin), penicillin combinations (amoxicillin/clavulanate, ampicillin/sulbactam, piperacillin/tazobactam, and ticarcillin/clavulanate), quinolones (ciprofloxacin, levofloxacin, moxifloxacin, nalidixic acid, and ofloxacin), polypeptides

Table 1: Antimicrobial susceptibility testing

Isolate	Aminoglycosides	Carbapenems	Cephalosporins	Monobactams	Nitrofurans	Penicillins	Penicillin combinations	Quinolones	Polypeptides (colistin)	Sulfonamides	Tetracyclines	Tigecycline
A.b† 1 ur‡	R#	R	R	R	R	R	R	R	S	R	R	S
A.b 2 ws	R	R	R	R	NT	R	R	R	S	R	R	S
A.b 3 ws	R	R	R	R	NT	R	R	R	S	R	R	S
A.b 4 bl	R	R	R	R	NT	R	R	R	S	R	R	S
A.b 5 ur	R*	R	R	R	R	R	R	R	S	R	R	S
A.b 6 bl	R	R	R	R	NT	R	R	R	S	R	R	S
E.c 1 bl	R	R	R	S	NT	R	R	R	S	R	S	S
E.c 2 ws	S	R	R	R	NT	R	R	R	S	S	S	S
E.c 3 bl	R	R	R	R	NT	R	R	R	S	R	R	S
E.c 4 bl	R	R	R	R	NT	R	R	R	S	R	S	S
K.p 1 ca	R	R	R	R	NT	R	R	R	R****	R	R	R****
K.p 2 ws	R	R	R	R	NT	R	R	R	S	R	R	R****
K.p 3 ur	R	R	R	R	R	R	R	R	R****	R	R	R****
K.p 4 ur	R	R	R	R	R	R	R	R	S	R	R	S
K.p 5 ur	R	R	R	R	R	R	R	R	S	R	R	R****
K.p 6 bl	R	R	R	R	NT	R	R	R	R****	R	R	R****
K.p 7 bl	R	R	R	R	NT	R	R	R	R****	R	R	R****
P.a 1 bl	R	R	R	R	NT	R	R	R	S	R	R	R
P.a 2 ca	R	R	R	R	NT	R	R	R	S	R	R	R
P.a 3 bl	R**	R	R	I	NT	R	R	R	S	R	R	R
P.a 4 ws	I***	R	R	R	NT	R	R	R	S	R	R	R
P.a 5 ws	R**	R	R	R	NT	R	R	R	S	R	R	R

†A.b: *Acinetobacter baumannii*, E.c: *Escherichia coli*, K.p: *Klebsiella pneumoniae*, P.a: *Pseudomonas aeruginosa*, ‡bl: Blood cultures, ur: Urine, ca: Vascular catheters, ws: Wound swabs, #S: Sensitive, R: Resistant, I: Intermediate, NT: Not tested, *Gentamicin: S, **Gentamicin: I, Tobramycin: I, ***Amikacin: R, ****confirmed by E-test, Aminoglycosides: Amikacin, Gentamicin, Netilmicin, Tobramycin, Carbapenems: Doripenem, Imipenem, Meropenem, Cephalosporins: Cefalotin, Cefepime, Cefixime, Cefotaxime, Cefoxitin, Ceftazidime, Ceftriaxone, Cefuroxime, Cefuroxime axetil, Monobactams: Aztreonam, Nitrofurans: Nitrofurantoin, Penicillins: Ampicillin, Piperacillin, Ticarcillin, Penicillin combinations: Amoxicillin/Clavulanate, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Ticarcillin/Clavulanate, Quinolones: Ciprofloxacin, Levofloxacin, Nalidixic Acid, Moxifloxacin, Ofloxacin, Polypeptides: Colistin, Sulfonamides: Trimethoprim/Sulfamethoxazole, Tetracyclines: Minocycline, Tetracycline, Tigecycline

(colistin), sulfonamides (trimethoprim/sulfamethoxazole) tetracyclines (minocycline and tetracycline), and tigecycline.

Essential Oils

The following five essential oils supplied by Sigma-Aldrich Co (Germany) were tested for antimicrobial properties:

Basil oil (FCC, comorictype, W211907), *Ocimum basilicum* L. (Lamiaceae).

Chamomile blue oil (W227307), *Matricaria chamomilla*, L. (Asteraceae).

Origanum oil (FCC, W282812), *Thymus capitatus*, L. (Labiatae).

Tea tree oil (W390208, *Melaleuca alternifolia*, (Myrtaceae).

Thyme oil-white (FCC, Kosher, W306509), *Thymus vulgaris*, L. (Lamiaceae).

For the used commercial oils, the supplier provided no data about their contents or chemical analysis, which is presumed to be the company's copyright. However, a simple chemical analysis was performed in order to have a gross estimate of the components of the employed essential oils. For the identification of the components a QP 5000 Shimadzu instrument, equipped with a capillary column DB-5-MS, 30 × 0.32 mm, 0.25 μm, containing 5% phenyl-methylpolysiloxane (J&W Scientific, Folsom, CA, USA) was employed. The gas chromatography oven temperature was programmed as follows: initial temperature 55°C ramped at 5°C/min to 200°C, ramped 1°C/min to 210 (held for 2 min), and finally rampedto 270°C at 20°C/min and held for 3 min. The injector was set to 240°C in the splitless mode. The ion source and transfer were kept at 240°C and 290°C, respectively. In the full-scan mode, electronic ionization mass spectra at m/z of 50-450 were recorded at 70 eV. Helium was used as the carrier gas at 1.5 mL/min.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Broth macrodilution assays were performed to determine the MIC and MBC for each essential oil, according to the Clinical and Laboratories Standards Institute (CLSI) protocol M7-A8 with some modifications [10].

Each essential oil was dispersed in a sterile tube containing Mueller-Hinton broth (MHB, Oxoid, UK) and was vortexed at room temperature, to obtain an initial stock solution of 8% (v/v). Subsequently, serial double-fold dilutions were prepared in sterile tubes containing MHB supplemented with 0.5% (v/v) Tween 20 (Serva, Germany). The final concentrations of each essential oil were 4, 2, 1, 0.5, 0.25, and 0.125 (v/v).

Overnight bacterial cultures on Mueller-Hinton agar (MHA, Oxoid, UK) were used to prepare the bacterial inocula. Each inoculum was adjusted with sterile saline to obtain the final suspension with turbidity analogous to that of 0.5 McFarland Standards, which equals to a concentration of

Table 2: Range of MIC and MBC values (% v/v) of selected essential oils against the tested bacteria

Bacterial strains	Basil oil		Origanum oil		Tea tree oil		Thyme oil		Chamomile blue oil	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
A. baumanni	0.50-1.25	0.50-1.5	0.25-0.37	0.25-0.37	0.12-0.25	0.25	0.25-0.50	0.25-0.50	>4	>4
E. coli	1-3	1-3	0.37-0.75	0.37-0.75	0.50-0.75	0.50-0.75	1-2.50	1-2.50	>4	>4
K. pneumoniae	1.50-3	1.50-4	1	1-2	0.50-0.75	0.50-0.75	0.50-1.50	1-2	>4	>4
P. aeruginosa	4->4	4->4	2-4	2-4	1-1.50	1-2	4	4	>4	>4

E. coli: Escherichia coli, K. pneumoniae: *Klebsiella pneumoniae, P. aeruginosa*: *Pseudomonas aeruginosa, A. baumannii: Acinetobacter baumannii,* MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

1-1.5 × 10^8 cfu/ml [11,12]. About 10 μl of the prepared bacterial inoculum were transferred to each tube containing the serial double-fold dilutions of the essential oil, giving a final bacterial concentration of 5 × 10^5 cfu/ml. The tubes were incubated aerobically at 37°C for 48 h. After the end of incubation, 10 μl of each dilution was inoculated onto MHA plates and incubated at 37°C for 24 and 48 h in order to determine the MIC and MBC, respectively. The MIC and MBC values were determined by viable counts in MHA, and the MIC was defined as the lowest concentration at which the inoculum viability was reduced up to 90% and MBC was defined as the lowest concentration at which the inoculum viability was reduced up to 99.9% or no apparent growth occurred [13].

Statistical Analysis

Statistical analysis was performed in SPSS (version 22.0. Armonk, NY: IBM Corp). The exhibited MICs and MBCs were grouped according to oil type and checked for normality by the Shapiro-Wilk test. Comparison between oil types was performed by one-way ANOVA, whereas differences between oil types were estimated by the Turkey's test.

RESULTS

The antimicrobial susceptibility of the tested clinical isolates is presented in Table 1, and the MIC and MBC values of the selected essential oils against the tested drug-resistant isolates are presented in Tables 2 and 3. Basil, origanum, tea tree, and thyme essential oils presented antibacterial activity, but chamomile blue oil demonstrated no antibacterial action at all. The tea tree oil demonstrated consistent antimicrobial activity against all the tested clinical isolates and all four oils inhibited growth of *A. baumanii* isolates. However, origanum, thyme, and basil oils antigrowth effect on *P. aeruginosa* was poor [Tables 2 and 3].

Statistically significant differences between the tested essential oils were determined by one-way ANOVA ($F[3,133] = 7.403$, $P = 0.002$). The Basil oil's MIC and MBC values were significantly higher than the origanum and tea tree oil respective values ($P < 0.05$), but not statistically different than the thyme oil's values. The thyme oil MICs and MBCs were significantly higher than the tea tree oil relevant values ($P < 0.05$), but not statistically different than the origanum oil corresponding values. For the tea tree oil, the recorded MIC and MBC values were not statistically different ($P < 0.05$), than the origanum oil respective values.

Table 3: MIC and MBC values (% v/v) of selected essential oils against *A. baumannii* (1-6), *E. coli* (7-10), *K. pneumoniae* (11-17) and *P. aeruginosa* (18-22)

	Basil oil		Origanum oil		Tea Tree oil		Thyme oil		Chamomile blue oil	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	0.50	0.50	0.25	0.37	0.25	0.25	0.25	0.25	>4	>4
2	0.75	1.25	0.37	0.37	0.25	0.25	0.37	0.37	>4	>4
3	1.25	1.50	0.37	0.37	0.25	0.25	0.50	0.50	>4	>4
4	1	1	0.25	0.25	0.25	0.25	0.37	0.37	>4	>4
5	0.50	0.50	0.25	0.25	0.12	0.25	0.25	0.50	>4	>4
6	0.50	1	0.25	0.25	0.25	0.25	0.25	0.25	>4	>4
7	2	3	0.50	0.50	0.50	0.50	1	1.50	>4	>4
8	2	2	0.50	0.50	0.50	0.75	1	1	>4	>4
9	3	3	0.37	0.37	0.50	0.50	1	1	>4	>4
10	1	1	0.75	0.75	0.75	0.75	2.50	2.50	>4	>4
11	2	2	1	1	0.50	0.50	1.50	1.50	>4	>4
12	2	2	1	1	0.50	0.50	1	1.50	>4	>4
13	3	3	1	1.50	0.50	0.75	1	1.50	>4	>4
14	2	2	1	2	0.50	0.50	0.50	1	>4	>4
15	1.50	1.50	1	1.50	0.50	0.75	1	1	>4	>4
16	2	2	1	1.50	0.50	0.50	1	1	>4	>4
17	2	4	1	1	0.75	0.75	1	2	>4	>4
18	>4	>4	2	2	1	1	4	4	>4	>4
19	>4	>4	3	3	1	1	4	4	>4	>4
20	4	4	4	4	1.50	1.50	4	4	>4	>4
21	4	4	2	2	1	2	4	4	>4	>4
22	4	4	4	4	1	1	4	4	>4	>4

E. coli: Escherichia coli, K. pneumoniae: *Klebsiella pneumoniae, P. aeruginosa*: *Pseudomonas aeruginosa, A. baumannii: Acinetobacter baumannii,* MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration

Regarding the chemical composition of the essential oils used in this study, the most abundant component in the case of basil oil was estragole. Carvacrol and thymol were identified as main constituents of origanum oil. The composition of tea tree oil presented high contents of terpinen-4-ol and p-cymene. The prevailing molecules of thyme oil were thymol, p-cymene, and linalool. Chamomile blue oil was rich in bisabolol and trans-b-farnesene. Typical chromatograms of the essential oils examined are shown in Figure 1.

DISCUSSION

The rapid evolution and spread of resistance among clinically important bacterial species constitutes a significant issue of outmost importance for public health. The emergence of antimicrobial resistance is the consequence of selective pressure imposed to microorganisms by the excessive use of antimicrobials mostly in the medical and veterinary

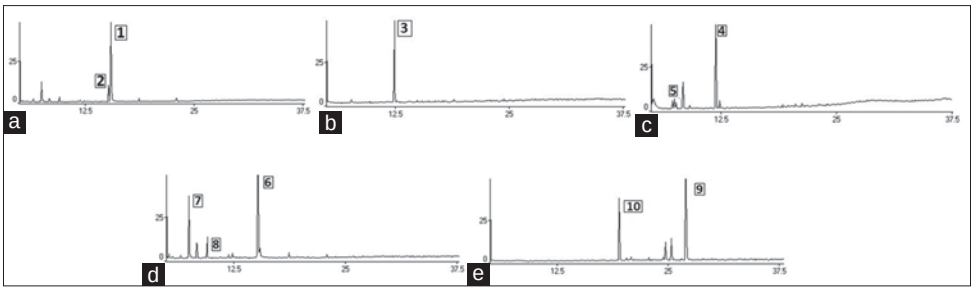

Figure 1: (a-e) Chromatograms of origanum oil (peak 1: Carvacrol, peak 2: Thymol), basil oil (peak 3: Estragole), tea tree oil (peak 4: Terpinen-4-ol, peak 5: p-cymene), thyme oil (peak 6: Thymol, peak 7: p-cymene, peak 8: Linalool), and chamomile blue oil (peak 9: Bisabolol, peak 10: Trans-b-farnesene)

practices. The major issue of this important health problem is that the appearance of resistance to antibiotics reduces the currently available therapeutic options for the treatment of infectious diseases signifying the need for the development of new antibiotic compounds. Plants produce a vast variety of phytochemicals that demonstrate a diversity of medicinal properties including antimicrobial effects. The principal phytochemicals present in plants are essential oils, phenolic compounds, alkaloids, polypeptides, and polyacetylenes [9].

Essential oils have shown antimicrobial properties against a number of Gram-negative and Gram-positive bacteria and in overall, their activity against the microbial cells of the same genera and species determined under the same conditions appears to be similar. However, some bacterial isolates may show a different response in comparison to the type strains [14,15]. Hence, to reach a decision on the antimicrobial activities of essential oils, it is important to use strains from different origins in order to simulate a more realistic situation instead of just using reference strains that may not reflect the actual behavior of the strains that can be found in nature, particularly in the clinical practice. The majority of the available published studies make use of reference strains, not clinical multidrug resistant isolates, and variable findings are recorded due to the diversity of the used methodologies.

Pertaining to the specific essential oils and isolates used in the present study, there are only two publications concerning the antimicrobial effect against clinical isolates. According to da Costa *et al*. [16] origanum oil inhibited *A. baumannii*, *E. coli*, and *K. pneumoniae* clinical isolates at MIC 0.12% (v/v) and *P. aeruoginosa* at 0.5% (v/v), while in our study it was significantly less effective (by 2SD). Hammer *et al.* [17] testing the tea tree oil toward clinical isolates, reported for *A. baumannii* and *P. aeruoginosa* MIC values 1% and 3% (v/v), while in our study the antibacterial performance of this oil was significantly better (by 2SD). However, for the *K. pneumoniae* the MIC values described by Hammer *et al.* [17] were significantly better (by 1SD) than those reported in the present study [Tables 2 and 3].

Diverse antimicrobial activities against *A. baumanii*, *E. coli*, *K. pneumonia*, and *P. aeruginosa* have been reported by researchers experimenting with reference strains. For *E. coli*, the MIC values reported for reference strains are ranging from 0.12% (v/v) for origanum oil to 0.5% (v/v) for basil [3,18-21]. In our study, the reported MIC values [Table 3] were much different (by 2SD) denoting much less successful antimicrobial activity of the origanum and Basil oils against multidrug resistant clinical isolates.

The MIC values reported in the present study and the reciprocal values reported for *K. pneumoniae* reference strains [3] are different by 2SD and 1SD for origanum and thyme oils, respectively, indicating less successful antimicrobial effect against resistant clinical isolates. Concerning the basil and tea tree oil's activity [Table 3] against *K. pneumoniae* no difference was observed between the tested resistant clinical isolates and the reference strains tested by Hammer *et al.* [3].

Regarding the *P. aeruginosa* strains and the activity of tea tree oil, a significant difference (by 2SD) was observed with this oil performing much better against the tested resistant clinical isolates than the reference strains tested by other researchers [3,22-24]. The antimicrobial activity of the origanum and basil oil against *P. aeruginosa* was poor and our findings coincide with those reported on reference strains [3,25].

Based on the afore-mentioned literature data and the results of the present study, much different MIC values are recorded between the reference and clinical resistant isolates. Studies employing reference strains are showing more efficient performance of the tested essential oils; however, in the case of clinical isolates and particularly in the case of the multidrug resistant isolates used in the present study, essential oils are less efficacious. This finding can be attributed to the strain origin rather than to the methodological differences reported by other researchers [4,13,18,26-29].

In the present study, we used multidrug resistant strains of Gram-negative bacteria isolated from hospitalized subjects. The Gram-negative bacteria are considered to be more resistant to essential oils than the Gram-positives [30]. This is largely attributed to the different structure of their cell wall which is more complex in Gram-negatives, not allowing the easy penetration of antibiotics and drugs, including the phenolic compounds (e.g., thymol, carvacrol, and eugenol) which are present in the essential oils [31,32]. Thus, the possible mechanism of action of the essential oils and their compounds is based on their ability to disrupt the bacterial cell wall and

the cytoplasmic membrane; this mode of action consequently leads to cell lysis and leakage of intracellural compounds [33]. Considering that an intact external cell envelope is a prerequisite for the bacterium survival protecting the cell cytoplasm from the external environment, any changes in the permeability of the cell wall and cytoplasmic membrane can influence the bacterial growth. Whenever antibacterial compounds are present in the environment surrounding microorganisms, the bacteria are forced to react by altering the synthesis of fatty acids and membrane proteins to modify the permeability of the membrane [34,35]. The essential oils have the potential to alter both the permeability and the function of the membrane proteins, particularly the essential oils, which are rich in phenolics, can penetrate into the phospholipids layer of the bacterial cell wall, bind to proteins and block their normal functions. Because of their lipophilic nature, essential oils and their compounds can influence the percentage of unsaturated fatty acids and their structure [30,36]. However, because of the variety of molecules present in plant extracts, the antimicrobial activity of the essential oils cannot be attributed to a single mechanism but to a number of diverse biochemical and structural mechanisms at various sites of the bacterial cell outer and inner components affecting the functions of cell membrane, cytoplasm, enzymes, proteins, fatty acids, ions, and metabolites.

CONCLUSIONS

A detailed examination of all the factors potentially influencing the antimicrobial activity of the essential oils should be ideal, but it is rather difficult to implement as evidenced by the existing relevant literature. However, any additional data do contribute to the increase of knowledge in the field. Concerning our study significant differences were observed between our results and the results of other researchers who experimented with non-clinical/non-resistant isolates. Our findings indicate that the essential oils' antimicrobial activities are influenced by the strain origin (wild, reference, drug sensitive, or resistant) and this observation should be taken into consideration whenever investigating the plants' potential for developing new antimicrobials. Nevertheless, the identification of the exact compounds encompassing a true antimicrobial effect is a prerequisite in order to optimize their potential therapeutic use. Yet, microbes are very good survivors having a remarkable ability to adapt to hostile environments, such as being surrounded by antimicrobials, thus meticulous investigation of their resistance mechanisms is necessary in order to encounter successfully the emergence of antibiotic resistance.

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Original Research

An *in vitro* evaluation of the Native American ethnomedicinal plant *Eryngium yuccifolium* as a treatment for snakebite envenomation

Joseph A. Price III

ABSTRACT

Aim: At least seven North American tribes specifically mention the use of *Eryngium* (typically roots) as an anti-snake venom therapy. As snake envenomation is an endemic, life-threatening medical risk, is there a scientific basis for the Native American ethnomedicine? Could this be demonstrated in an assay amenable to mechanistic evaluation and high throughput screening for later isolation and possible evaluation as a source for a lead drug? **Materials and Methods:** Proteases, mainly metalloproteases, are thought to be the main pathological agents in most American snake venoms. Water extracts of four plant parts of *Eryngium yuccifolium* were tested for enzyme inhibition in three highly sensitive *in vitro* protease assays, with multiple venoms. **Results:** Interestingly, activity was found in all plant parts, not just the roots, in the general protease assay, also in the most specific assay for collagenases, but less so for elastases where enzymatic activity was low, and against five species of American snake venoms. Inhibition spared the activity of a mammalian elastase, suggesting it has some specificity. In dose response assays, inhibitory activity in extracts of *Eryngium* was noticeably more effective than randomly chosen plants and comparable to some others. **Conclusions:** All data shown here are consistent with pharmacological inhibition of proteases in at least selected venoms of common venomous snakes by *Eryngium* extracts. Moreover, as the genus is widely distributed in America, the ethnological practice of using this plant as an anti-snake venom treatment is supportable, may have been common, and suggests further bioactivity and phytochemical studies are warranted.

KEY WORDS: *Eryngium*, ethnopharmacology, protease inhibition, snakebite, toxins, venoms

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INTRODUCTION

Many people now (and historically) world-wide depend on plants and plant extracts as the main source of their medications. The World Health Organization itself has endorsed the use of native medicines and initiated a strategy in 2002 and again 2014-2023 [1] for world-wide development and safe use, which includes extracts as well as pure compounds. Some ethnomedicines have led to lead drugs [2-6] for further development.

Snake envenomation has historically been a life-threatening major challenge to indigenous and other rural peoples worldwide, and as a result, cultures often have anti-venom treatments using local botanicals that are of both pharmacological and anthropological interest to study. In 2009, the World Health Organization officially designated snakebite envenomation an important but neglected tropical disorder [7], with a recognized need for an integration of snakebite envenoming within a global strategy to combat the neglected tropical diseases [8]. Estimates indicate thousands of people in the U.S receive hospital treatment [9-11], with an estimated 4000 deaths in the Americas per year and many more worldwide [12,13].

Although snake envenomation remains a world-wide problem, treatment is limited. If the victim has access to high-level medical care, therapies are supportive, palliative, and may rely on antivenoms. Antivenoms, made as antisera, can be life-saving therapeutics but are expensive, not always readily available [14], requiring as well a hospital setting for administration. Antivenoms are aimed at specific species, which often requires knowing what species is involved, which can be unclear. Even with identification, treatment failures occur and successful treatment does not always prevent significant tissue loss. Thus, the biomedical interest in plants inhibiting venoms as available drugs to the general population and as possible sources of lead drugs continues and for which this paper adds useful information on *Eryngium*.

As this is an endemic, life-threatening medical risk, how did Native American peoples treat snake envenomation, and is there a scientific basis for the practice, a pharmacological validation

that would also support the study of this genus for possible lead drugs? Several plants in North America have been reputed to have anti-venom properties but have been unverified. Unusually, one genus of plants has a world-wide reputation and ethnomedicinal use for both as an antitoxin and anti-inflammatory, *Eryngium*. In the U.S, these are wild flowers [15,16] of distinctive appearance found in a variety of natural habitats while some varieties are available through commercial plant suppliers. Some may be endangered or threatened in many locales including where habitat is being lost for both coastal and native prairie species [17]). American *Eryngium*, usually *Eryngium aquaticum* or *Eryngium yuccifolium* (often var. *synchaetum* or *Michigan*), are referred to as "rattlesnake master" and ethnomedicinally used (usually topically) to treat snake envenomations (Crotalid). As published, *Eryngium* for this purpose was used by at least six tribes, the indigenous Choctaw, Chickasaw, and Creek [18,19], Meskwaki [20], Cherokee [21], and Mikasuki [22]. One might expect even more accounts, but as Taylor [19] pointed out in her study of five tribes, even if the plants are available to neighboring tribes, they typically did not share medicinal information [18]. Further, the removal of eastern "civilized" tribes to Oklahoma Territory caused their herbalists to adapt to a new ecosystem, and reculturization (often forced) of almost all U.S tribes has caused much of the herbal knowledge to become isolated or lost. Moreover, unlike Arab countries, since North American peoples did not keep written records, ethnological accounts as cited are likely an underestimate of use. Ethnological phytomedicinal uses for *Eryngium* have been reported among other indigenous peoples as well, including as a topical antidote to scorpion envenomation [23,24].

To test the likely efficacy of the ethnological practice, one can ascertain if the pharmacology supports the use of the plant for that therapeutic application with a pathologically relevant *in vitro* system. The venom of most North American snakes is degradative not neurotoxic, and it is argued that their main pathophysiological agents are metalloproteases [25], largely collagenases. Recently, research has included seeking low molecular weight natural antagonists from plants to snake venom, e.g. as reviewed recently [26], or to venom metalloproteases proteases specifically [27], or to proteolytic and phospholipase activities in Bothrops venoms [28]. This is aside from naturally occurring factors in venom-resistant animals, anticipated to often be polypeptides, and thus possibly immunogenic and not therapeutically suitable. Libraries of antiprotease compounds can be screened for their inhibition of protease and collagenase activities using a variety of North American venoms [29]. Moreover, as others have stated, the possible antivenom mechanisms of action by this plant genus should be explored [30].

To evaluate *Eryngium* antivenom activity, the approach was to secure unrelated plants, make aqueous extracts, and test extracts for antiprotease activity. Further, was the activity limited to roots as reflected in historical and popular use; to any one species of snake; and was this a particularly potent activity in this plant compared to other unrelated plants? Is there an *in vitro* activity that would support the ethnomedicinal use of the plant for envenomation? If so, this would also provide a basis for later *in vivo* studies and any phytochemical compound isolations.

MATERIALS AND METHODS

Laboratory chemicals were from Sigma-Aldrich®. Water was 18 mΩ filtered on site. Venoms were obtained from Miami Serpentarium Laboratories (Punta Gorda FL, USA), dissolved in saline at 2 mg/mL, cleared at 10,000 × g for 5 min, aliquoted then frozen at −80°C until use. Venoms are mixtures of many enzymes and other agonists and may vary substantially in activity and composition between species and individuals, even to some extent between batches from the same animal.

E. yuccifolium Michx. plants were obtained from an established commercial supplier (Ion Exchange, 1878 Old Mission Drive - Harpers Ferry, Iowa). The common herbs dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), and sage (*Salvia officinalis* L.) were obtained from a local established commercial supplier (Southwood Nurseries, Tulsa, OK, USA). The plant names have been checked with www.theplantlist.org on June 5, 2015. Plants were grown to maturity locally and voucher specimens submitted for identification and archiving. Harvested *E. yuccifolium* plants were separated into flowers, rootlets, leaves, and corm, while aerial parts were used from other plants. Samples were washed in water, dried, and stored at −80°C until used. The composition of crude extracts from different plant tissues would be expected to have some components in common, but otherwise vary, as has been widely reported. Algae names were checked with algaebase (www.algaebase.org). Dried commercially prepared *Chlorella* (Beijerinck) and *Spirulina platensis* (Gamont) Geitler (also listed as *Arthrospira platensis* Gomont) were each also purchased from commercial sources: (*Chlorella* sp. [Paragon Plus; Paragon, Torrance, CA, USA] and *S. platensis* [Solaray, Park City, Utah; Earthrise, Calipatria, CA, USA]). The dried algae were rehydrated in water at room temperature briefly before extraction. For all samples, 5 g of each ground tissue or algae were extracted with 15 mL 18 mΩ water at 42°C for 2 h, then re-extracted with 10 mL. Extractions were pooled, prefiltered, filtered through a 1.2 um 32 mm filter (Supor Pall), aliquoted, and stored at −80°C for later assay. For each species, one pool of extract was made from multiple plants and assayed in replicates.

Assays were conducted as published [29] using EnzChek® Protease Assay, EnzChek® Elastase Assay, and EnzChek® Gelatinase/Collagenase Kits from InVitrogen according to kit instructions. The gelatinase substrate has been used for venoms previously [31]. Briefly, experiments were done by manual micropipetting, typically within a single black 96 well (Costar) microplate (typically $n = 3$ unless otherwise noted), always with plate-wide controls per plate. Reactions consisted of 100 uL of reactants (e.g., venom in buffer, plus plant extract) followed by 100 uL of substrate in buffer.

Due to the known variability of the composition of venoms between various species and within species, batches would be expected to show different levels of activity. In preliminary experiments (not shown), various concentrations of venom

from several different species were incrementally increased until all gave usefully active readings at the same concentration of venom, conditions later used to compare inhibition of the venoms by extracts.

Concentrations of venom and extract vary with the experiment and are shown in the Results section. For assays testing the inhibition of the enzyme activity in venoms, a concentration of enzyme was incubated for 15 min with the inhibitor, then substrate added and the reaction conducted for a sufficient length of time to demonstrate effective enzyme activity in the positive controls, typically 1-2 h. The highest practical concentration of extracts, as noted, were used for qualitative inhibition tests while in the later experiment a dose response was used to compare activities. Controls receiving substrate plus buffer served as the blanks and were included on each microplate. Appropriate wells with enzyme but no inhibitor served as activity controls. Data were always blank adjusted. None of the venoms were autofluorescent at the concentrations tested, i.e. never significantly fluorescent above the buffer blank. Reactions were conducted in triplicate, with the exception of blanks, which were done at either $n = 3$ or $n = 6$.

Measurements were done using a Perkin-Elmer HTS 7000 spectrofluorometer. Fluorescence data are in arbitrary units. Gain settings were customized for each assay to keep the substrate control values low and the active samples within measurable limits producing a workable dynamic range. Reactions were monitored with periodic measurements while single time point fluorescence values were used as per instructions in the kits for activity data unless otherwise noted. Preliminary data handling was done with Microsoft® Excel (Microsoft Office 2010) including macros programmed by me, while graphing and statistical analysis was done with GraphPad Prism version 6.04 for Windows (GraphPad Software, La Jolla, California, USA). All figures showing error bars are plotted as the mean plus/minus the standard error of the mean (SEM).

RESULTS

Most proteases will degrade casein while elastin and gelatin are preferentially degraded by elastases and collagenases, respectively. Each of these substrates was evaluated with a selection of venoms at several venom concentrations. Due to the heterogeneity of venoms, if the samples had been adjusted by dilution to have equal apparent activity, it would still be likely that the variety, number and specific activities of the enzymes would vary. Figure 1 shows the progress of an example reaction over time in each well with casein substrate and *Crotalus viridis viridis* venom. For each assay used, a substrate blank control treatment was always done on the same microplate, although decay of fluorescence was always minimal, as seen here.

A comparison of activity at the same venom concentration as shown in Figure 2 showed all venoms active, including a comparison control, the neurotoxic but the comparatively mildly degradative venom of *Naja naja siamensis* (Thailand). At an equivalent concentration (4 ug/mL) to other venoms tested, the activity of *Naja naja siamensis* was not significantly different from the negative controls (substrate blanks, data not shown). It required approximately 25 times the concentration of *Naja naja siamensis* venom (100 ug/mL) to display comparable protease activity.

It could be supposed that like many ethnomedicinal claims for plants as anti-venom medicines that the venom inhibitory activity of *E. yuccifolium* is found in the roots. Testing this hypothesis, extracts made from different parts of the plant were first examined to ensure extraction occurred, seen by the dry weight of extracted materials. As seen in Figure 3, except for the corm extract, which was very different from the other plant part extracts (Tukey's multiple comparisons test, $P < 0.01$), all were similar, thus verifying the extraction and revealing relatively comparative concentrations of material for later activity analysis.

As seen in Figure 4, contrary to common anti-venom expectations from cultural beliefs, under conditions tested the extracts of leaves, flowers, rootlets, and corm all showed strong protease inhibition activity (different than positive controls,

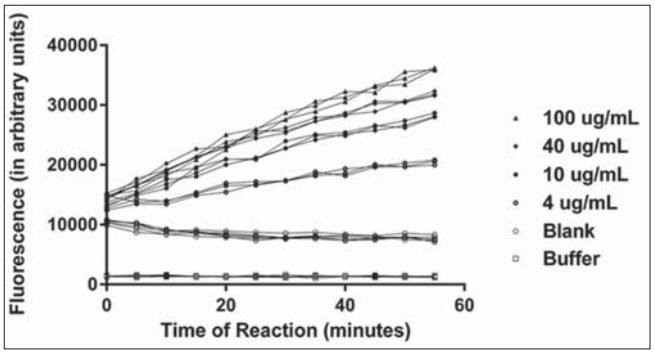

Figure 1: Progress of the reaction with *C. v. horridus* venom at four concentrations in the casein digestion assay shown for each individual microplate well (*n*=3). For buffer and blank controls *n*=6

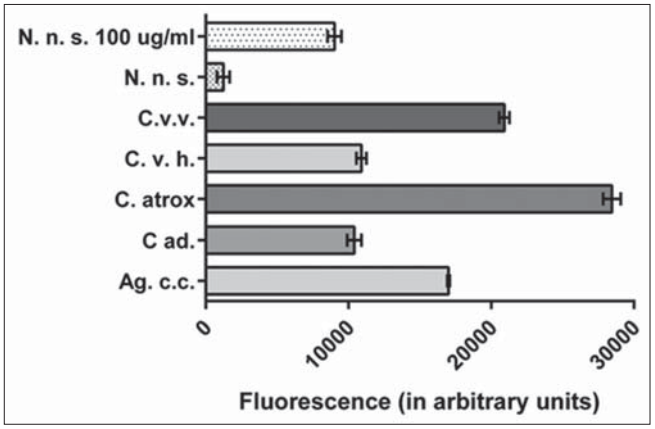

Figure 2: A comparison of protease (caseinase) activity of six venoms (means with SEM). All venoms were at 4 ug/mL except N. n. siamensis which was assayed at two concentrations as noted. Ag.c.c. : *Agkistrodon contortrix contortrix* (copperhead), C.ad. : *Crotalus adamanteus* (Eastern Diamondback rattlesnake), C. atrox : *Crotalus atrox* (Western Diamondback rattlesnake), C. v. h. : *Crotalus viridis* helleri (southern pacific rattler), C. v. v. : *Crotalus viridis viridis* (Prairie rattlesnake), N. N. s.: *Naja naja siamensis* (Thailand cobra)

Tukey's multiple comparisons test, $P < 0.001$, but not different from each other). For subsequent experiments, a pool of extract from aerial parts was used.

To ask if the inhibition was limited to the venom of one species of snake, or to only one type of assay, a panel of venoms was used in the casein (general protease) assay, collagenase (collagenase/gelatinase, often metalloproteases) assay, and elastase assay, respectively. In evaluating inhibition at two levels of five venoms in the casein assay, the inhibition of each venom tested was seen to varying extents, shown in Figure 5, always statistically significant (unpaired *t*-tests per species, controls and treated, $P < 0.05$). Tests at lower concentrations of venom were unrevealing due to marginal enzymatic activity in the venom. Thus, the inhibitory component(s) were active with much of the protease(s) activity in the variety of venoms.

Similarly, inhibition of gelatinase/collagenase at two concentrations of five venoms was also seen, shown in Figure 6, always statistically significant (unpaired *t*-tests per species, controls and treated, $P < 0.05$). Patterns of inhibition, shown here for the first time and by direct within experiment comparisons, were similar to the general protease assay data (casein). These results might mean that most of the activity seen in the casein assay was due to enzymes with collagenase activity, although this need not be exclusively true.

Next, reactions tested if elastase activity, a degradative factor in venoms, is readily measured in this assay, and if it is inhibited by the plant extract. Porcine elastase (phylogenetically unrelated) served as a comparison control for the five venoms tested to see if inhibition showed specificity. As seen in Figure 7, levels of elastase activity were low compared to the other assays. Under these conditions, only inhibition of *Crotalus atrox* and *C. v. viridis* venom activity was seen ($P < 0.01$). There was no significant inhibition of the porcine elastase, and inhibition was not limited to the least active venoms. Furthermore, the observed inhibition is selective, sparing the porcine enzyme and several venoms' elastase activity as opposed to a non-discriminant general inhibitor of enzymes, as is the case for many small molecular weight compounds.

To ask if the inhibition of venom activities is particularly strong from *E. yuccifolium* compared to other plants, extracts from five

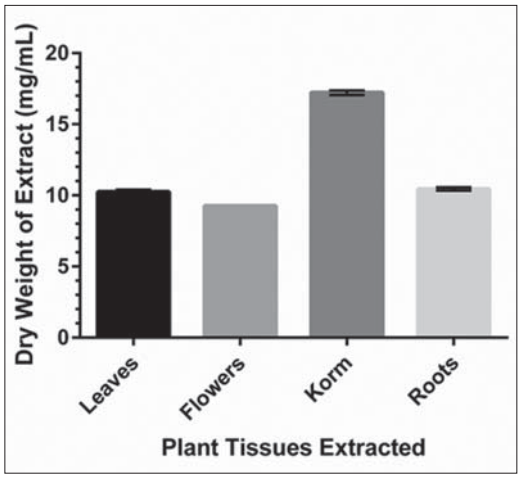

Figure 3: Tissues from four parts of *E. yuccifolium* were extracted identically, and the yield shown as dry weight per mL of extract (means with SEM)

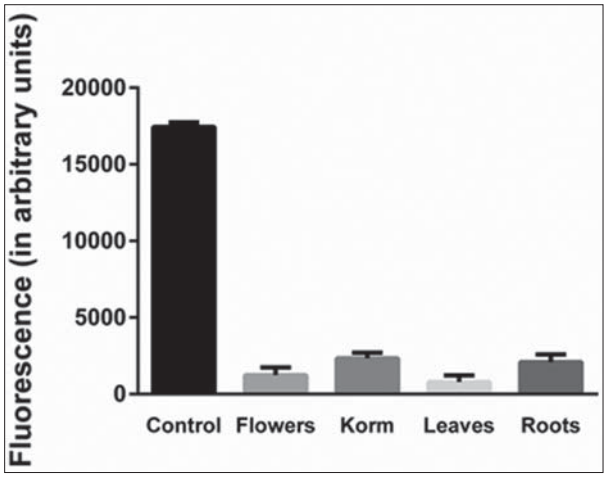

Figure 4: Inhibition of the protease (caseinase) activity of 125 ug/mL of *C. adamanteus* venom by 2 mg/mL extracts of four parts of *E. yuccifolium* (means with SEM)

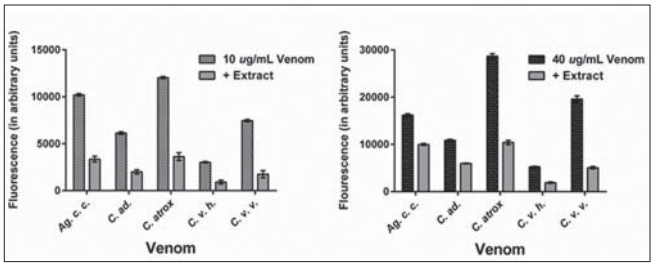

Figure 5: Inhibition of the protease activity (caseinase) of five venoms (means with SEM) after treatment with 2 mg/mL of *E. yuccifolium* extract, repeated at 10 ug/mL and 40 ug/mL of venom. Ag.c.c. : *Agkistrodon contortrix contortrix* (copperhead), C.ad. : *Crotalus adamanteus* (Eastern Diamondback rattlesnake), C. atrox : *Crotalus atrox* (Western Diamondback rattlesnake), C. v. h. : *Crotalus viridis helleri* (southern pacific rattler), C. v. v. : *Crotalus viridis viridis* (Prairie rattlesnake)

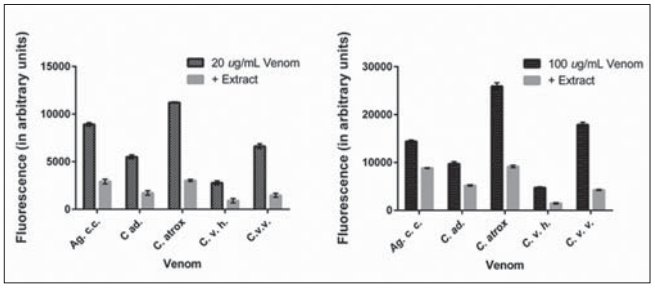

Figure 6: Inhibition of the collagenase activity of five venoms (means with SEM) after treatment with 2 mg/mL of *E. yuccifolium* extract, repeated at 20 ug/mL and 100 ug/mL of venom. Ag.c.c. : *Agkistrodon contortrix contortrix* (copperhead), C.ad. : *Crotalus adamanteus* (Eastern Diamondback rattlesnake), C. atrox : *Crotalus atrox* (Western Diamondback rattlesnake), C. v. h. : *Crotalus viridis helleri* (southern pacific rattler), C. v. v. : *Crotalus viridis viridis* (Prairie rattlesnake)

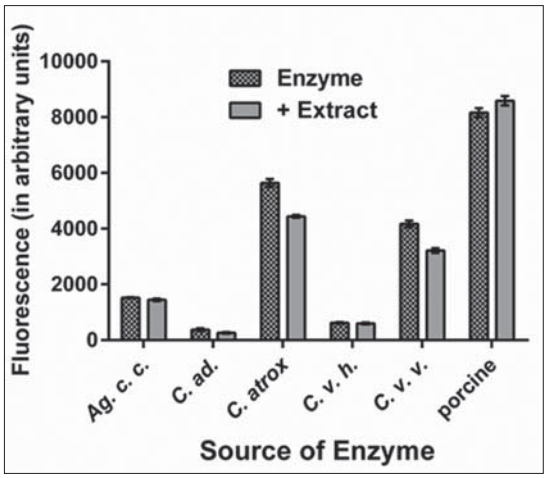

Figure 7: Inhibition of the elastase activity of five venoms (means with SEM) after treatment with 2 mg/mL of *E. yuccifolium* extract. Venoms were at 40 ug/mL, porcine elastase control at .2 U/mL. Ag.c.c.: *Agkistrodon contortrix contortrix* (copperhead), C.ad. : *Crotalus adamanteus* (Eastern Diamondback rattlesnake), C. atrox : *Crotalus atrox* (Western Diamondback rattlesnake), C. v. h. : *Crotalus viridis helleri* (southern pacific rattler), C. v. v. : *Crotalus viridis viridis* (Prairie rattlesnake), and porcine : porcine elastase positive control

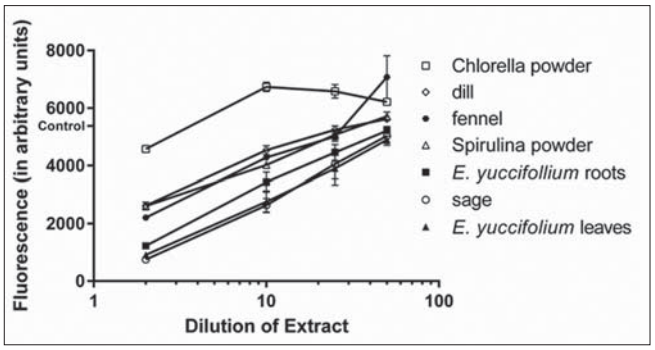

Figure 8: 2-fold to 50-fold dilutions of seven similarly made extracts of unrelated plants in a dose response inhibition of *C. atrox* (7.5 ug/mL) venom protease activity (caseinase). The mean for the positive controls was 5374, and the standard deviation was 645

unrelated sources, including two algae, were made exactly as had been done for *E. yuccifolium*. Figure 8 shows data from dose-response titrations of extracts using *C. atrox* venom (7.5 ug/mL) as a model. Activity was found for these sources, as might be expected, but some were much more active. Using a comparison at a dilution of 2 as an example, the statistical groupings by Tukey's multiple range test ($P < 0.05$) were: *E. yuccifolium* leaves/sage were the most potent, *E. yuccifolium* roots (different from sage but not E. leaves), fennel dill/Spirulina , and Chlorella. The fennel-dill-Spirulina grouping of extracts required about 5 times the concentration to get the same level of inhibition as the most potent extracts. Differences in activity became less at lower concentrations (higher dilutions). Complete inhibition was not observed with concentrations tested which is not uncommon with crude extracts and could be an effect of the prevalence of the inhibitor(s) affinity, or mechanisms.

DISCUSSION

Worldwide, many plants contain extractable compounds that may have *in vitro* activity as enzyme inhibitors, or at times reported to reduce *in vivo* envenomation pathology [23,24,32-35]. While mechanisms are generally unclear, many low molecular weight nonnitrogenous antivenom compounds in plant extracts may mimic natural inhibitors of the venoms [36], explaining this use [37], while others are nonspecific inhibitors of enzymes, either as direct inhibitors of venom components or maybe anti-inflammatory [38,39].

Eryngium genus displays anti-inflammatory [40-49], anti-microbial and anti-oxidant effects [30,50]. Anti-venom protection by *Eryngium* water extract significantly prolongs survival time of guinea pigs challenged with scorpion (*Leiurus quinquestriatus*) venom [51-53]. This type extract also inhibited the hemolytic activity of *Cerastes cerastes* (desert horned viper) highly proteolytic venom [54]. The aqueous root extract of *Eryngium creticum* reduces the hyperglycemic response caused by *Cerastes ceras* in rats [55]. Given the variety of activities documented for *Eryngium*, and that there is a documented effect of the *E. creticum* plant inhibiting both snake and on scorpion venoms, there seems good support for the *Eryngium* extracts as an anti-venom treatment, to which this study adds.

The work reported here adds *in vitro* protease inhibition, a specific mechanism for the actions of the *Eryngium* extracts, as shown with a panel of North American venomous snakes, most noticeable in the general protease and the collagenase assays and provides a system to further study the inhibitors. The presence of the enzyme inhibition activity in all the plant parts tested and not only the roots, should be of interest to phytochemists. The specificity of inhibition, sparing the porcine enzyme, argues against the trivial case in which the *Eryngium* extract is simply a source of nonspecific inhibitors of enzymes. This makes the results more interesting to enzymologists. It will be of interest to later pursue this point further both more thoroughly and widely and to further study the phytochemistry of *Eryngium* species.

As *Eryngium* is widely distributed in America, and all data shown here are consistent with pharmacological inhibition of proteases in at least selected venoms of common local venomous snakes, this validates the effectiveness of this plant's extract as a source of antivenom agent(s) that may have some specificity, the ethnological practice of using this plant among any others as an anti-snake venom treatment is supportable and may have been common, and suggests further bioactivity and phytochemical studies are warranted. As proteases serve as the major virulence factors of many snakes worldwide, the findings in this paper suggest laboratory and later *in vivo* studies would be appropriate for members of this plant genus in various countries and select venoms of the local snakes.

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Antimicrobial and cytotoxicity properties of the organic solvent fractions of *Clerodendrum myricoides* (Hochst.) R. Br. ex Vatke: Kenyan traditional medicinal plant

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ABSTRACT

Background/Aim: *Clerodendrum myricoides* is a Kenyan herbal plant used in the management of respiratory diseases. In the current study, we investigated *in vitro* antimicrobial activity, cytotoxicity, and phytochemical screening of *C. myricoides.* **Materials and Methods:** Antimicrobial activities of *C. myricoides* organic fractions against array of microorganisms including: (i) *Mycobacterium tuberculosis* (MTB) H37Rv, (ii) *Staphylococcus aureus,* (iii) *Klebsiella pneumoniae,* (iv) *Escherichia coli,* (v) *Candida albicans,* (vi) *Pseudomonas aeruginosa,* (vii) *Cryptococcus neoformans,* (viii) *Salmonella typhi,* (ix) *Shigella sonnei, and* (x) Methicillin-resistant *S. aureus* (MRSA) were investigated by disc diffusion and microdilution techniques. Antituberculous activity was investigated using BACTEC MGIT 960 system while cytotoxicity was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on HEp-2 cells. Finally, phytochemicals were screened using standard procedures. **Results:** Methanolic fractions exhibited a broad spectrum activity inhibiting 75% of test pathogens. It had the highest activity with minimal inhibition concentration (MIC) values of $\leq$62.5 μg/ml recorded against 62.5% tested microbes. It yielded the highest zone of inhibition of 20.3 mm (*S. aureus*), lowest MIC of $<$12.5 μg/ml (MTB), and the lowest minimal bactericidal concentration of 62.5 μg/ml (*C. albicans*), within the acceptable toxicity limit (CC_{50} $>$90 μg/ml). The phytochemicals largely believed to be responsible for the observed activity included: Alkaloid, phenols, anthraquinones, terpenoids, and flavonoids. **Conclusion:** Methanolic fraction had remarkable activity against MRSA, *S. aureus, E. coli, S. sonnei, C. albicans,* and MTB, which are of public health concerns due to drug resistance and as sources of community and nosocomial infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of *C. myricoides* and thence a major output in search of novel, safe drug leads to mitigate the global tuberculosis threat.

KEY WORDS: *Clerodendrum myricoides*, cytotoxicity, ethnopharmacology, herbal medicine, infectious diseases, phytochemicals, tuberculosis

INTRODUCTION

The emergence and spread of drug resistance has severely compromised the efficacy of antimicrobial therapy and increased the threat of therapeutic failure [1], which now has become a matter of urgent attention [2]. This is particularly important because of the growing numbers of susceptible people such as: those with compromised immunity and the elderly [1]. In addition, the spread of resistance among infectious pathogens has revealed intricate mechanisms by which resistance coding genes are passed from one microbe to another, species difference notwithstanding. This has led to spread of resistance among Gram-positive, Gram-negative, acid-fast bacterial, and fungal pathogens. These pathogens are a cause for hospital- and community- acquired infections including and not limited to fluoroquinolone-resistant *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae*, *Escherichia coli*, and drug-resistant *Mycobacterium tuberculosis* (MTB) [1]. Furthermore, therapeutic options for emerging pathogenic strains are limited, forcing the use of a second and third line of antimicrobials agents. These second and third line classes of drugs are associated with serious adverse effects on the users [1-4]. Consequently, there is a need for concerted effort in prospecting for novel formulations which are lethal to resistant infectious microorganisms yet safe for humans to combat this on-going health scourge.

Plants provide unlimited prospects for novel potent antimicrobial agents because of their unmatched chemical diversity. However, despite their wide use and the fact that they form an integral part of the primary health care in many countries, there is paucity of information regarding their efficacy and more importantly, their safety levels. For this reason, the current study primarily aimed to evaluate the *in vitro* antimicrobial potency of *Clerodendrum myricoides* organic fractions against a panel of pathogenic microorganisms including *S. aureus*, MRSA, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Salmonella typhi*, *Shigella sonnei*, *Candida albican*, *Cryptococcus neoformans*, and acid-fast MTB strain H37RV and also determine their safety by assaying for their *in vitro* acute toxicity on HEp-2 cells (human laryngeal carcinoma cell line ATCC CCL-23).

C. myricoides (Hochst.) R. Br. ex Vatke, locally known as "Munjuga-iria" among Mbeere community of Embu County in Kenya, is a member of Lamiaceae family. It is a shrub or small multi-stemmed tree characterized with ovate velvety alternate or 3-4-whorled leaves that often crowd near the ends of branches. The shrub is occasionally punctuated with few- purple-blue and white flowers with long exerted stamens terminal and axillary heads.

The selection of this plant was based on its ethnopharmacological use. *C. myricoides* roots are used in the management of respiratory infections, arthritis, malaria, tonsillitis, eye infections, and gonorrhea [5-7].

MATERIALS AND METHODS

Plant Organic Solvent Fractions Preparation

The plant for this study was identified through ethnobotanical approach whereby information regarding its use among the Mbeere community, Kenya, was gleaned from local herbalist and confirmed from literature [5,6]. This plant is not an endangered species, and it was collected in open community field, and therefore, no prior permission was required. The location for collection was around 0°46'27.0"S 37°40'54.9"E; −0.774156, 37.681908 of GPS co-ordinates. The identity was also confirmed by Botanists at Egerton University (Prof. Kariuki S. T. and Mr. Peter Amwogah) where voucher specimen number NSN13 was deposited, and the name checked as acceptable [8].

The plant roots were harvested, cleansed with tap water, shade dried at room temperature (23°C ± 2°C), and finely powdered using a mechanical grinder. Successive fractionation was done using different solvents of increasing polarity. 50 g of powdered sample was soaked in 200 ml of petro ether (PET) with intermittent shaking for 48 h then filtered and the resulting residue further re-extracted using the same fresh solvent for 24 h and the filtrates pooled together for further use. The resulting residue was air dried and further extracted with dichloromethane (DCM) followed by ethyl acetate (EA) and lastly methanol (MOH) following the same procedure. The solvent was vaporized from each filtrate using a rotary evaporator under conditions of reduced temperature and pressure, and the resulting dry extract weighed and stored in airtight sample bottles at −20°C. The extracts were reconstituted in dimethyl sulfoxide (DMSO) to form stock solution of different concentrations.

Antimicrobial Activity Assessment

One Gram-positive; *S. aureus* (ATTC 25923) strain and MRSA strain (clinical isolate); five Gram-negative; *E. coli* (ATTC 25922), *K. pneumoniae* (clinical isolate), *P. aeruginosa* (ATCC 27853), *S. typhi* (clinical isolate), and *S. sonnei* (clinical isolate); two Fungi; *Candida albicans* (ATTC 90028), *C. neoformans* (ATTC 66031), and acid-fast MTB strain H37Rv (ATCC 27294) were investigated for antimicrobial activity. These organisms were sourced from Kenya Medical Research Institute (KEMRI) – Nairobi-Kenya.

Disc Diffusion Assay

The antimicrobial activity was screened using agar disc diffusion method according to Clinical and Laboratory Standard Institute [9] and Mbaveng *et al.* [10] with slight modification. Briefly, fresh bacteria and fungi inoculums were prepared by suspending activated colonies in physiological saline water (0.85% NaCl). Using 0.5 McFarland turbidity standard, the bacteria and fungi suspensions were adjusted to 1.5×10^6 CFU/ml after which they were inoculated aseptically by swapping the surfaces of the Mueller-Hinton agar plates and Sabouraud dextrose agar (SDA) plates. Whatman filter paper (No. 1) discs of 6 mm diameter were made by punching the paper, and the blank discs sterilized in the hot air oven at 160°C for 1 h. They were then impregnated with the different organic solvent extracts (5 μg/disc for PET, DCM, and MOH fractions and 2.5 μg/disc for EA), carefully placed on the surface of the test plate at equidistant points using a sterile forceps and then culture incubated at 37°C (18 h) for bacteria and 27°C for fungi (48 h). Three standard drugs were used as positive controls: Oxacillin 10 μg/disc (Oxoid Ltd., Tokyo-Japan) and Gentamycin 10 μg/disc (Oxoid Ltd., Tokyo-Japan) for Gram-positive and Gram-negative bacteria, respectively. Nystatin 100 μg/disc (Oxoid Ltd, Tokyo-Japan) was used as the standard drug for all fungi while discs loaded with 10 μl of DMSO was used as negative controls. Antimicrobial activity was evaluated by determining the size of the zone of inhibition to the nearest (mm) using a ruler [11]. Extracts that gave a zone of more than 10 mm were considered to be active [9], and therefore, their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determined [11]. All tests were done in triplicate.

Determination of MIC and MBC

The MIC and MBC of the organic solvent fractions that exhibited antimicrobial activity against the test microorganisms were determined using broth microdilution technique. Stock solutions of PET, DCM, and MOH fractions were reconstituted using DMSO to form final stock concentration of 500 μg/ml while that of EA due to merger

quantity available at 250 μg/ml. To 100 μl of nutrient broth in a sterile 96 well plate, 50 μl of varying concentrations of the organic solvent fractions (PET, DCM, and MOH fractions) obtained by serially diluting the active plant fractions resulting in concentrations ranging from 500 to 3.91 μg/ml while EA at 250-1.95 μg/m was added followed by 50 μl of test organisms previously diluted to equivalent of 0.5 McFarland standard. The choice of concentrations profiles was guided by the limiting material available for the study and care to have possible MIC within the acceptable limit of 100 μg/ml. Addition of the test organisms was done in all the wells with the exception of column 11 wells which contained neat DMSO and broth; this served as control to check for purity. The adequacy of the media to support the growth of the test organism was interrogated by putting the broth and the test organism in wells of column 12. The plates were then covered with a sterile "cling-on" sealer and incubated for 24 h at 37°C. Bacterial growth was evaluated by addition of 40 μl of 0.2 mg/ml p-iodonitrotetrazolium chloride (INT, Sigma) to each well and incubated for 30 min. The growth of bacteria was detected by formation of a pink-red coloration while inhibition of growth was signaled by persistence of a clear coloration. The lowest concentration that exhibited color change was considered as the MIC. MBC was determined by streaking a loopful of broth from wells that exhibited no color change onto sterile nutrient agar and SDA for bacteria and fungi, respectively, and thereafter incubated at 37°C for 24 h. The lowest concentration that exhibited no growth was considered as the MBC [12].

Antituberculous Activity

The H37Rv strain of MTB ATCC 27294 was sourced from the KEMRI, Nairobi. Before its use, it was rejuvenated on Lowenstein-Jensen slants for 14 days at 37°C following standard procedures [13]. The efficacy of the plant fractions against MTB was carried out using the BACTEC MGIT 960 system (BD, New York, USA). This is a fully automated, high volume, non-radiometric instrument that offers continuous monitoring of culture growth. Growth supplement (0.8 ml) containing a mixture of OADC - oleic acid, bovine albumin, dextrose, and catalase was added to five 7 ml BBL™ MGIT™ tube and labeled appropriately to provide essential substrates for rapid growth of mycobacteria. 100 μl of BBL™ MGIT™ streptomycin, isoniazid, rifampicin, ethambutol (SIRE) prepared aseptically according to the manufacturer's instructions was added to corresponding labeled BBL™ MGIT™ tube followed by addition of 0.5 ml of 1% *Mycobacterium* suspension. *Mycobacterium* suspension was prepared by pipetting 0.1 ml Middlebrook 7H9 Broth containing *Mycobacterium* adjusted to 0.5 McFarland standard into 10 ml sterile saline aseptically. The BACTEC MGIT™ 960 system (BD, New York, USA) was then loaded following the manufacturer's instructions and incubated at 37°C [14]. These served as the positive SIRE control (streptomycin at 1.0 μg/ml, isoniazid at 0.1 μg/ml, rifampicin at 1.0 μg/ml, and ethambutol at 5.0 μg/ml), whereas DMSO was used as a negative control. The procedure was repeated using PET, DCM, EA, and MOH organic solvent fractions. The organic solvent fractions were tested at varying concentrations ranging from 50 to 6.25 μg/ml (PET, DCM, and MOH) or 25-3.125 μg/ml (EA, due to limited amounts) to determine the MIC.

Phytochemical Analysis

Phytochemical analysis was done to establish the class(es) of ingredient(s) present in the active organic solvent fractions that could be responsible for activity and/or cytotoxicity using thin layer chromatography (TLC). TLC plates were developed with EA:PET (2:8) with few drops of glacial acetic acid before spraying with TLC visualizing reagent to give characteristic color changes [7,15-18]. The results were reported as (+) for presence and (−) for absence based on color intensity.

Dragendorff's reagent for tests for alkaloids

The reagent was prepared by mixing two portions: Reagent A - 0.85 g of bismuth substrate prepared in a solution of 10 ml acetic acid and 40 ml water. Reagent B - 8 g of potassium iodide dissolved in 20 ml of water (stock solution was prepared by mixing volumes of solution A and B). The spray reagent was constituted by mixing 1 ml of the stock solution with 2 ml of fresh acetic acid and 10 ml of water. Formation of orange-brown spots on yellow background indicated the presence of alkaloids and other nitrogen compounds.

Ferric ferrocyanide reagent for phenolic test

About 10% iron chloride ($FeCL_3$) was mixed with iron cyanide ($FECN_6$) (0.1 g/10 ml). Ferric chloride (0.1 g) and potassium ferricyanide ($K_3F_3CN_3$) (0.1 g) were freshly prepared by dissolving in 10 ml of distilled water. Equal portions of $FeCL_3$ and $FECN_6$ were mixed then sprayed to the plates and heated at 110°C. The instant blue change indicates the presence of phenols.

Vanillin reagent for terpenoids test

About 10% vanillin was dissolved in acidified ethanoic acid in ratio of 2:1, and thereafter sprayed onto the plates. The plates were then dried in the oven for 15 min. The presence of terpenoids was indicated by formation of different colors: Brown or dark green or purple.

Tests for flavonoids

TLC plate loaded with samples was exposed to ammonia. The presence of flavonoids was confirmed by colored spots, e.g., yellow, pink, gray, and brown spots.

Tests for anthraquinones

Sample loaded TLC plate was sprayed with a solution of 10 ml MOH and 10 g potassium hydroxide. Change of the original yellow-brown to purple confirms presences of anthraquinones.

Cytotoxicity Testing

In vitro toxicity of active plant organic solvent fractions were tested using HEp-2 cells (human laryngeal carcinoma cell line ATCC CCL-23) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). HEp-2 cells were cultured in growth media comprising of 100 ml DMEM, 10 ml fetal bovine serum (FBS), 1 ml penstrep, 1 ml amphotericin B, and 1 ml L-glutamine. They were incubated at 37°C in 5% CO_2 until they attained confluency. Thereafter, they were harvested by trypsinization and then 2 ml transferred into a 50 ml vial and adjusted to 1×10^5 cell/ml by topping up to 50 ml mark using growth media. 100 μl of the cell suspension was plated into wells of a microtiter plate in duplicates from A-H and then incubated for 48 h at 37°C and 5% CO_2 in 100 μl growth media to form a confluent monolayer. Rows of cells containing medium without plant extracts were included to act as a negative control. The growth medium was replaced with 100 μl of maintenance medium (100 ml DMEM, 2 ml FBS, 1 ml penstrep, 1 ml amphotericin B, 1 ml L-glutamate, and 0.1 ml gentamycin). HEp-2 cells were then treated with varying concentrations of plant extracts (from 2.0 μg/ml to 500 μg/ml) for 48 h at 37°C after which 10 μl of 5 mg/ml MTT solution was added to all wells after aspirating off the plant extracts and further incubated for 4 h. Then, 100 μl of acidified isopropanol (0.04 N HCl in isopropanol) was added to dissolve formazan. The absorbance was read at 562 nm and 690 nm used as a reference using ELISA Scanning Multiwell Spectrophotometer (Multiskan Ex labs systems) and the percentage cell viability determined.

$$\text{Cell viability } (\%) = \frac{\left(OD_{\text{sample } 562} - OD_{690}\right)}{OD\ \text{control}_{562} - OD_{690}} \times 100$$

Statistical Analyses

Mean zones of inhibition of the plant extracts on the microbial samples were determined. One-way ANOVA was used to determine the effect of plant extracts on microbial samples. Mean zones of inhibitions were separated by Tukey's test ($P < 0.05$). Cytotoxicity data were calculated as a percentage of untreated controls. Regression analysis was used to determine CC_{50} (concentration that kills 50% of the Vero cells). A sample was considered toxic if it had CC_{50} value of <90 μg/ml. All data were analyzed using SPSS version 20.

RESULTS

Antimicrobial Activity of *C. myricoides*

The methanolic solvent fraction exhibited broad spectrum activity inhibiting 75% (six out of eight of tested pathogens) while EA registered the least activity; 12.5% (inhibiting one out of eight test pathogens). The inhibitory effect differed significantly among the fractions ($P < 0.05$). The diameter of the zone of inhibition ranged from (20.3 ± 0.3) to (6.3 ± 0.3) mm. The activity was a broad spectrum with Gram-positives bacteria being more susceptible to the organic fractions than Gram-negative bacteria. *S. aureus* was most sensitive Gram-positive bacteria with the highest zone of inhibition of 20.3 ± 0.3 and the lowest MIC of 15.3 μg/ml. *E. coli* was the Gram-negative bacteria that gave the highest zone of inhibition of 14.7 ± 0.3 mm and MIC of 31.25 μg/ml. Among the fungi, *C. albicans* had the highest zone of inhibition of 12.3 ± 0.3 mm, MIC of 31.25 μg/ml, and MBC of 62.5 μg/ml [Tables 1 and 2]. The methanolic solvent fraction exhibited the best activity with MIC values of ≤62.5 μg/ml recorded against 62.5% (five of the eight) tested microbes which is less than 100 μg/ml; the set threshold for plant extract fractions [19]. DCM and EA solvent fractions gave inhibition zones of less than 10 mm. Therefore, these 2 fractions were not considered for MIC and MBC determination [Table 2].

Antimycobacterial Activity of the Organic Solvent Fractions

The antitubercular activity assay was done using varying concentrations of organic solvent extracts of *C. myricoides* by BACTEC MGIT™ 960 system (BD, New York-U.S.A) to determine MIC values. The results revealed that methanolic solvent extract was the most lethal to the tubercle with an MIC of <12.5 μg/ml. This was comparable to SIRE (positive control) which gave Zero GU, and it is indicative of a strong

Table 1: Antimicrobial effects of *C. myricoides* extract fractions

Diameter of zones of inhibition (mm)*									
Fraction	Gram-positive		Gram-negative					Fungi	
	SA	MRSA	PA	EC	KP	SS	ST	CA	CR
MOH	20.3 ± 0.3^{Aa}	13.3 ± 0.3^{Ba}	6.0 ± 0^{Da}	14.7 ± 0.3^{Ba}	6.0 ± 0^{Da}	11.7 ± 0.9^{Ba}	8.7 ± 0.3^{Ca}	12.3 ± 0.3^{Ba}	NT
DCM	7.7 ± 0.3^{Ac}	6.3 ± 0.3^{Ab}	6.0 ± 0^{Ba}	7.7 ± 0.3^{Ab}	6.0 ± 0^{Ba}	7.0 ± 0.6^{Ab}	6.0 ± 0^{Bb}	8.0 ± 0^{Ab}	6.0 ± 0^{Ba}
PET	15.3 ± 0.3^{Ab}	NT	6.0 ± 0^{Ca}	9.3 ± 0.3^{Bb}	6.0 ± 0^{Ca}	6.0 ± 0^{Cc}	6.0 ± 0^{Cb}	8.7 ± 0.3^{Bb}	NT
EA	8.0 ± 0^{Ac}	6.0 ± 0^{Bb}	6.0 ± 0^{Ba}	6.0 ± 0^{Bc}	NT	6.0 ± 0^{Bc}	6.0 ± 0^{Bb}	6.0 ± 0^{Bc}	6.0 ± 0^{Ba}
PC	33.7±0.3	24.3±0.3	23.7±0.6	17.0±0.6	15.7±0.3	19.7±0.3	21.3±0.3	16.3±0.3	20.3±0.6
NC	6.0±0	6.0±0	6.0±0	6.0±0	6.0±0	6.0±0	6.0±0	6.0±0	6.0±0

*Diameter of inhibition zone includes the diameter of the disc (6 mm); values are represented as mean±SD; Means followed by different letters are significantly different ($P<0.05$); MOH: Methanol fraction at 5 μg/disc, EA: Ethyl acetate fraction at 2.5 μg/disc, DCM: Dichloromethane fraction at 5 μg/disc, PET: Petro ether fraction at 5 μg/disc, PA: *Pseudomonas aeruginosa,* EC: *Escherichia coli,* SA: *Staphylococcus aureus,* KP: *Klebsiella pneumoniae,* MRSA: Methicillin-resistant *Staphylococcus aureus,* SS: *Shigella sonnei,* ST: *Salmonella typhi,* CA: *Candida albicans,* CR: *Cryptococcus neoformans,* PC: Positive control (oxacillin 10 μg/disc and gentamycin 10 μg/disc for Gram-positive and Gram-negative bacteria, respectively. Nystatin 100 μg/disc for fungi); NC: Negative control discs loaded with 10 μl of DMSO; NT: Not tested, $n=3$, DMSO: Dimethyl sulfoxide

antituberculous activity. DCM solvent extract exhibited antituberculous activity with MIC of 50 μg/ml while EA and PET fractions had MIC of >50 μg/ml [Table 3].

Preliminary Phytochemical Analysis

The qualitative phytochemical analysis results revealed that EA and DCM solvent extracts contained all the major classes of phytochemicals assayed for whereas PET and methanolic solvent extracts contained two and three classes, respectively [Table 4].

Cytotoxicity Results of *C. myricoides*

The cytotoxicity results indicated that the PET, DCM, and EA solvent extracts were all not within the acceptable toxicity limits (CC_{50} >90 μg/ml) against HEp-2 cells. Interestingly, toxicity of the methanolic extract (which yielded the highest antibacterial and antitubercular activity) was within the acceptable toxicity limit with CC_{50} of >500 μg/ml) [Table 5].

Table 2: MIC and MBC for *C. myricoides* extract fractions

MIC and MBC for the fractions in μg/ml										
Fraction	Gram-positive				Gram-negative				Fungi	
	SA		MRSA		EC		SS		CA	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MOH	15.3	-	31.25	-	31.25	-	62.5	-	31.25	62.5
PET	15.3	125	-	-	15.3	-	-	-	-	-

MOH: Methanol fraction, PET: Petro ether fraction, EC: *Escherichia coli,* SA: *Staphylococcus aureus,* MRSA: Methicillin-resistant *Staphylococcus aureus,* SS: *Shigella sonnei,* CA: *Candida albicans,* MIC: Minimum inhibitory concentration, MBC; Minimum bactericidal concentration, -: >500 μg/ml, *C. myricoides: Clerodendrum myricoides*

Table 3: Antituberculous activity for *C. myricoides* extract fractions

Plant	Fraction	Antituberculous testing concentration (μg/ml)	GU	Status (R/S)	MIC (μg/ml)
C. myricoides	MOH	50	0	S	12.5
		25	0	S	
		12.5	0	S	
		NC	400	R	
		SIRE	0	S	
	EA	25	400	R	-
		12.5	400	R	
		6.25	400	R	
		3. 125	400	R	
		SIRE	0	S	
	DCM	50	0	S	50
		25	400	R	
		12.5	400	R	
		NC	400	R	
		SIRE	0	S	
	PET	50	400	R	-
		25	400	R	
		12.5	400	R	
		NC	400	R	
		SIRE	0	S	

MOH: Methanol fraction, EA: Ethyl acetate fraction, DCM: Dichloromethane fraction, PET: Petro ether fraction, SIRE: Positive control of streptomycin at 1.0 μg/ml, isoniazid at 0.5 μg/ml, rifampicin at 1.0 μg/ml, and ethambutol at 5.0 μg/ml, GU: Growth unit, NC: Negative control of media treated with DMSO, -: MIC of>50 μg/ml, R: Resistant, S: Sensitive, MIC: Minimum inhibitory concentration, MBC; Minimum bactericidal concentration, DMSO: Dimethyl sulfoxide, DMSO: Dimethyl sulfoxide

DISCUSSION

The search for potent antimicrobial ingredients from natural sources has recently received much attention. There are concerted efforts from scientific community to identify safe innovative therapeutic agents for various conditions including infectious diseases that can act as suitable pharmaceuticals agent to replace synthetic ones. Plant-derived phytocompounds can serve as molecular prototype to synthesize less toxic, yet highly effective drugs to combat the ever-evolving plethora of bugs. These phytochemicals have been demonstrated to have remarkable therapeutic potential against human microbes including fungi, bacteria, parasites, and viruses. Many studies have been conducted using various plants' extracts to screen for antimicrobial activity as well as to unearth novel antimicrobial compounds [15].

In the present investigation, four different organic solvent fractions of *C. myricoides* were sequentially prepared using organic solvents of increasing polarity (PET, DCM, EA, and MOH fraction). Each of the organic solvent extracts was evaluated for their antimicrobial activity against selected Gram-positive, Gram-negative, acid-fast bacteria, and fungi, which are considered to be of public health concern. The efficacy of each fraction against Gram-positive, Gram-negative bacteria, and fungi was determined by disc diffusion and broth microdilution assays to evaluate zones of inhibition, MIC, and MBC. Efficacy against acid-fast bacteria was evaluated using BACTEC MGIT™ 960 system (BD, New York, USA) to determine MIC.

The antimicrobial activity results varied remarkably with respect to the organic solvent extract used. The observed variation

Table 4: Phytochemical composition of *C. myricoides* extract fractions

Chemical compounds					
Fraction	Terpenoids	Flavonoids	Anthraquinones	Alkaloids	Phenols
MOH	+	+	+	-	+
EA	+	+	+	+	+
DCM	+	+	+	+	+
PET	-	-	+	+	+

MOH: Methanol, EA: Ethyl acetate, DCM: Dichloromethane, PET: Petro ether, -: Absent phytochemicals, +: Present, *C. myricoides: Clerodendrum myricoides*

Table 5: Cytotoxicity of *C. myricoides* extract fractions

Fractions	CC_{50} (μg/ml)	Remark
Methanol	>500	Not toxic
Dichloromethane	20.53	Highly toxic
Petro ether	14.05	Highly toxic
Ethyl acetate	4.22	Highly toxic

CC_{50}: Concentration of extract fraction that kills 50% of HEp-2 test cells, *C. myricoides: Clerodendrum myricoides*

in terms of efficacy among the fractions most probably may be due to differences in polarity of the organic solvents used during the extraction process that resulted in the differential distribution of bioactive ingredient among the extracts. This suggests that root part of *C. myricoides* contains several antibacterial and antifungal compounds of different polarities as supported by phytochemical studies [Table 4]. These phytochemicals are sequestered at different levels of polarity explaining the varied activity and cytotoxicity demonstrated by extracts. In general, the methanolic solvent extract was the most potent of all fractions with the highest zone of inhibition of 20.3 mm (*S. aureus*), lowest MIC of <12.5 μg/ml (MTB), and the lowest MBC (*C. albicans*). These results demonstrated that the most polar (MOH) solvent was capable of extracting active antimicrobial component(s) from the roots of *C. myricoides*. This strongly suggests that solvents play a key role in the extraction of active phytochemicals. In this case, MOH solvent seems to be a superior solvent for plant antimicrobial molecules extraction, and this is in agreement with previous studies [20-23].

The antimicrobial activity testing revealed that the organic solvent extract of *C. myricoides* has broad spectrum activity inhibiting the growth of Gram-positive, Gram-negative, acid-fast bacteria, and fungi. The potency against Gram-positive was significant ($P < 0.05$). For instance, methanolic fraction gave inhibition zone diameter of 20.3 mm and MIC of 31.25 μg/ml. The sensitivity of the Gram-positive microbes could be attributed to their cytoplasmic membrane which is simple, composed of a lipid bilayer, hence not an effective permeability barrier for most amphipathic compounds, and therefore, can be easily traversed by antibacterial. In contrast, Gram-negative bacteria have evolved a sophisticated, strong permeability barrier with an additional outer membrane comprising a highly hydrophilic lipopolysaccharide layer. This layer restricts penetration of hydrophobic and amphipathic compounds, which encompasses many drug compounds making them less sensitive [24,25]. The difference in sensitivity among tested strains also could be due to genetic differences between different strains, and this proofs the necessity of antibiogram before prescription as a precautionary measure in mitigating drug resistance development [26].

The qualitative screening of *C. myricoides* root extract's revealed presence of alkaloids, phenols, anthraquinones, terpenoids, and flavonoids. Although there are several sub-classes of these phytochemicals, research has shown that members of a particular sub-class inflict damage on microbes by employing almost the same mechanism [22]. For instance, alkaloids are known to interfere with microbial cell wall and DNA besides enhancing the role of immune cells resulting in microbicidal activity. On the other hand, flavonoids, which are an effective antimicrobial agent, inactivate microbial enzymes and interfere with the bacterial cell wall by forming complexes with soluble proteins and the bacterial cell wall, respectively. Other studies have shown flavonoids to have antituberculous activity, and here, they function mechanistically by inhibiting *de novo* biosynthesis of fatty acid in *Mycobacteria*, inhibiting mycolic acid biosynthesis, proteasome inhibition, topoisomerase inhibition, inhibition of phosphatidyl-inositol 3-kinase, induction of cell cycle arrests, accumulation of p53 or enhanced expression of c-fos and c-myc genes [13,27]. The observed antitubercular activity of the MOH fraction could be due to presences of terpenoids, which have been shown to have capacity to traverse the highly hydrophobic tubercle envelope and interfere with the integrity of microbial membranes [28,29]. Similarly, anthraquinones and phenols, present in some of the fractions, are useful antimicrobial phytochemicals. Phenols demonstrate antimicrobial activity through enzyme inhibition by oxidized molecules (probably through reaction with sulfhydryl group(s) or through non-specific interaction with proteins), whereas anthraquinones exhibit their antimicrobial activity by inhibiting nucleic acid synthesis [22].

On the other hand, it was of significant to assess the safety of *C. myricoides* because locally its root extracts are chewed by the human subject. Cytotoxicity assay results indicated that methanolic fraction of *C. myricoides* is within the acceptable toxicity limit (CC_{50}> 500 μg/ml). However, EA (4.22 μg/ml), PET (14.05 μg/ml), and DCM (20.53 μg/ml) fractions were found to be highly toxic. Thus, MOH fraction is a good candidate for the antibacterial, antifungal, and antituberculous agent because of its observed remarkable, selective activity within the acceptable toxicity limits.

CONCLUSION

A major output of the current study is the identification of the methanolic solvent fraction which exhibited a broad spectrum activity inhibiting 75% (six out of eight of test pathogens). It had the best bioactivity, with MIC values of ≤62.5 μg/ml recorded against 62.5% (five of the eight) tested microbes which was less than 100 μg/ml; the set threshold for plant extract fractions. It yielded the highest zone of inhibition of 20.3 mm (*S. aureus*), lowest MIC of <12.5 μg/ml (MTB), and the lowest MBC (*C. albicans*). Of particular excitement is its high activity against MRSA, *S. aureus*, *E. coli*, *S. sonnei*, *C. albicans*, and MTB, which are currently posing great public health challenge due to drug resistance development and as major sources for community and hospital based infections. To the best of our knowledge, this is the first report exploring the antituberculous activity of *C. myricoides* and thence a major output in search of new safe drug leads to mitigate the global tuberculosis threat. However, more work is still required with a view of isolating pure compounds, determining the efficacy of the pure compound as well as elucidate the probable mechanism of action.

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Original Research

Nigerian propolis improves blood glucose, glycated hemoglobin A1c, very low-density lipoprotein, and high-density lipoprotein levels in rat models of diabetes

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ABSTRACT

Objective: According to our previous studies, propolis of Nigerian origin showed some evidence of hypoglycemic and hypolipidemic activities in addition to its ability to ameliorate oxidative-stress-induced organ dysfunction. This study was carried out to determine whether an ethanolic extract of Nigerian propolis (EENP) improves glycated hemoglobin A1c (HbA1c), fasting plasma glucose, very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL) concentrations in rats that have alloxan diabetes. **Materials and Methods:** Diabetes was induced with alloxan (110 mg/kg). Animals were divided into 5 groups ($n = 5$); Group 1 was non-diabetic receiving normal saline and Group 2 was diabetic but also received only normal saline. Groups 3, 4, and 5 were diabetic receiving 200 mg/kg propolis, 300 mg/kg propolis, and 150 mg/kg metformin, respectively, for 42 days. **Results:** Hyperglycemia, elevated serum level of VLDL, elevated plasma level of HbA1c, and decreased levels of HDL were observed in the diabetic untreated animals. Nigerian propolis decreased blood glucose level and serum level of VLDL but elevated HDL level. These changes were significant ($P < 0.05$). The levels of plasma HbA1c were also reduced in the propolis-treated groups, and the reduction was significant ($P < 0.05$). **Conclusion:** Nigerian propolis contains compounds exhibiting hypoglycemic, antihyperlipidemic, and HbA1c reducing activities.

KEY WORDS: Glycated hemoglobin, high-density lipoprotein, Nigerian propolis, plasma glucose, very low-density lipoprotein

INTRODUCTION

Diabetes mellitus is a disease that can be a severe threat to public health as the number of adults that will be suffering from the disease by the year 2030 is estimated to be 439 million [1]. It was estimated that about 387 million people have diabetes worldwide, as of 2014, representing 3.8% of the adult population [2]. Alternative therapies with antihyperglycemic properties are more and more sought by diabetic patients for blood sugar control [3].

Propolis is a resinous substance that bees produce using materials collected from the buds and other parts of certain trees. It is used as a traditional herbal medicine in many countries. Over 300 components have been isolated from propolis. Moreover, they are mainly compounds of phenol (e.g., flavonoids and aromatic compounds), terpenes, and essential oil [4]. Propolis has been proven to have many bioactivities such as antipathogenic, antioxidative, immunoregulatory, antitumor, hepatoprotective, and anti-inflammatory and is now the focus of many biomedical research projects [5]. In general, propolis contains phenolic aldehydes, sesquiterpene, polyphenols, quinines, steroids, coumarins, amino acids, and inorganic compounds [6]. Plant origin and the region where it is collected bear a great significance to what the composition of propolis will be [7], hence, necessity of the prefix of source country (e.g., Nigerian propolis). The Nigerian propolis used for this study was obtained from south-western part of Nigeria – Abeokuta, Ogun State, Nigeria. Location coordinates of the site of collection goes thus: 7.15° North and 3.35° East. The propolis was collected during the rainy season. Despite the chemical differences, it is well known that samples of different geographical origins and chemical compositions usually demonstrates similar pharmacological properties [8]. *Baccharis dracunculifolia* shrub is the main plant source of Brazilian propolis, and prenylated p-coumaric acids are the prevalent biologically active substances in this propolis [9]. Chinese propolis is a propolis derived from poplar tree; and cinnamic acids, flavonoids, and their esters are the predominant active components in this propolis [10]. Earlier studies show

that Chinese propolis helped in reducing fasting blood glucose and ameliorated oxidative stress and lipid metabolism in alloxan-induced diabetic rats [11]. Clinical application of propolis was useful in the control of blood glucose [12].

Chemical analysis of Nigerian propolis used for this study revealed that it is composed of alkaloids in appreciable amounts and also steroids, glycosides, saponins, and tannins in moderate amounts while other flavonoids and phlobatannins are in minute amounts. It also contains phenol compounds. The main plant source of this propolis is, however, yet to be determined.

Glycated hemoglobin (hemoglobin A1c or HbA1c) is a type of hemoglobin primarily used to identify the average plasma glucose concentration over extended periods of time (at least 6-8 weeks) [13]. It is formed in a non-enzymatic pathway by hemoglobin's normal exposure to high plasma glucose levels [14]. HbA1c is implicated in the genesis of diabetic complications such as retinopathy, nephropathy, and neuropathy [15]. It is estimated that the risks of these complications are decreased by approximately 3% for every 1 mmol/mol decrease in HbA1c [16]. Monitoring the HbA1c level in type-1 diabetic patients may help to improve treatment [16].

Diabetes mellitus is almost always associated with changes in plasma lipoproteins. To understand the mechanism of the changes in lipoproteins that occur in diabetes mellitus and how they may influence the complications that accompany this disorder, we must examine lipoprotein metabolism. There are multiple abnormalities of lipoprotein metabolism, primarily in very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) but also to some extent in low-density lipoprotein (LDL), which can potentially explain the increased atherogenesis in human diabetics.

Alloxan (2,4,5,6-pyrimidinetetrone) is a pyrimidine derivative which can bond with H_2O molecules when in aqueous solution to form alloxan hydrate [17]. Alloxan is a toxic glucose analog that selectively destroys insulin-producing pancreatic β cells when administered to rodents and many other animal species [18,19]. This causes an insulin-dependent diabetes mellitus known as "Alloxan diabetes" in these animals, with features similar to Type 1 diabetes in humans [20]. Alloxan is selectively toxic to the β cells as it preferentially accumulates in these cells through uptake by the glucose transporter 2; hence, its usage in the induction of diabetes in laboratory animals [21].

This study showed the effect of ethanolic extract of Nigerian propolis (EENP) on plasma glucose, HbA1c, and some blood lipids (VLDL and HDL) in Type 1 diabetic rats.

MATERIALS AND METHODS

Drugs and Reagents

Nigerian propolis was obtained from south-western part of Nigeria, in Abeokuta town, Ogun State, during rainy season. The alloxan and metformin used were products of Sigma-Aldrich (Sigma, St. Louis, USA). The glucometer used in evaluating blood glucose was on-call-plus (ACON Biotech, Hang Zhou, China). A standard weighing scale was used for weight determination. The kits used for lipid profile was by Randox (UK). HbA1c was determined using the diabetes control and complications trial (DCCT) aligned clover A1c Analyzer (Infopia Co. Ltd., Korea) with a test range of 4-14%.

Extract Preparation

The fresh propolis was frozen (this makes it brittle) and broken into small pieces. 100 g of propolis was soaked in 500 ml of 95% ethanol. A total of 250 g of raw propolis was soaked. The mixture was allowed to stay for a week. This allowed for proper extraction of the propolis into the solvent (ethanol). After 1-week, the mixture was then filtered using Whatman No.1 filter paper. The filtrate was evaporated to dryness under low pressure and a maximum temperature of 60°C yielding a crude (EENP) weighing 29 g. The method used here, in the preparation of propolis extract is similar to that described in a publication by Musa *et al*. [22].

Laboratory Animals

The animals used for this study were bred in the animal house of the Department of Physiology, University of Ibadan, Nigeria, under standard laboratory conditions and were allowed free access to standard rat food and water. The study was performed in accordance with the ethical guidelines stipulated by the Ethical Committee of the College of the Institution. These guidelines are in accordance with the Helsinki Principles for laboratory animal use and care. Rats, weighing 160-200 g, were selected for the experiment.

Induction of Diabetes

Animals were fasted for 12 h. Diabetes was then induced by intraperitoneal injection of alloxan (110 mg/kg) dissolved in sterile normal saline (0.9%) after which they were allowed free access to food and water. The diabetic state was determined 72 h after alloxan administration. A pinch of blood collected from the tail was analyzed for glucose level (in each animal) with the aid of on-call-plus glucometer. Animals chosen for the diabetic model were identified and selected on the basis of blood glucose levels – higher than 230 mg/dl [21].

Experimental Design

There were five groups of 5 rats per group:

- Group 1: Control (non-diabetic); received normal saline
- Group 2: Diabetic untreated; received normal saline
- Group 3: Diabetic experimental; received 200 mg/kg of EENP as used by Bhadauria *et al.* [23]
- Group 4: Diabetic experimental; received 300 mg/kg of EENP as used by Zamami *et al.* [24]
- Group 5: Diabetic treated; received 150 mg/kg of metformin.

Drug Administration

The EENP was mixed with Tween 80 (polysorbate 80) for solubility [23], at the ratio of 200 mg propolis to 1 ml of Tween 80 before oral administration. The metformin and control groups were also given Tween 80 (proportionately mixed in) before oral administration. The administration continued for 42 days (a period sufficient for the formation of HbA1c). The fasting blood glucose and weight of all rats were measured weekly for 6 weeks.

Animal Sacrifice

After 42 days of treatment, animals were anesthetized and sacrificed by cervical dislocation and then dissected. Blood was obtained directly from the heart into heparinized and plain centrifuge bottles for plasma and serum samples, respectively.

Blood Sampling and Biochemical Analysis

Whole blood was used in the analysis of HbA1c with the aid of DCCT aligned clover A1c analyzer (Infopia Co. Ltd., Korea) that has a test range of 4-14%.

For serum preparation, blood samples (in plain centrifuge bottles) were allowed to stand for 30 min and then centrifuged at 3000 r/min for 15 min at 19°C to obtain the serum.

Obtained serum was analyzed for HDL and VLDL by an enzymatic method using commercial diagnostic kits (Randox, UK).

To determine the weekly levels of glucose, pinches of blood were obtained from the tip of the tails of the rats and dropped on the glucometer-strip which was prior inserted into the glucometer. The readings were then noted. The weekly amounts of weight of the rats were taken using a standard scale.

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by Tukey-Kramer post-hoc test. The software used was GraphPad Prism (version 5, XML Project, November 2010) by GraphPad Software, Inc. California, USA. Results were presented as mean ± standard error of mean with statistical significance accepted at $P \leq 0.05$.

RESULTS

To measure the effect of EENP on alloxan-induced diabetic rats, various biochemical assays were done in all experimental animals to approximate the levels of plasma glucose, serum VLDL, serum HDL, and plasma HbAlc. The following are the pharmacological effects discovered:

Glycemic Control

From the results, it can be seen that the 200 mg/kg propolis and the 300 mg/kg propolis caused a 67.98% and 72.76% (significant) drop in blood glucose, respectively ($P < 0.001$), compared to the 6.36% fluctuation of blood glucose level in the untreated group. The blood glucose level of the untreated group hovered on the high side while the blood glucose levels in the treated groups (including metformin-treated group which caused a 60.30% significant reduction ($P < 0.001$) in blood glucose level) significantly dropped over the period of administration with the highest percentage drop recorded in the 300 mg/kg propolis-treated group [Table 1]. Figure 1 is showing and comparing the weekly changes in blood glucose levels in the different groups from the 1st to 5th week. Alloxan administration had increased blood glucose level by the 1st week in both treated and untreated groups. Blood glucose level was significantly reduced ($P < 0.001$) in the metformin and propolis (both doses) treated groups from the 2nd week onward when compared to the diabetic control group.

Weight Amelioration

Table 2 compares change in body weight of the propolis-treated groups with the untreated group. The 200 mg/kg propolis group caused just a 0.5% increase in body weight, whereas the 300 mg/kg propolis caused an 11% increase in body weight. Both increments were significant ($P < 0.01$) when compared to the 21.91% weight loss in the untreated group as indicated by the negative sign. Metformin also significantly prevented weight loss when compared to untreated group. The untreated animals lost weight throughout the duration of the experiment while the treated ones gained weight with the most weight gain registered in the metformin and 300 mg/kg propolis-treated groups. Figure 2 is depicting and comparing the weekly change-in-weight of animals in the different groups. Note that the change-in-weight (Δ weight) is plotted against duration (in weeks) and not the absolute weight values. This is because the weight of the animals was not uniform at the start of the experiment, so it is reasonable working with and plotting the Δ weight versus duration, instead of absolute weight values. A plummet is seen in the untreated group indicating loss of weight over the period while a spike is seen in the normal control group indicating continual weight gain over the period. All

Table 1: Effects of propolis extracts on blood glucose level of alloxan diabetic rats

Treatment group	Initial blood glucose (mg/dl)	Final blood glucose (mg/dl)	Change in blood glucose (mg/dl)	% Change in blood glucose
Control (normal saline)	96.80±9.43	89.6±3.22	7.20±7.28*	7.44*
Diabetic untreated	486±5.30	455.1±2.03	30.90±12.60	6.36
200 mg/kg (EENP)	437.80±15.16	140.18±22.20	297.62±11.66*	67.98*
300 mg/kg (EENP)	462.80±13.80	126.08±4.44	336.72±10.90*	72.76*
Metformin (150 mg/kg)	390.40±19.35	155.0±12.43	235.40±8.90*	60.30*

*Indicates significant (reduction) change at $P<0.001$, compared with the untreated group, EENP: Ethanolic extract of Nigerian propolis

Table 2: Effects of propolis extracts on body weight of alloxan diabetic rats

Treatment group	Initial weight (g)	Final weight (g)	Change in weight (g)	% Change in weight
Control (normal saline)	181.20±3.65	220.10±3.95	38.90±0.31[a]	21.47[a]
Diabetic untreated	156±4.41	123.00±2.00	−33.00±1.29[b]	−21.91[b]
200 mg/kg (EENP)	201±3.21	202±1.10	1±1.39[a]	0.50[a]
300 mg/kg (EENP)	159±4.24	177.70±3.82	18.70±2.01[a]	11.76[a]
Metformin (150 mg/kg)	154±7.96	172.10±9.00	18.10±1.26[a]	11.75[a]

[a]Significantly different from diabetic untreated; [b]Significantly different from normal control at $P<0.01$, EENP: Ethanolic extract of Nigerian propolis

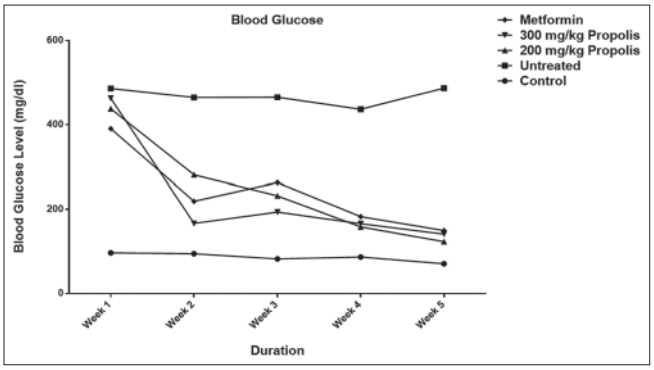

Figure 1: Effect of ethanolic extract of Nigerian propolis on blood glucose

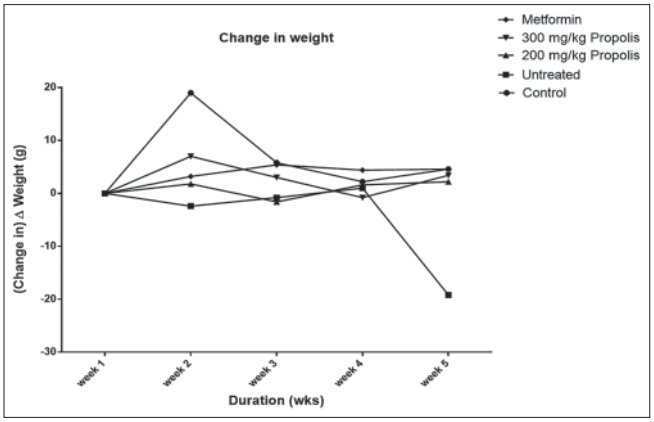

Figure 2: Effect of ethanolic extract of Nigerian propolis on body weight

treated groups ameliorated weight loss significantly, compared to the untreated group.

HbA1c

Table 3 shows comparison between HbA1c levels in the treated groups, untreated group, and control group and at varying levels of significance. It is seen that high levels of $HbA1_C$ in all the treated groups (150 mg/kg, metformin; 200 mg/kg, propolis; 300 mg/kg propolis) are significantly prevented ($P < 0.05$) when compared to the untreated group. This indicates regulation of blood glucose level of the treated animals over the course of the experiment (6 weeks).

VLDL and HDL

Only the 300 mg/kg propolis caused a significant drop ($P < 0.01$) in VLDL level when compared with the untreated group, while 200 mg/kg propolis, 300 mg/kg propolis, and metformin caused significant increases ($P < 0.001$) in HDL levels when compared with the untreated group [Table 3].

DISCUSSION

Hyperglycemia is considered one of the primary causes of diabetes complications, and the presence of hyperglycemia is associated with increased morbidity and mortality [25]. HbA1c is highly correlated with long-term hyperglycemia, and hence, the presence of diabetic microvascular complications [26]. HbA1c is as effective a predictor of microvascular complications as fasting plasma glucose [27,28]. Measurement of HbA1c has been recommended for the diagnosis of diabetes and prediabetes [29]. Significant weight loss accompanies high level of blood glucose that occurs in diabetes. This is due to excessive gluconeogenesis that includes usage of body fats (lipolysis) for the synthesis of new glucose. The result of this study showed that Nigerian propolis was able to ameliorate the rise in blood glucose and the drop in weight of diabetic rats. This is in accordance with our earlier studies [30]. It also shows amelioration of dyslipidemia by propolis to a significant level. The level of HbA1c was also reduced significantly in the propolis-treated groups suggesting amelioration of hyperglycemia over the period of 6-week (period of diabetes and treatment) as against the untreated group. The period of 6 weeks was chosen to allow for proper glycation of hemoglobin to form HbA1c.

Earlier studies carried out on Croatian propolis [31], Chinese propolis [32], Brazilian propolis [32], and Egyptian propolis [33] among many others, revealed their diabetes ameliorating effects.

In this study, 200 mg/kg and 300 mg/kg EENP caused a significant decrease in blood glucose level when compared to the untreated diabetic group with the higher dose causing a more rapid decrease in blood glucose level. The significant hypoglycemic activity of this propolis suggests that propolis exacts its activity either by a direct or indirect mechanism in rats [34]. If the propolis had acted indirectly as a hypoglycemic agent, the effect observed when administered to alloxan-treated rats would have been minimal or absent due to the severe destructive effect of alloxan on the β cells of the pancreas [18]. Furthermore, propolis may have acted indirectly by rejuvenating the surviving β cells to secrete more of insulin instead of aiding the regeneration of necrotic pancreatic β cells. Furthermore, both doses of propolis ameliorated weight loss and caused a significant increase in rat weight when compared to the untreated diabetic group. Both the 200 mg/kg EENP and 300 mg/kg EENP significantly ameliorated

Table 3: Effect of treatment of alloxan-induced diabetic rats with ethanolic extract of Nigerian propolis (EENP) on glycated hemoglobin, VLDL, and HDL

Treatment group	HbA1c (%)	VLDL (mg/dl)	HDL (mg/dl)
Control (normal saline)	5.01±0.42	26.46±0.88	38.14±0.78
Diabetic untreated	8.98±1.40^{b}**	50.85±1.57^{b}***	19.21±1.07^{b}***
200 mg/kg (EENP)	6.76±0.50^{a}*^{b}*	45.90±1.64^{b}***	41.00±1.51^{a}***
300 mg/kg (EENP)	6.58±0.02^{a}*^{b}*	37.36±1.25^{a}**^{b}***	33.00±0.73^{a}***
Metformin (150 mg/kg)	6.85±0.22^{a}*^{b}*	48.53±1.97^{b}***	32.55±2.42^{a}***

^{a}Significantly different from diabetic untreated; ^{b}Significantly different from normal control. *$P<0.05$, **$P<0.01$ and ***$P<0.001$, EENP: Ethanolic extract of Nigerian propolis, VLDL: Very low-density lipoprotein, HDL: High-density lipoprotein, HbA1c: Glycated hemoglobin A1c

HbA1c in diabetic rats; in correlation with the blood glucose lowering effect observed.

Diabetes mellitus is almost always associated with changes in plasma lipoproteins. To understand the mechanism of the changes in lipoproteins that occur in diabetes mellitus and how they may influence the complications that accompany this disorder, we must examine lipoprotein metabolism. There are multiple abnormalities of lipoprotein metabolism, primarily in VLDL and HDL but also to some extent in LDL, which can potentially explain the increased atherogenesis in human diabetics. In this study, increase in serum levels of VLDL and decreased level of HDL that were observed in the diabetic untreated groups agrees with the findings of Douillet *et al*. [35] and Naziroglu *et al*. [36]. A number of observations indicate that plasma HDL are low in untreated Type 1 diabetics [37] and HDL increases with degree of glycemic control. On the other hand, VLDL in individuals with Type 1 diabetes receiving adequate therapy need not be elevated [38], and it is now well-established that VLDL elevations in Type 1 diabetes are often well-correlated with degree of diabetic control [39]. The levels of VLDL in the 200 mg/kg EENP group rats did not change significantly when compared with the untreated group rats, whereas rats in the 300 mg/kg EENP group had their VLDL levels decreased significantly. HDL levels, however, increased in both 200 and 300 mg/kg EENP group rats significantly when compared with untreated group.

The prevalent biologically active substances in Brazilian propolis are prenylated p-coumaric acids [9] while in Chinese propolis it is cinnamic acids, flavonoids, and their esters that are the predominant active components [10]. On carrying out chemical screening assay, Nigerian propolis though also contains glycosides, steroids, flavonoids, saponins, and tannins among others (see introduction above); alkaloids appear to be the predominant constituent compounds. Moreover, this group of compounds has been demonstrated to possess antihyperglycemic activities [40]. Research is, however, ongoing to determine which actual compounds are responsible for the antihyperglycemic and hypolipidemic effects.

CONCLUSION

Nigerian propolis significantly reduced the rise in blood glucose of alloxan-induced diabetic male rats and alleviated weight loss caused by this disease state. It also reduced the rate of HbA1c formation significantly over the period of its administration and ameliorated diabetic dyslipidemia by increasing the HDL levels. More research is, however, ongoing to determine which actual compound or compounds is responsible for the antihyperglycemic and hypolipidemic effects, thus creating chance for future components isolation and drug manufacture and to reveal a possible mechanism of action of this propolis.

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Original Research

ScopeMed

Ethnopharmacological studies on the uses of *Euphorbia hirta* in the treatment of dengue in selected indigenous communities in Pangasinan (Philippines)

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ABSTRACT

Aim: *Euphorbia hirta* is the most widely used plant in the folkloric treatment of dengue in the Philippines. This study documents the anecdotal uses of *E. hirta* in the treatment of dengue in 3 indigenous communities in Pangasinan from April to June of 2015. **Materials and Methods:** The number of use reports pertaining to symptoms of dengue and other diseases were tallied from 82 informants living in Anda Island, Mt. Colisao and Mt. Balungao. The demographics of the informants as well as the corrected major use agreements (cMUAs) and fidelity levels (FLs) of each reported symptom of dengue were calculated. The major dosage forms used during treatment were also tallied. **Results:** Respondents, dominated by the age group 60-80 and mostly females with at least primary and secondary education, provided information on the use reports of *E. hirta*. High FL values and cMUA of at least 35% were obtained for cardinal symptoms of dengue-related to bleeding episodes while low cMUAs (i.e. 2-4%) were obtained for symptoms during the recovery phase. High FL values were obtained for symptoms observed during the febrile phase. The most widely used dosage forms are decoctions of the leaves and barks of *E. hirta*. **Conclusion:** This study was able to qualify the uses of *E. hirta* in the treatment of dengue in the 3 communities surveyed.

KEY WORDS: Dengue, ethnopharmacological, *Euphorbia hirta*, Pangasinan

INTRODUCTION

The Philippine archipelago, consisting of about 7107 islands, is a center of endemicity and biodiversity where a large number of endemic plants have been reported to exhibit medicinal properties. Previous ethnobotanical studies of medicinal plants in this country were conducted in the archipelagos of Batanes and Visayas and the southern island of Mindanao. There are limited ethnobotanical studies conducted in Luzon, the largest island in the Philippines. An online survey of ethnobotanical studies conducted in the Philippines showed that up to date, no documentations have been done on the anecdotal therapeutic uses of plants in Pangasinan, a province which is situated in the central–eastern part of Luzon.

Pangasinan is one of the most populated provinces in the Philippine archipelago, with a population of about 3 million as of the 2011 census and a total area of 5369 km^2. It is located in the northern Ilocos region of the island of Luzon [Figure 1]. The predominant climate is a wet season from June to October followed by a dry season from November to May. The average monthly temperature is 27.91°C with the highest occurring in May and the lowest in January [1]. About 95% of the people embrace Christianity with minorities belonging mainly to Islam, Hinduism, and Buddhism [2]. Comprising the province are six political districts which have different dialects, cultures, and traditions, including the self-care use of medicinal plants which have been passed to them from previous generations.

The asthma weeds plant, *Euphorbia hirta*, also known as *Chamaesyce hirta* (L.) Millsp. and identified by its vernacular names "tawa-tawa," is a hairy herb grown in open grasslands, roadsides, and pathways and with a pantropic distribution. Tungol-Paredes *et al*. [3] considered this indigenous plant to be the most popular folkloric treatment for dengue in the Philippines. Apostol *et al.* [4] and Arollado

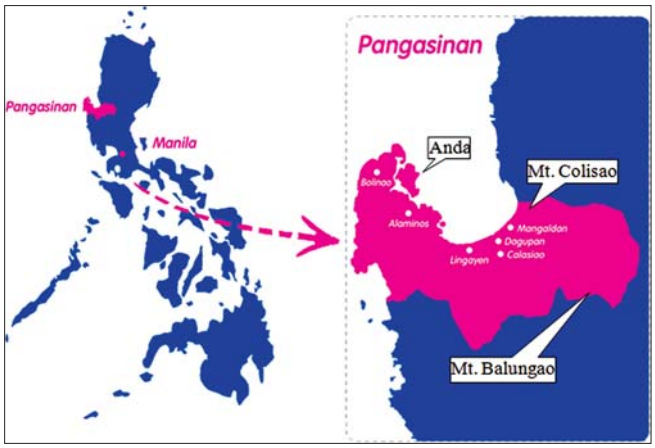

Figure 1: Map of the Philippines showing the province of Pangasinan and the three surveyed areas

et al. [5] demonstrated how the lyophilized leaf decoction of *E. hirta* augmented platelet count in thrombocytopenic rats. Practitioners of traditional medicines believe that "tawa-tawa" leaf decoction can reverse viral infection and prevent the fever from moving into critical stages. Mir *et al.* [6] described how "tawa-tawa" water was effective in increasing platelet count and improving the symptoms in dengue patients. In a cohort study, Paredes *et al.* [7] reported that the beneficial effect of *E. hirta* in dengue patients depends on the degree of changes in platelet levels. Despite these developments, there is no concrete evidence that reveals the effectiveness of this plant in humans infected with the dengue virus. Since little information is known about the purported therapeutic claim of *E. hirta* against dengue and other illnesses, this study seeked to assess, quantify, qualify and compare the ethnobotanical uses of *E. hirta* from three selected indigenous communities in the province of Pangasinan.

MATERIALS AND METHODS

Local of the Study and Vegetation

Three different indigenous areas in Pangasinan [Figure 1] where *E. hirta* was widely propagated were selected as sampling sites on the basis of the existence of traditional herbalists (herbolarios) who depend on medicinal plants to treat illnesses due to the absence of modern health care facilities, clinics and pharmacies as well as the lack of access to electricity. Most plants were seen growing wild in places adjacent to homes, making collection possible, instead of cultivation. It must be considered that the therapeutic uses of plants and the synthesis of bioactive constituents they contain are most optimized when these plants grow more slowly, particularly in the wild.

The indigenous communities surveyed are:

1. The island municipality of Anda (surveyed on April 2015) at the northwestern tip of Pangasinan, which is characterized by shallow white sand coastal lines with lush forested and semi-forested vegetations that spread on plains that are traversed by streams. The locals speak Bolinao, a minority dialect, and Ilocano and thrives on farming and deep sea fishing;
2. Mt. Balungao (surveyed on April 2015), a dormant volcano at the southeastern boundary of Pangasinan with Nueva Ecija with secondary dipterocap forests, hot springs and a mossy peak. The locals who speak mostly Ilocano depend on fruit crops, farming, and logging; and,
3. Mt. Colisao municipal park (surveyed on May and June 2015) in the municipality of San Fabian, described by the shortage of water supply and the presence of denuded hilly forests brought about by illegal mining and logging, and a variety of both wild and cultivated plants. The locals in this area speak mostly Ilocano and Pangasinense.

Plant Collection and Herbarium Preparation

The survey, data gathering and analysis was conducted from April to June of 2015. The aerial part of *E. hirta* were collected in the 3 aforementioned areas. The specimens in the field were pressed in between newspapers and treated with denatured alcohol. In the laboratory, the specimens were soaked in 100 mL of 95% ethanol-phenol (60:40) and subsequently oven-dried [8]. Properly oven-dried specimens were mounted in herbarium sheets with official label. Herbarium specimens and photographs of the plants in their natural habitats were submitted to the Philippine National Herbarium for authentication.

Questionnaire Design and Sampling

Each specimen of *E. hirta* collected from the three sampling sites surveyed, together with their photographs in their natural habits, was shown to each respondent. A validated semi-structured questionnaire was used to determine the number of use report for each specimen collected. The questionnaire and the objectives of the study were relayed to each respondent, in either the Pangasinense or Ilocano dialect, whichever was appropriate. No appointments were made prior to these visits. Information on the manner by which plant parts were prepared into dosage forms before administration was obtained per use report corresponding to symptoms of dengue.

Each use reports maybe a specific disease state or a symptom. Use reports pertaining to symptoms of dengue were properly documented and tallied while use reports pertaining to other symptoms were tallied only. For each of the 3 indigenous areas surveyed, at least 20 respondents aged 20-80 were randomly selected. The sampling and survey were conducted from January 15 to March 15, 2015. An interview of municipal health officers in the Poblacion or town centers of the municipalities of Anda, Balungao, and San Fabian was also conducted to know the prevalence of patients infected with dengue from January to March 2015.

To ensure no issues on the infringement of biodiversity rights arise, the local government in all areas surveyed, in collaboration with the Department of Environment and Natural Resources, are consulted on the details of the sampling and collection of plant materials and the interview of local respondents on the use reports of these plants.

Calculations

The major uses agreement (MUA) was determined as the ratio between the number of informants that independently cited *E. hirta* for a single use report pertaining to symptoms of dengue and the total number of informants [*N* = 76, Table 1] that mentioned the plant for any use reports. A correction factor (CF) was applied to calculate the corrected MUA (cMUA), given by the formula: cMUA = MUA × CF × 100. The CF is the ratio between the number of informants citing *E. hirta* for any use report pertaining to a symptom of dengue and the highest number of informants citing the most employed medicinal plant. In this study, *Vernonia cinerea* L. was the most employed medicinal plant with 57 out of 82 informants.

The fidelity level (FL) of *E. hirta* in each of the 3 areas surveyed was computed, thus: $\% FL = I_p/I_u \times 100$, where I_p is the number of informants who independently suggested a use report pertaining to symptoms of dengue and I_u is the number of informants who mentioned the same plant for any use report [*N* = 76, Table 1].

RESULTS

Demographics

Tables 2-4 show the demographic data of the 82 informants in this study according to sex, age group, their distributions among the three indigenous areas surveyed and educational attainment.

The questionnaires were evenly distributed to each of the 3 sampling sites surveyed, but more allocations were given to female respondents because females are perceived to have higher knowledge over males in the traditional self-care uses of medicinal plants, a tradition that could have been passed to them from previous generations. In this study, significantly higher number of use reports was generated by female respondents compared to their male counterparts ($P < 0.0001$, Chi-square).

The majority of use reports pertaining to symptoms of dengue were given by respondents aged 60-80, a significantly higher number as compared to younger counterparts (227 vs. 79, $P < 0.0001$, Chi-square). This is because the respondents within this age bracket are more experienced than younger informants in the self-care uses of medicinal plants for dengue based on anecdotal information that is consistent with the rapid rise in the epidemics of this disease for the previous years. The use of medicinal plants also increased with increasing age to imply that the high dependence of these three indigenous communities on plants to treat illnesses is still consistent with time.

Elementary and secondary schools were found to have important roles in the dissemination of information on the self-care uses of medicinal plants in the three areas surveyed. Respondents who finished primary and secondary schoolings generated higher use reports related to dengue symptoms ($P < 0.001$, Chi-square). Interviews with teachers in these areas reveal that the study of medicinal plants on the scientific and anecdotal points of view has been incorporated in elementary and high school science curriculums. There is a low number of respondents with higher educational attainment because most of the informants surveyed have lower income compared to inhabitants in more populated municipalities, in addition to the great distances of colleges which are mostly located in Pangasinan city propers – findings showing similarities to the observations of Abe and Ohtani [9] among Ivatans in Batan Island north of Luzon.

Table 1 shows the number of informants interviewed and the frequency of use reports relative to any symptom in the 3 sampling sites surveyed. There is overlapping citation of use reports in Table 1. For instance, 3 respondents in Mt. Colisao cited *E. hirta* for at least 5 symptoms of dengue as well as 7 other symptoms. In Mt. Balungao, 4 respondents cited the

Table 1: Tally of use reports of *E. hirta* for any symptoms in three indigenous communities in Pangasinan

Sampling site	Total number of informants	Number of informants citing *E. hirta* for any use report for any symptom	Total number of use reports related to dengue	Total number of use reports for any symptom
Anda Island	26	25	100	197
Mt. Balungao	24	22	91	111
Mt. Colisao	32	29	115	109
Total	82	76	306	417

E. hirta: Euphoribia hirta

Table 2: Distribution of informants according to area surveyed

Sampling site	Gender		Total number of informants (%)
	Male	Female	
Anda Island	5	21	26 (31.70)
Mt. Balungao	6	18	24 (29.30)
Mt. Colisao	6	26	32 (39.00)
Total number of use reports related to dengue	251	55	306

Table 3: Demographic data according to age group and sex

Age group	Gender		Total number of informants (%)	Total number of use reports related to dengue
	Male	Female		
20-30	1	4	5 (6.10)	9
30-40	2	7	9 (11.00)	11
40-50	2	9	11 (13.40)	24
50-60	3	11	14 (17.10)	35
60-70	5	19	24 (29.30)	116
70-80	4	15	19 (23.20)	111
Total	17	65	82 (100.00)	306

Table 4: Educational attainment of informants

Educational level	Number of informants (%)	Number of use reports related to dengue
Illiterate	5 (6.10)	54
Elementary	25 (30.50)	117
Highschool	45 (54.90)	104
Some college	5 (6.10)	22
College	2 (2.40)	9
Total	82 (100.00)	306

same plant for use in 7 symptoms of dengue in addition to 10 other symptoms.

The relatively high number of use reports for any symptoms generated in Anda Island ($N = 197$; $P < 0.001$) can be due to a larger population that is dependent on medicinal plants in treating diseases, the higher density and diversity of plants available for use as medicinal agents and the high prevalence of acute diseases, including dengue. Furthermore, the absence of modern health facilities, clinics, hospitals and pharmacies in Anda Island may explain for the high dependence of its residents on medicinal plants.

An interview of municipal health officers within the vicinities of the 3 sampling sites surveyed shows a high prevalence of dengue infections in Anda Island from January to March of 2015, with 36 reported cases, including 2 casualties that affected mostly children ages 2 years and above. Within this period, 17 and 15 cases of dengue were reported in the municipalities of Balungao and San Fabian (i.e. where Mt. Colisao is located), respectively, with 1 reported casualty in Balungao. The high prevalence of dengue infection in the 3 sampling sites surveyed can be due to the lack of proper hygiene and sanitation in possible breeding places of mosquito vectors. From January 1 to September 5, 2015, the Department of Health reported 78,808 cases of dengue in the entire country, a 16.5% increase from the same time period last year, with about 8.2% accounted for the Ilocos Region (Region 1) including Pangasinan which was declared under a state of dengue outbreak at the time of survey.

MUA

Table 5 tallies and compares the number of use reports generated as symptoms of dengue in the 3 samplings sites surveyed and their cMUAs and FLs.

The cMUA reflects the relative importance of *E. hirta* in the 3 communities surveyed for a given symptom. cMUA of at least 35% were obtained for cardinal symptoms of dengue related to blood thinning episodes (i.e. nose bleeding, skin blisters and mouth bleeding). The febrile symptoms fever, headache, and joint pains have relatively high cMUAs as well. Low cMUAs (i.e. 2-4%) were obtained for symptoms in the recovery phase, namely, skin rashes, itching, and diarrhea partly because inhabitants do not follow-up or consult with respondent herbalists after the febrile phase. Symptoms such as hypotension, capillary fragility, and tachycardia have not been reported because the respondents are not familiar with the use of manual apparatus such as sphygmomanometers and torniquets. It should be noted that the symptoms of dengue enumerated in Table 5 may be symptoms of other diseases as well, such as bacterial and viral infections and certain inflammatory conditions.

A high FL is reflected when *E. hirta* is the most preferred medicinal plants to treat a particular symptom while a low FL is obtained when the plant is indicated for several symptoms and other diseases. Relatively high FL values were obtained for similar symptoms with high cMUAs such as the bleeding episodes as well as symptoms observed in the febrile phase. Consistently, lower FLs were reflected for symptoms with lower cMUAs.

Dosage Preparations

From the 76 use reports related to dengue symptoms, about 132 corresponding dosage forms of *E. hirta* are compounded extemporaneously as shown in Table 6.

About 111 dosage preparations were administered internally by mouth while the rest were applied topically to affected areas. Topical dosage forms (i.e. paste, poultice, cataplasm, rubifacient, or emollient) are applied to the skin and the forehead for headache, fever, joint pain, muscle pain, stomachache, skin rashes and itching while preparations for internal use (i.e. infusion and decoction) are indicated for symptoms related to bleeding of the skin, nose, oral cavites and the gastrointestinal tract. Some respondents in Anda Island and Mt. Colisao recommended the use of at least 3 preparations for a single use report or symptom as well as the use of both internal and external preparations for a single symptom.

Several ethnomedicinal studies have also reported that leaves are the most frequently used part because of their remarkable identity and accessibility in addition to the fact that most biosynthesis of therapeutically active constituents occur in leaves [10-12]. On the other hand, the use of leaf sap and latex was founded from the belief that these exudates from plants

Table 5: Prevalence of dengue symptoms in three indigenous communities in the province of Pangasinan

Symptoms	Number of informants			cMUA[a] (%)	FL[b] (%)
	Anda Island	Mt. Balungao	Mt. Colisao		
Nose bleeding	18	14	17	55.40	64.50
Skin blisters	16	11	15	40.70	55.30
Mouth bleeding	11	12	16	35.10	51.30
Fever	11	12	12	28.30	46.10
Headache	10	11	9	20.80	39.50
GIT bleeding	9	7	10	15.60	34.20
Joint pain	5	7	8	9.20	26.30
Muscle pain	4	3	5	3.30	15.80
Stomachache	4	3	5	3.30	15.80
Vomiting	3	3	2	1.50	10.50
Skin rashes	3	2	5	2.30	13.20
Itching	4	1	5	2.30	13.20
Diarrhea	2	5	6	3.90	17.10
Seizures	0	0	0	-	-

[a]cMUA: Corrected major use agreement, [b]FL: Fidelity level, GIT: Gastrointestinal tract

Table 6: Dosage preparation of *Euphorbia hirta* compounded according to plant part

Plant part	Number of dosage forms (%)	Predominant dosage forms
Leaves	67 (50.8)	Decoction, infusion, sap/latex
Barks	33 (25.0)	Decoction, paste, poultice
Stem barks	11 (8.3)	Infusion, cataplasm
Roots	12 (9.1)	Decoction, rubefacient, emollient
Flowers	9 (6.8)	Decoction, wine infusion, chewed
Total	132 (100.0)	

belonging to the family Euphorbiaceae possess antibacterial and antiviral properties and finds great application as antiseptics in the treatment of wounds [13]. The respondents did not described how cataplasms, poultices, paste, emollients and rubefacients are compounded although the issue of hygiene in their preparation are important, particularly if these dosage forms are to be applied externally to infected sites.

DISCUSSIONS

The global incidence of dengue has grown dramatically in recent decades, particularly in urban areas in countries with tropical climates, and becoming a leading cause of death among children [14]. Since there is no specific cure against dengue, treatment modalities include supportive measures to address specific symptoms. In rural areas, such as the three indigenous communities surveyed in this study, limited access to modern health care facilities limits early detection of dengue which is important to avoid long-term complications. The lack of vector control against dengue-carrying mosquitoes in these areas further complicates the situation.

The most commonly-used plants in the folkloric treatment of dengue in the Philippines include *E. hirta* and *Carica papaya* [14]. Although the people of Pangasinan have a long history of the traditional uses of medicinal plants, no ethnobotanical studies have been undertaken to document the traditional knowledge on their self-care uses of these plants. Documentation of the traditional uses of *E. hirta* against dengue indicates the relative folkloric importance of the plants in the three surveyed indigenous communities in Pangasinan where dengue continues to be an epidemic.

The current supportive therapies for hospitalized dengue patients include hydration and blood transfusion as well as treatment with acetaminophen since most non-steroidal anti-inflammatory drugs are contra-indicated due to their anti-platelet augmenting properties. Antiviral drugs and vaccines against dengue have yet to be fully developed. Since *E. hirta* was found in this study to be effective against most symptoms of dengue in the initial, febrile and recovery stages, the findings warrant the development of the plant into dosage forms that can be utilized in clinical trials aimed at ensuring the efficacy and safety of *E. hirta* in the supportive therapy of dengue.

CONCLUSION

This study was able to document the anecdotal and traditional self-care uses of *E. hirta* in the treatment of dengue in three indigenous communities in Pangasinan according to demography, relative importance, and FL.

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Original Research

Coconut water of different maturity stages ameliorates inflammatory processes in model of inflammation

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ABSTRACT

Aim: Coconut water is a natural beverage that is a part of daily diet of many people. This study was designed to explore the anti-inflammatory activity of coconut water of different maturation stages (young and mature) with rat paw edema model of inflammation using plethysmometer. **Methodology:** For this study, albino rats were selected and divided into four equal groups (10 rats in each group). Group 1 was set as control and administered distilled water 1 ml orally; Groups 2 and 3 were treated with young and mature coconut water, respectively, at 4 ml/100 g dose orally. Group 4 was treated with the standard drug (ibuprofen) at 400 mg/70 kg. 0.1 ml of 1% w/v acetic acid was administered in the subplantar tissue of rat paw 30 min after oral treatments of groups. Plethysmometer was used to measure rat paw edema. **Results:** Results revealed that both coconut water possess significant anti-inflammatory activity ($P < 0.001$). In comparison to control, percent inhibition by young coconut water was 20.22%, 35.13%, 42.52%, and 36% at 1, 2, 3, and 4 h of acetic acid administration, respectively. However, maximum percent inhibition (42.52%) was observed in the second phase of the inflammatory process. On the other hand, percent inhibition by mature coconut water was 18.80%, 25.94%, 24.13%, and 18.66% at 1, 2, 3, and 4 h of acetic acid administration, respectively. However, maximum percent inhibition (25.94%) was observed in the first phase of the inflammatory process. **Conclusions:** This study strongly suggests the use of young coconut water for potent anti-inflammatory effect and mature coconut water for moderate anti-inflammatory effect.

KEY WORDS: Anti-inflammatory, coconut water, paw edema, salicylic acid

INTRODUCTION

Inflammation is a normal response that takes place in the presence of tissue injury. It is a frequent clinical observation [1]. Pain, swelling, local redness, edema, and loss of function are considered as classic signs of inflammatory process [2]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are considered as most commonly used anti-inflammatory drugs worldwide. However, in addition to their high costs such drugs also present a high profile list of adverse effects and toxicity. Furthermore, one of the most common disadvantages of currently available drugs is that the symptoms of inflammation reappear after their discontinuation. Therefore, development and screening of new anti-inflammatory drugs are the need of time. For new drug discovery, plants are considered a rich source [3].

In the prevention of chronic ailments, nutrition can play a significant role. It is because most of the chronic ailments may be related to diet. The concept of "functional foods" explains that food should be considered not only for living purposes but also be considered as a great source of physical and mental health. It is because this consideration will aid in the reduction and prevention of risk factors for different diseases. It may also help in enhancing different physiological functions [4]. Food may be termed as "functional" if it satisfactorily demonstrates to affect different functions of the human body in a beneficial way. Moreover, food is termed as functional if it is beyond adequate nutritional effects, in such a way that is relevant to health or reduction in the risk of diseases [5]. Simplest examples of functional food include tomatoes, carrots, turmeric, and mustard. These items are considered as functional food items because these contain a high content of physiologically active composition [6]. Phytochemicals found in vegetables and fruits provide a synergistic and additive effects as a result of their significant antioxidant potential [7,8].

Phytochemicals are explained as bioactive components found in grains, vegetables, and fruits. Synergistic and additive effects of phytochemicals in vegetables, fruits, and whole grains are held responsible for its potent anticancer and antioxidant activities. Use of a wide range of vegetables, fruits, and whole grains on a daily basis is considered as a practical strategy to optimize health [9].

Coconut (*Cocos nucifera*) is among significant plants that possess nutritional and medicinal properties. It is frequently cultivated for medicinal and nutritional purposes especially in tropical areas [10]. It belongs to the family Arecaceae. Total cultivation area of the coconut tree is 11 million hectares worldwide [11]. However, it may grow where ever there is adequate rainfall and warmth. Coconut water is a refreshing and nutritious beverage in its natural form. It is traditionally used all around the world due to its beneficial effects on human health. Coconut water [Figure 1] has a variety of health-related effects including antihyperlipidemic [12], antiulcerogenic, and cardioprotective effects [13]. The composition and quantity of coconut water changes as the fruit mature [Figures 4 and 5]. There is no research study conducted to compare the anti-inflammatory effects of young and mature coconut water. Rat is a commonly used animal species for preclinical model studies [14]. History shows that different people have been using plants to treat their ailments. For instance, there are more than 13,000 reports of plant extracts that possess anti-inflammatory effect [15]. The spectrum of biochemical effects from various phytochemicals might be able to influence different processes in the organism such as anti-inflammatory activity. Therefore, this study was designed to explore and compare anti-inflammatory effects of both young and mature coconut water in an animal model of inflammation.

METHODOLOGY

Collection of Coconut Water

Coconut fruits, both young and mature (Figures 2 and 3), were harvested from the coconut trees grown in Karachi city. Water from young and mature coconut fruits was collected after carefully cutting the fruits from the top, and the water was stored in the refrigerator for further use [18]. University Board of Advanced Studies and Research approved this study with no. 02181/Pharm.

Selection of Animals

To conduct this study, 40 rats of either sex were selected and divided into 4 equal groups. Their weights ranged from 200 to 250 g. Rats were housed with 12/12 h dark and light cycle and 21°C ± 1°C temperature. Animals were kept on standard animal diet and water *ad libitum*. Rats were handled according to Helsinki Resolution 1964 and divided into 4 groups of 10 rats each.

Experimental Protocol

All four groups received 0.1 ml of 1% acetic acid in the subplantar tissue of the rat's paw 30 minutes after oral treatment of distilled water, young coconut water, mature coconut water, and ibuprofen. Group 1 received distilled water (1 ml orally) and served as control. Groups 2 and 3 served as treated groups for young coconut water and mature coconut water, respectively, and received respective coconut water orally at the dose of 4 ml/100 g [19]. Group 4 served as a standard group and received ibuprofen orally at the dose of 400 mg/70 kg.

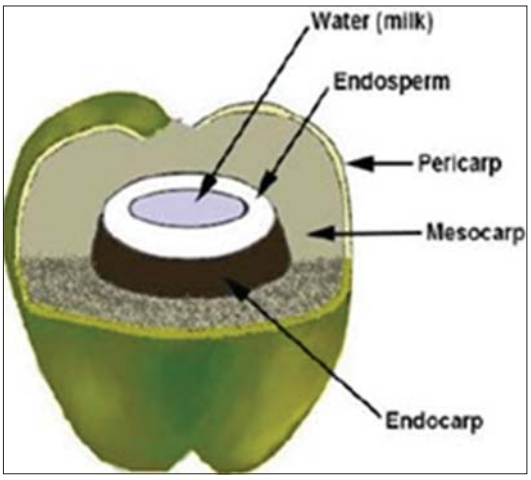

Figure 1: Coconut fruit (cross section)

Figure 2: Young coconut fruit

Figure 3: Mature coconut fruit

Component	Young *Cocos nucifera* Water	Mature *Cocos nucifera* Water
Proximates	**g per 100 g**	**g per 100 g**
Water	94.18	94.45
Dry	5.82	5.55
Protein	0.12	0.52
Total lipid (fat)	0.07	0.15
Ash	0.87	0.47
Carbohydrate, by difference	4.76	4.41
Fiber, total dietary	Non-detectable	Non-detectable
Sugars	**g per 100 g**	**g per 100 g**
Total	5.23	3.42
Sucrose	0.06	0.51
Glucose	2.61	1.48
Fructose	2.55	1.43
Inorganic Ions	**mg per 100 g**	**mg per 100 g**
Calcium	27.35	31.64
Iron	0.02	0.02
Magnesium	6.40	9.44
Phosphorus	4.66	12.77
Potassium	203.70	257.52
Sodium	1.75	16.10
Zinc	0.07	0.02
Copper	0.01	0.03
Manganese	0.12	0.08
Aluminum	0.07	0.06
Boron	0.05	0.08
Vitamins	**mg per 100 dm^3**	**mg per 100 dm^3**
Vitamin C, Total Ascorbic acid	7.41	7.08
Thiamin (B1)	Trace	0.01
Riboflavin (B2)	0.01	0.01
Niacin (B3)	Non-detectable	Non-detectable
Pyridoxine (B6)	Non-detectable	Non-detectable

Figure 4: Chemical composition of coconut water [16,17]

Animal Model of Inflammation

1% w/v acetic acid induced paw edema model was used to study anti-inflammatory activity of young and mature coconut water. Prepared acetic acid (0.1 ml) was administered parenterally in the subplantar tissue of rat's paw 30 min after oral treatments. Edema (sign of inflammation) in the paw of the animal was observed with the use of plethysmometer (Ugo Basile, Italy). The observations were made at different time intervals, i.e. before administration of acetic acid (baseline observation), immediately after administration of acetic acid, and after 1, 2, 3, and 4 h of acetic acid administration.

Plethysmometer

Paw of each rat was immersed in measuring tube of plethysmometer. The moment the paw is immersed in water, it is displaced, and this displacement is sensed by electrodes of platinum. Plethysmometer detects changes in conductance and an output signal is transmitted on the digital display that indicates measured volume displacement (resolution: 0.01 ml) [20]. Following formula was used to calculate percent inhibition in edema [21,22]:

$$\frac{\text{Paw edema of control animal at given time} - \text{Paw edema of treated animal at same time}}{\text{Paw edema of control group at given time}} \times 100$$

Statistical Analysis

Collected data are presented as mean ± standard deviation and analyzed by one-way ANOVA followed by *post-hoc* Bonferroni multiple comparisons. $P < 0.001$ is considered highly significant.

RESULTS

The results of this study are presented in Table 1. Young and mature coconut water showed remarkable anti-inflammatory effect. However, the intensity of the effect is varied.

Component	Young *Cocos nucifera* Water	Mature *Cocos nucifera* Water
Fatty Acids	**g per 100 g**	**g per 100 g**
Total monounsaturated	0.03	0.02
Total polyunsaturated	0.0128	0.0054
Amino Acids	**mg per g (defatted sample)**	**mg per g (defatted sample)**
Alanine	1.13	3.88
Arginine	0.13	0.81
Aspartic acid	1.60	0.76
Glutamic acid	3.44	3.75
Glycine	0.43	0.11
Histidine	0.39	0.67
Isoleucine	0.26	0.27
Leucine	0.66	0.58
Lysine	4.72	3.41
Methionine	0.22	0.21
Phenylalanine	0.26	0.00
Proline	0.52	0.95
Serine	0.64	1.06
Tyrosine	0.00	0.00
Tryptophan	0.00	0.00
Threonine	0.20	0.33
Valine	0.91	0.82
Organic acids	**mg per 100 g (dry matter)**	**mg per 100 g (dry matter)**
Tartaric	1.6	2.4
Malic	317	307
Citric	Non-detectable	24.8
Chemical Properties		
pH	4.7+0.1	5.2+0.1

Figure 5: Chemical composition of coconut water [16,17]

Table 1: Anti-inflammatory effect of young and mature coconut water

Groups	Mean±SD		Results shown as displacement in 'ml' and percent inhibition – after acetic acid treatment (%)			
	Before drug treatment (displacement in ml)	Immediately after acetic acid treatment (displacement in ml) (0 h)	1 h	2 h	3 h	4 h
Acetic acid only	2.27±0.33	2.25±0.16	3.51±0.03	3.70±0.06	4.35±0.16	3.75±0.79
Acetic acid+ Young coconut water	2.33±0.21	2.45±0.31	***2.80±0.42 (20.22)	***,++,##2.40±0.31 (35.13)	***,+,###2.50±0.52 (42.52)	***,#2.40±0.51 (36.00)
Acetic acid+ Mature coconut water	2.12±0.09	2.05±0.20	***2.85±0.26 (18.80)	***2.74±0.28 (25.94)	***3.30±0.42 (24.13)	*3.05±0.26 (18.66)
Acetic acid+ Ibuprofen	2.35±0.12	2.85±0.19	***2.70±0.21 (23.07)	***2.80±0.04 (24.32)	***2.98±0.14 (31.49)	*2.97±0.33 (20.8)

n=10, Data are presented as mean±SD. $P<0.001$*** is considered highly significant, $P<0.05$* is considered significant in comparison to control group; $P<0.01^{++}$ is considered more significant, $P<0.05^{+}$ is considered significant in comparison to standard drug (ibuprofen treated) group; $P<0.001^{\#\#\#}$ is considered highly significant, $P<0.01^{\#\#}$ is considered more significant, $P<0.05^{\#}$ is considered significant in comparison of young coconut water v/s mature coconut water groups. SD: Standard deviation

Effects after 1 h of Acetic Acid Administration

Ibuprofen (standard drug group), young coconut water and mature coconut water showed a highly significant decrease ($P < 0.001$) in rat paw edema after 1 h of acetic acid administration in comparison to control group. Moreover, the effects of both young and mature coconut water were similar to ibuprofen showing insignificant differences in rat paw edema between ibuprofen and young and mature water at 1 h.

Effects after 2 h and 3 h of Acetic Acid Administration

After 2 h and 3 h of acetic acid administration, ibuprofen, young coconut water, and mature coconut water showed a highly significant decrease ($P < 0.001$) in rat paw edema. The decrease of rat paw edema by mature coconut water is similar to ibuprofen as the difference in rat paw edema of these two groups is statistically insignificant. However, young coconut water showed better results than ibuprofen (standard drug

group) at 2 h ($P < 0.01$) and 3 h ($P < 0.05$) of acetic acid administration.

Effects after 4 h of Acetic Acid Administration

After 4 h of acetic acid administration, young coconut water showed a highly significant decrease ($P < 0.001$) in rat paw edema. However, mature coconut water and ibuprofen showed an only significant decrease in paw edema ($P < 0.05$). Furthermore, at 4 h of acetic acid administration, the decrease in paw edema by young coconut water and mature coconut water is similar to ibuprofen as the difference between them is statistically insignificant.

Percent Inhibition of Rat Paw Edema

In comparison to control, percent inhibition of rat paw edema by young coconut water was 20.22%, 35.13%, 42.52%, and 36% at 1, 2, 3, and 4 h of acetic acid administration, respectively. However, maximum percent inhibition (42.52%) was observed in the second phase of the inflammatory process. On the other hand, percent inhibition by mature coconut water was 18.80%, 25.94%, 24.13%, and 18.66% at 1, 2, 3, and 4 h of acetic acid administration, respectively. However, maximum percent inhibition (25.94%) was observed in the first phase of the inflammatory process.

Comparison of Anti-inflammatory Effect of Young and Mature Coconut Water

At 1 h, the difference between rat paw edema of young and mature coconut water groups was found insignificant.

At 2, 3, and 4 h of acetic acid administration, the difference of rat paw edema between young and mature coconut water was more significant ($P < 0.01$), highly significant ($P < 0.001$), and significant ($P < 0.05$), respectively [presented by # in Table 1]. This difference in the results at 2, 3, and 4 h of inflammation shows the better anti-inflammatory effect of young coconut water than mature coconut water.

DISCUSSION

This study explored the anti-inflammatory effect of young and mature coconut water using acetic acid induced rat paw edema model. The effects were also compared with the standard drug, ibuprofen. Inflammation is induced by acetic acid as a result of inflammatory mediators. These inflammatory mediators are released in two phases. Platelet activation factor, serotonin, and histamine are released in the first phase. The time duration of this phase is first 90 min. However, prostaglandins (PGs), lysosomes, proteases, and kinins are released in the later phase (after 90 min) [23,24]. Synthesis of PGs, i.e. prostaglandin F2 alpha, prostaglandin E2 and synthesis of free radicals along with interleukin-1 (IL-1), IL-2 and tumor necrosis factor-alpha induces nociception and inflammation via stimulation of nociceptors [25]. Moreover, in addition to this induction, it may also stimulate cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase [26]. This eventually increases oxidative stress in inflamed tissue of rats paw. Edema at the site of inflammation is due to the release of bradykinin, histamine, and serotonin [27]. According to Panthong *et al*., NSAIDs produce its effect in the later phase of inflammation [28]. In this phase, they suppress the expression of COX-2 and hence reduce the synthesis of PGs [29].

Young and mature coconut water showed excellent anti-inflammatory effects [Table 1]. Coconut water contains flavonoids. These components are responsible for potent anti-inflammatory effect as they inhibit the synthesis of PGs [1,30,31]. Moreover, it is reported that if plants possess antioxidant activity, they show significant anti-inflammatory response [32,33]. Coconut water is reported to have antioxidant potential due to its unique composition including kinetin and micronutrients [16,34]. Coconut water is also reported to have the antihistaminic effect that further contributes to anti-inflammatory activity [35]. Furthermore, coconut water contains abscisic acid (ABA). ABA may contribute to the anti-inflammatory activity of water by acting on peroxisome proliferator-activated receptor-gamma (PPAR-γ). According to Kelly *et al.*, the activation of PPAR-γ agonist produces direct inhibition of inflammation via action on nuclear factor-kappa B [36]. In addition to this, it may also inhibit monocyte chemoattractant protein-1 induced migration of monocytes [37,38].

Young coconut water decreased paw edema right from the first hour of inflammation (early phase). However, the percent inhibition of edema from young coconut water was found to be maximum in the second phase of inflammation. This result hence reveals that young coconut water showed the effect on histamine and serotonin (agents released in the first phase). However, since the maximum effect was observed in the third hour (second phase) of inflammation, it may also act on COX-2 and inhibits synthesis of PGs. This effect may be attributed to the presence of salicylic acid in young coconut water [39].

Mature coconut water also showed a decrease in rat paw edema right from the first hour of inflammation and maximum percent inhibition of edema was observed at the second hour (first phase). This proposes that mature coconut mainly acts via action on histamine and serotonin [23,24].

Difference in Effect of Young and Mature Coconut Water

The concentration of salicylic acid in coconut water starts decreasing as the fruit matures [39] suggesting that young coconut water contains more salicylic acid content than mature coconut water. It is probably because of this concentration difference; only young coconut water has shown a maximum effect in the second phase of inflammation as generally observed by NSAIDs also. Salicylic acid is one of the active components of aspirin (an example of NSAIDs).

CONCLUSION

Young and mature coconut water both possess significant anti-inflammatory activity. As the coconut water is widely used worldwide and is a part of daily diet of many people, this study could serve as a positive finding in using coconut water for

their anti-inflammatory potential. Since young coconut water showed better results than mature coconut water and standard drug ibuprofen, this study strongly suggests the use of young coconut water for potent anti-inflammatory effect and mature coconut water for moderate anti-inflammatory effect. This proposed mechanism for anti-inflammatory effect is based on the composition of coconut water. However, further molecular level research may be conducted to uncover the more precise anti-inflammatory mechanism of action of young and mature coconut water.

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Original Research

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Investigation the efficiency of various methods of volatile oil extraction from *Trichodesma africanum* and their impact on the antioxidant and antimicrobial activities

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ABSTRACT

Background: Currently, there is an increasing interest in developing more efficient techniques for the extraction of phytochemicals. Microwaves and ultrasonic extraction methods are promising techniques that can be used for this purpose. **Objectives:** The purpose of this study was to investigate the impact of different extraction methods on yield, antioxidant and antimicrobial activities of volatile oil extracted from *Trichodesma africanum*. **Materials and Methods:** Volatile oil was extracted using microwave, ultrasonic, microwave-ultrasonic, and conventional hydrodistillation methods. The extracted oil was evaluated for antioxidant and antimicrobial activities. The antioxidant activity was assessed by 2,2-Diphenyl-1-picrylhydrazyl scavenging assay, whereas the antimicrobial activity was assessed by broth microdilution method. The antimicrobial activity of the volatile oils was examined against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* American type culture collection reference strains, as well as against methicillin-resistant *S. aureus* (MRSA) and *Candida albicans* clinical isolates. **Results:** The volatile oil obtained by the four extraction methods in this study exhibited both antioxidant and antimicrobial activities. Among the four extraction methods used, the microwave-ultrasonic method yielded the largest amount (1.8% v/w) and the yield exhibited the highest antioxidant activity in terms of inhibition (91.83% ± 1.1). The minimum inhibitory concentrations for *E. coli*, *P. aeruginosa*, *S. aureus*, MRSA, and *C. albicans* were 3, 5, 6, 3, and 9, respectively. **Conclusion:** Among the extraction techniques used in this study, the microwave-ultrasonic method showed the best results. Moreover, this study suggests that *T. africanum* volatile oils contain active substances that could potentially be used both as natural preservatives in food and pharmaceutical industries as well as in developing new antimicrobial and antioxidant agents.

KEY WORDS: Antimicrobial, antioxidant, microwave-ultrasonic, *Trichodesma africanum*

INTRODUCTION

Medicinal plants are among the main sources of phytochemicals. These phytochemicals are secondary metabolites that plants can use to defend themselves against microbial infections, insect infestations, and herbivorous animals. Fortunately, many of these phytochemicals have been found to be pharmaceutically and medicinally important such as eugenol, ascaridole, cineole and many others [1]. Unfortunately, a lack of safety is a major problem that limits the use of various herbal products [2]. Many regulatory agencies including the United States Food and Drug Administration and the European medicines agency require that herbal extracts are standardized to ensure safety and efficacy of their medicinal use [3]. Accordingly, the method used for extraction is crucial because it may impact the final quality and the yield of the phytochemical constituents, especially when these constituents are sensitive and may undergo degradation [2]. Therefore, optimized extraction methods are important for reducing processing costs, time, energy, and improving yield. Conventional extraction methods,

such as heating, boiling, or refluxing, have a negative impact on the quality and yield. Phytochemicals might be lost due to hydrolysis, oxidation and/or ionization during these extraction processes [4]. Recently, new extraction methods including ultrasound-assisted extraction and microwave-assisted extraction have emerged as promising techniques for the extraction of phytochemicals from plants and herbals [5].

Trichodesma africanum (L.) Sm., also known as African barbell in English and *Lozeka* in Arabic, belongs to the Boraginaceae family. This erect, harshly scabrid, annual, short-lived perennial herbaceous plant is of about 1 m in height. It forms branches mainly from the base and has a fistular stem which is densely covered with prickly stiff hairs of about 2 mm in length. The leaves of this plant are simple with entire margins that are covered on both surfaces with erect-patent, prickles [6-8]. *T. africanum* has been reportedly used in the folk medicine of several countries for the treatment of many clinical conditions including a cough and common cold [9]. In Iran, roots and leaves of *T. africanum* have been used for the treatment of several clinical conditions including common cold, chest congestions, chickenpox, scarlet fever, measles, bone fractures, headache, abdominal pain, mouth ulcer, and constipation [10]. In Nigeria, it has been used as used to induce diuresis [11]. In Pakistan, it has been described for the treatment of severe respiratory tract diseases [12]. Many studies have investigated the medicinal importance of the phytochemicals of *T. africanum*. It was shown that the methanolic extract of this plant has a remarkable inhibitory effect against the growth of many clinically important bacterial and fungal pathogens such as *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Candida albicans* [13]. In addition, it has been shown that *T. africanum* methanolic extract exhibits antitumor activity in cell lines derived from several human cancers such as breast, colon, liver, and lung cancers [14].

Phytochemical analysis of aerial parts of *T. africanum* has shown the presence of several phytochemicals of clinical importance such as alkaloids, sterols, triterpenes, tannins, anthraquinones, and pyrrolizidine [15]. *T. africanum* volatile oils have been found to consist mainly of caryophyllene oxide, γ-eiudesmol, α-muurolene, elemol, carvone, and β-caryophyllene and to a lesser extent of α-pinene [16]. Based on the above, the purpose of this study was to investigate the impact of extraction methods, on extraction yield, antioxidant, and antimicrobial activities of *T. africanum* volatile oils.

MATERIALS AND METHODS

Chemical Reagents

The following reagents were used to evaluate the antioxidant activity of *T. africanum* extracts: Methanol (Lobachemie, India), *n*-hexane (Frutarom LTD, Haifa), trolox ((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) (Sigma-Aldrich, Denmark), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany).

Reagents used for the screening of the antimicrobial activity of *T. africanum* volatile oil included nutrient broth that was ordered from Himedia, India, and dimethyl sulfoxide (DMSO), which was purchased from Riedeldehan, Germany.

Instrumentations

During this study, the following instruments were used: Ultrasonic-microwave cooperative extractor/reactor (CW-2000, China), rotary evaporator (Heidolph OB2000, VV2000, Germany), UV-visible spectrophotometer (Jenway 7315, England), grinder (Moulinex model, Uno. China), balance (Radw ag, AS 220/c/2, Poland), filter paper (Machrery-Nagel, MN 617 and Whatman no.1, USA), micropipettes (Finnpipette, Finland), incubator (Nuve, Turkey), syringe filter 0.45 μm pore size (Microlab, China), and 96-well plates (Greiner bio-one, North America).

Collection and Preparing Plant Materials

The leaves of *T. africanum* were collected during its flowering time from Bethlehem region (Palestine) during May 2015. Botanical identification was carried out by Pharmacognosist Dr. Nidal Jaradat from the Pharmacognosy and Herbal Products Laboratory, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus. The identification process was conducted using live herbal specimens and photographs from books [17]. Voucher specimens were deposited in the pharmacognosy and herbal products laboratory under the code number: Pharm-PCT-2457.

To extract volatile oil, the leaves of *T. africanum* were separated carefully and then washed twice with distilled water. The washed leaves were dried for 10-14 days in the shade at room temperature to avoid damage and to minimize cross contamination of the separated leaves. Finally, the dried leaves were grounded well and the powder obtained was stored in cloth bags for future use.

Volatile Oils Extraction

Volatile oils from *T. africanum* leaves were extracted using several methods (shown below). The main aim behind using different extraction methods was to evaluate the efficiency of these methods in terms of the obtained yield as well and the extraction time. *T. africanum* volatile oils obtained by the different extraction methods used in this study were then examined for both of their antimicrobial and antioxidant activities.

Volatile Oil Extraction Methods

Hydro distillation

Simple hydrodistillation was carried out using clevenger apparatus with some modifications [18]. About 100 g of the *T. africanum* dried leaves were placed in a round-bottom flask. The leaves were mixed with about 500 ml distilled water and then boiled for 120 min at 100°C. Volatile oils were collected into a clean beaker (this procedure was repeated three times). The obtained volatile oil was chemically dried using $CaCl_2$. The purified volatile oil was weighed and stored in tightly-closed amber-colored bottles at 4°C.

Microwave method

T. africanum volatile oil was extracted using microwave oven as described by Bousbia *et al*. with some modifications [19]. The power of the microwave oven was set at 1000 W. Clevenger apparatus with a 1 L round-bottom flask containing about 100 g of *T. africanum* dried leaves powder was placed inside the microwave oven. About 500 ml distilled water was then added into the flask containing the powder. The flask was then connected to clevenger apparatus. Microwave distillation was carried out three times for 15 min each at 100°C. The obtained volatile oil was collected into a clean beaker, chemically dried, weighed, and stored as mentioned above.

Ultrasonic-assisted extraction method

During this method, a suspension of the dried leaves powder was prepared in a screw-caped class bottle using about 100 g of the dried leaves powder, which was mixed with about 500 ml hexane. The screw-capped bottle containing the suspension was placed in the ultrasonic extractor apparatus. Volatile oils were extracted for 120 min at 100°C. During the extraction process, the ultrasonic extractor apparatus was adjusted at its maximum power (50 W and frequency of 40 kHz). Finally, the hexane was removed in a rotary evaporator at 30°C and the obtained volatile oil was stored as mentioned above [20].

Microwave-ultrasonic method

Microwave-ultrasonic is a recently developed method that is used for the extraction of volatile oil from medicinal plants. In this method, volatile oil was extracted using a microwave oven with ultrasonication. However, during the extraction process, the powder suspension being extracted was exposed to ultrasonic waves to improve the extraction process. In this study, the apparatus consisting of a microwave oven combined with an ultrasonic extractor was used (ultrasonic-microwave cooperative extractor/reactor (CW-2000, China). A 1 L round-bottom flask containing about 100 g of the dried leaves powder was placed in this apparatus. In this flask, the powder was suspended in about 500 ml deionized water. Then, the flask was connected with Clevenger apparatus, which was placed in the same apparatus. While carrying out the extraction process, the power of the microwave-ultrasonic extractor apparatus was adjusted at 1000 W. The ultrasonic power of the apparatus was adjusted at its maximum power as well (50 W and frequency of 40 kHz). The extraction process using this apparatus was conducted for 10 min at 100°C. This process was repeated for three times. The obtained volatile oil was collected into a clean beaker, chemically dried and stored as motioned above.

DPPH Radical-scavenging Activity

Trolox standard and plant working solutions

A stock solution of *T. africanum* volatile oil that was extracted by the four extraction methods used in this study was used to prepare a stock solution, in methanol and Trolox, at a concentration of 1 mg/ml. Each of these stock solutions was diluted in methanol to prepare 12 working solutions with the following concentrations: 1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 μg/ml.

Spectrophotometric Measurements

A freshly prepared DPPH solution (0.002% w/v) was mixed with both methanol and with each of the above-mentioned working solutions at 1:1:1 ratio. In addition, a negative control solution was prepared by mixing the above-mentioned DPPH solution with methanol in 1:1 ratio. Then, all of these solutions were incubated at room temperature in a dark cabinet for 30 min. By the end of the incubation period, the optical density of these solutions was determined spectrophotometrically at a wavelength of 517 nm using methanol as the blank solution.

Antioxidant Activity of *T. africanum* Volatile Oils

The antioxidant activity of *T. africanum* volatile oil and Trolox standard was determined in terms of inhibition percentage of DPPH activity using the following formula as follows:

Percentage of inhibition of DPPH activity (%) = (A-B)/A × 100%, where: A = Optical density of the blank and B = Optical density of the sample.

The antioxidant half-maximal inhibitory concentration (IC_{50}) for each of the *T. africanum* volatile oil and Trolox standard solution as well as their standard deviations, were calculated by using BioDataFit edition 1.02 (data fit for biologist).

Data Analysis

The antioxidant activities of *T. africanum* volatile oil at different concentrations mentioned above were expressed in terms of the antioxidant activity of the Trolox standard. This was determined using the BioDataFit fitting program as follows:

Inhibition percentage according to Trolox = (Trolox IC_{50}/volatile oil IC_{50}) × 100%

Antimicrobial Tests

T. africanum volatile oils obtained by the four different extraction methods used in this study were investigated for both of their antibacterial and antifungal activities. The antibacterial activities of *T. africanum* volatile oils were examined against the growth of four reference bacterial strains obtained from the American type culture collection (ATCC) (*S. aureus* [ATCC 25923], *E. coli* [ATCC 25922], *and P. aeruginosa* [ATCC 27853]) as well as against the growth of a diagnostically-confirmed methicillin-resistant *S. aureus* (MRSA) clinical isolates. The antifungal activity of *T. africanum* volatile oils was examined against the growth of a diagnostically-confirmed *C. albicans* clinical isolate.

The antimicrobial activities of *T. africanum* volatile oils obtained by the four extraction methods used in this study were

determined using broth microdilution method as described previously [21,22]. Briefly, *T. africanum* volatile oils were dissolved in 5% DMSO at a concentration of 132 mg/ml. The prepared *T. africanum* volatile oils solutions were filter-sterilized and then were serially micro-diluted (2 folds) 11 times in sterile nutrient broth. The dilution processes were carried out under aseptic conditions in 96 well plates. In the micro-wells that were assigned to evaluate the antibacterial activities of the extracted *T. africanum* volatile oils, the concentration of these oils ranged from 0.129 to 66 mg/ml. On the other hand, the concentrations of these volatile oils in the micro-wells assigned to evaluate their antifungal activities ranged from 55 to 0.065 mg/ml. In these plates, micro-well number 11 contained volatile oils-free nutrient broth, which was used as a positive control for microbial growth. On the other hand, micro-well number 12 contained volatile oils-free nutrient broth that was left uninoculated with any of the test microbes. This well was used as a negative control for microbial growth. Micro-wells numbers 1 to 11 were inoculated aseptically with the test microbes. At the time of inoculation, the final concentrations of microbial cells were about 5×10^5 and $0.5\text{-}2.5 \times 10^3$ colony-forming unit /ml for the tested bacterial pathogens and *C. albicans*, respectively. Each of the included microbes in this study was examined in duplicate for being inhibited by the obtained *T. africanum* volatile oils.

All the inoculated plates were incubated at 35°C. The incubation period lasted for about 18 h for those plates inoculated with the test bacterial strains and for about 48 h for those plates inoculated with *C. albicans*. The lowest concentration of *T. africanum* volatile oils, at which no visible microbial growth in that micro-well was observed, was considered as the minimal inhibitory concentration (MIC) of the examined *T. africanum* volatile oils.

Statistical Analysis

Volatile oil yield of different extraction methods and IC_{50} values was determined in triplicates. Results are expressed as means ± standard deviation (SD). Data were compared using ANOVA with multiple comparisons. The statistical significance was considered when the $P < 0.05$. Statistical significance is expressed in terms of *when the $P < 0.05$, **when the $P \leq 0.001$, and ***when the $P \leq 0.0001$.

RESULTS

Volatile Oil Yields

Comparing the amounts of volatile oil obtained from *T. africanum* by the four extraction methods used in this study, hydrodistillation method yielded the smallest amount with a dry weight yield of about 0.3% v/w after 2 h of extraction time, as shown in Table 1. The microwave method yielded a dry weight yield of about 1.2% v/w after about 15 min of extraction time. The amount of volatile oil obtained increased by ultrasonic-assisted method with a dry weight yield of about 0.43% v/w after 120 min of extraction time. The largest amount of volatile oils was obtained by the microwave-ultrasonic method, which gave a dry weight yield of about 1.8% v/w after only 10 min of extraction time.

Antioxidant Activity using Trolox as Standard Equivalent

The free radical scavenging-activity of *T. africanum* volatile oils obtained by each of the extraction methods used in this study, have been tested by DPPH method using Trolox as a reference standard. The concentrations of the used volatile oils extracts as well as the Trolox standard ranged from 1 to 100 μg/ml. For measurement of the baseline scavenging activity, DPPH was diluted in the corresponding methanol solvent without any plant extract.

The calculated half-maximal IC_{50} for the Trolox standard was about 3.06 ± 0.54 μg/ml. The IC_{50} and percentage of inhibition for *T. africanum* volatile oils obtained by each of the used extraction methods are shown in Table 2 and clarified in [Figure 1].

Table 1: Volatile oils yields obtained by four different extractions methods (at 100°C) versus extraction time

Extraction method	Yield, (% v/w)	Extraction time (min)
Hydrodistillation	0.3±0.69 (reference)	120
Microwave	1.2±0.47 (ns)	15
Ultrasonic-assisted	0.43±0.5 (ns)	120
Microwave-ultrasonic	1.8±0.59*	10

NS: Not statistically significant, *: p value < 0.05

Table 2: IC_{50} for Trolox standard and *T. africanum* volatile oils obtained by four different extraction methods with their inhibition percentages according to Trolox standard

Extraction methods	IC_{50}±SD	% of inhibition, ± SD
Hydrodistillation	21.5±0.43***	15.16±0.79***
Microwave	8.6±0.87***	37.91±0.84***
Ultrasonic-assisted	6.45±0.77***	50.54±0.88***
Microwave-ultrasonic	3.55±0.74 (ns)	91.83±1.1***
Trolox	3.06±0.54 (reference)	100±1.1 (reference)

NS: Not statistically significant, SD: Standard deviation, IC_{50}: Half-maximal inhibitory concentration, ***: p value < 0.0001

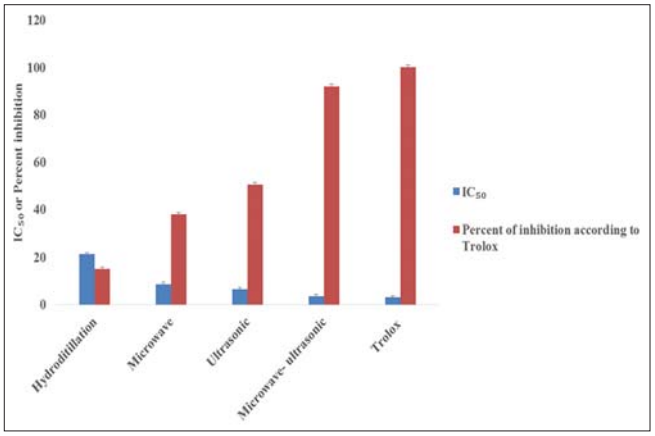

Figure 1: Half-maximal inhibitory concentration percentages of *Trichodesma africanum* volatile oils yields obtained by four different extraction methods and their antioxidant-inhibition activities according to Trolox standard

Antimicrobial Activity

Volatile oils extracted by the four methods used in this study exhibit bioactivity against the growth of all microbes examined in this study.

On comparing the MICs of the volatile oils extracted by the four different methods, with the exception of *C. albicans* [Table 3 and Figure 2], clearly showed that there was a gradual decrease in the values of the MICs of volatile oils extracted by hydrodistillation, microwave, ultrasonic and microwave-ultrasonic, respectively. This indicates that the concentration of substances that have potential inhibiting-activity against microbial growth gradually increases in the volatile oil extracts obtained by extraction methods mentioned above. In all cases, the highest antimicrobial activity (lowest MIC) against microbes examined in this study, was seen in the extract obtained by microwave-ultrasonic method, indicating that among the four methods used in this study for extraction of volatile oil, this method was the most efficient one.

DISCUSION

According to World Health Organization, about 80% of developing countries populations utilize herbal medicines for the treatment of many diseases. In addition, about 25% of modern pharmacopoeia includes medications that are either phytochemicals of medicinal plants or their semi-synthetic derivatives [23]. Extraction techniques play an essential role in food, cosmetic and pharmaceutical industries. It is estimated that these techniques require up to 50% of the financial investments of these industries. Moreover, about 70% of the used energy used in food industries, is consumed by extraction techniques [24]. Recently, there has been an increasing-interest in developing more efficient and environment-friendly extraction techniques. These new techniques that are being developed, must guarantee decreasing environmental pollution and technical hazards, be more efficient in terms of quantity and the quality of the extracts as well as minimizing the financial cost [25]. The several new extraction techniques have been described recently, among which are microwave, ultrasonic, and microwave-ultrasonic assisted methods [26]. These methods showed a positive effect on quality and yield of the obtained medicinal agents due to the mechanism involved during the extraction process. In fact, through shear forces created by ultrasonic cavitations and cell walls are broken mechanically which results in an easier transfer of the phytochemical materials especially volatile oils. In addition, there is no chemical relationship in the ultrasound-assisted extraction, which means no chemical degradation in the product of interest [27]. Moreover, the used ultrasonic waves allow better penetration of the solvent into the herbal matrix which increases the contact surface between solid and liquid phases [28]. Therefore, the selection of the appropriate extraction method will depend on the kind of herb species and also the nature of extracts [27].

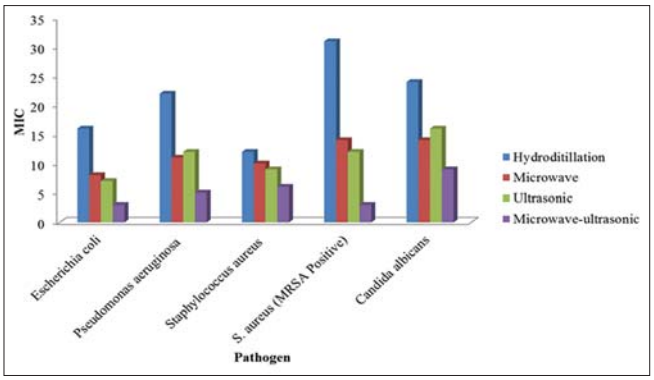

Figure 2: Minimum inhibitory concentrations of volatile oils extracted by hydrodistillation, ultrasonic, microwave and microwave-ultrasonic methods

The main aim of this study was to evaluate these new extraction techniques in terms of both the quantity and the quality *T. africanum* volatile oils and to compare them with same volatile oils obtained by conventional hydrodistillation method. The obtained data showed that ultrasonic-assisted extraction method was comparable to hydrodistillation extraction method in terms of the extraction time (about 120 min) needed for *T. africanum* volatile oil extraction. However, both microwave and microwave-ultrasonic extraction methods were remarkably more efficient in terms of the extraction times needed for *T. africanum* volatile oil extraction, which were 15 and 10 minutes, respectively. This indicates that volatile oil extraction by both microwave and microwave-assisted extractions methods require about 8-12% of the time needed for the extraction of the same oil by both ultrasonic-assisted and the hydrodistillation methods. The microwave and the microwave-ultrasonic methods gave about 4-6 times more than the amount of *T. africanum* volatile oils (% v/w), obtained by the hydrodistillation method and about 3-4 times more than the amount of volatile oils obtained by the ultrasonic-assisted methods.

Our data clearly indicated that both the microwave and the microwave-ultrasonic extractions methods were much more efficient than ultrasonic method as well as conventional

Table 3: MICs of *T. africanum* volatile oils obtained by different extraction methods

Extraction method	*E. coli* (ATCC 25922)	*P. aeruginosa* (ATCC 27853)	*S. aureus* (ATCC 25923)	MRSA	*C. albicans*
Hydrodistillation	16	22	12	31	24
Microwave	8	11	10	14	14
Ultrasonic	7	12	9	12	16
Microwave-ultrasonic	3	5	6	3	9

MICs: Minimum inhibitory concentrations, ATCC: American type culture collection, MRSA: Methicillin-resistant *Staphylococcus aureus*, *T. africanum*: *Trichodesma africanum*, *E. coli*: *Escherichia coli*, *P. aeruginosa*: *Pseudomonas aeruginos*, *S. aureus*: *Staphylococcus aureus*, *C. albicans*: *Candida albicans*

hydrodistillation extraction methods in terms of both of the extraction time and obtained yields of *T. africanum* volatile oils. Accordingly, it can be concluded that the microwave and the microwave-ultrasonic extractions methods are more efficient as well in terms of the consumed energy needed for the extraction process as well. In fact, our finding is in accordance with another similar study that has been conducted on the same plant where the extracted oils have been identified and analyzed using GC-MS techniques. According to this research, the major components of *T. africanum* volatile oils were caryophyllene oxide (15.6%), γ-eudesmol (13.7%), α-muurolene (10.5%), elemol (7.0%), carvone (6.8%), and β-caryophyllene (6.6%) while the minor component was α-pinene (0.1%) [16]. However, in a future study, this constituent will be extracted using different techniques and its quality and activity such as antioxidant and antimicrobial will be assessed. To evaluate the quality of the obtained *T. africanum* volatile oils by the four used extraction methods, their antioxidant activity as well as their antimicrobial activities were evaluated. Our results indicated that *T. africanum* volatile oils obtained by the microwave, the ultrasonic-assisted and the microwave-ultrasonic extraction methods, exhibited significant higher antioxidant activities in comparison to the antioxidant activity of same volatile oil obtained by the hydrodistillation extraction method. The antioxidant activities of the volatile oils obtained by the former mentioned three extraction methods were about 2.5, 3.3 and 6 times, respectively, higher than the antioxidant activity of same volatile oil obtained by the conventional hydrodistillation method. Antibacterial activity tests of the *T. africanum* volatile oils obtained by conventional hydrodistillation, microwave, ultrasonic-assisted and microwave ultrasonic, showed a gradual decrease, respectively, in their MICs against the growth of the tested bacterial pathogens. This gradual decrease in the MICs indicates that there is a gradual increase in the concentration of ingredients with potential antibacterial activity in these volatile oils. The lowest MICs against the growth of the tested bacterial pathogens were those of the volatile oil obtained the microwave-ultrasonic extraction methods, indicating that this extraction method was the best one in terms of the concentration of ingredients with potential antibacterial activity. Similar antimicrobial studies on this plant has been conducted by El-Ghazali *et al*. who tested the whole methanolic extract against *Proteus vulgaris*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, *S. aureus* bacteria and the results were 9.5 ± 0.5, 10.5 ± 0.5, 12.5 ± 0.5, 10.5 ± 0.5, and14.0 ± 0.0 respectively. However, these results cannot be compared with our methods since we worked on essential oils instead of the crude extract.

The antifungal activities of the obtained volatile oils indicated that the volatile oils obtained by microwave, ultrasonic-assisted, and microwave ultrasonic extraction methods showed a significantly lower MICs-thus higher antifungal activity than the same oil obtained by the conventional hydrodistillation method. The highest antifungal activity was exhibited by the volatile oil obtained by the microwave ultrasonic extraction method. Since both the highest antibacterial and antifungal activities (lowest MICs) were exhibited by the volatile oil obtained microwave-ultrasonic extraction method indicates that this extraction method is the best one among the used ones in this study and further investigations required to identify the changes in the constituents of these volatile oils which extracted by these four different methods. Although only one medicinal plant was included in this study (*T. africanum*), the obtained results are encouraging in terms of applying these recent extraction methods for volatile oil extraction from other medicinal plants.

CONCLUSION

Environment-friendly and recently-developed methods for the extraction of volatile oils from *T. africanum* were evaluated in this study in terms of their quantitative and qualitative efficiency in comparison to the conventional hydrodistillation method. Our results indicated that these recent extraction methods were significantly more efficient than the conventional hydrodistillation method in terms of the extraction time, the obtained yields as well as in terms of their antioxidant and antimicrobial activities. In addition, this implies that these recent extraction methods are more-likely to be more economical in terms of the consumed energy, water, and the financial cost needed for the extraction process. Among the four extraction methods that were evaluated in this study, the microwave-ultrasonic extraction method was the best one since volatile oil obtained by this method always gave the best one in terms of yield (1.8% v/w) and antioxidant activities (91.83% ± 1.1%) of inhibition, while the MICs for *E. coli*, *P. aeruginosa*, *S. aureus*, MRSA and *C. albicans* were 3, 5, 6, 3, and 9, respectively.

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Original Research

ScopeMed

Assessment of anti-inflammatory and anti-arthritic properties of *Acmella uliginosa* (Sw.) Cass. based on experiments in arthritic rat models and qualitative gas chromatography-mass spectrometry analyses

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ABSTRACT

Aim: The principle objective of the study was to explore the anti-arthritic properties of *Acmella uliginosa* (AU) (Sw.) Cass. flower in a rat model and to identify potential anti-inflammatory compounds derived from flower extracts. The synergistic role played by a combination of AU flower and *Aloe vera* (AV) gel crude extracts was also investigated. **Materials and Methods:** Male Wistar rats induced with Freund's complete adjuvant (FCA) were used as a disease model of arthritic paw swelling. There were three experimental and two control groups, each consisting of five rats. Paw circumference and serum biochemical parameters were evaluated to investigate the role of the flower extracts in disease amelioration through a feeding schedule spanning 21 days. Gas chromatography/mass spectrometry (GC/MS) analyses were performed to search for the presence of anti-inflammatory compounds in the ethanolic and n-hexane solvent extracts of the flower. **Results:** As a visual cue to the experimental outcomes, FCA-induced paw swelling decreased to the normal level; and hemoglobin, serum protein, and albumin levels were significantly increased in the treated animals. The creatinine level was estimated to be normal in the experimental rats after the treatment. The combination of AU and AV showed the best recovery potential in all the studied parameters, confirming the synergistic efficacy of the herbal formulation. GC/MS analyses revealed the presence of at least 5 anti-inflammatory compounds including 9-octadecenoic acid (Z)-, phenylmethyl ester, astaxanthin, à-N-Normethadol, fenretinide that have reported anti-inflammatory/anti-arthritic properties. **Conclusion:** Our findings indicated that the crude flower homogenate of AU contains potential anti-inflammatory compounds which could be used as an anti-inflammatory/anti-arthritic medication.

KEY WORDS: *Aloe vera*, Freund's complete adjuvant, rheumatoid arthritis

INTRODUCTION

Ethnic people have used herbal resources from ancient times to fulfill their nutritional and medicinal needs. A vast majority of the nutritional supplements and edible medicinal materials are either consumed as such from herbs or synthesized from herbal resources [1]. The interest of producing herbal formulations for medicinal purposes has developed due to their low side effects, low costing, and ready availability in countries such as India and China. In ethnobotanical knowledge, daily consumption of different plant parts and products are said to have disease-modifying and disease-improving activities. However, scientific explanations and working principles of such crude plant parts or herbal formulations are not well-established experimentally. *Acmella uliginosa* (AU) (Sw.) Cass. (Family Asteraceae) is a plant found in the Northern part of West Bengal (known as North Bengal) and has a worldwide distribution [Figure 1]. These herbs grow up to 1 m, generally creep or sometimes stand erect, rooting at nodes, and their stems are sub-glabrous to scabrid-pilose. It has been used as food by many human populations all through the world. The Malay people, as well as the Rajbanshi people from Northern part of West Bengal, consume the plant and its flowers for symptoms such as tooth ache, mouth ulcer, and mouth ache [2]. When consumed, the flower has a characteristic pungent taste which is soon followed by a characteristic tingling and numbness of the tongue. The antinociceptive activity, anti-

Figure 1: *Acmella uliginosa* flower and plant body in Medicinal Plant Garden of the University of North Bengal (Photograph courtesy: APD)

inflammatory, and immunomodulatory effects of this plant have been reported by few researchers [3-5]. Antimicrobial activity and antioxidant effect of the plant were also evaluated, and AU has been reported as an effective phytochemical-containing plant [6].

Rheumatoid arthritis (RA), which is a form of an auto-immune bone destructive disease, affects at least 1% of the population in the industrialized world with higher frequency in women. In severe cases of RA, the synovial inflammation leads to particular cartilage damage, bone erosion, and subsequent change in joint integrity. Usually, peripheral joints are involved [7,8]. Based on the results and observations made on the biological and analgesic activities of the plant, we assumed that the plant flower may have an anti-arthritic activity as well. To the best of our knowledge, such activities of AU have not been explored in appropriate model systems. Therefore, we have designed and performed animal model-based experiments to evaluate the anti-arthritic potential of the plant flower at the primary level and correlated the result with the gas chromatography/mass spectrometry (GC/MS) qualitative analyses.

MATERIALS AND METHODS

Preparation of Plant Extract

AU (Sw.) Cass. (Family Asteraceae) flowers were collected from the University Medicinal Garden during October-November, 2015. Because the flowers are chewed by the ethnic populations, the extract was prepared in a crude form, and no alterations were made in the unprocessed whole plant flower before monitoring its activity. Flowers were collected and washed thoroughly with water before the extract preparation. Flowers were then crushed and mixed with 3 ml of distilled water per gram of plant material thoroughly in a homogenizer. The mixture was kept for an hour at room temperature, and the process was again repeated. After homogenization, the extract was centrifuged at 3000 rpm and the supernatant was taken for experimentation. Every time the extracts were made freshly before the feeding of the animals. For GC/MS analysis, the flower was dried at room temperature for 6-7 days, crushed in a grinder, and the fine dust was collected in sterile capped Tarson tubes. It was followed by overnight stirring the powdered *Acmella* flower sample in appropriate solvents, i.e. ethanol and n-hexane, respectively. The supernatants were collected after 24 h and filtration was done using Whatman No 1 filter paper. The extracts were concentrated using nitrogen flow which facilitated enhanced diffusion of the solvent from the sample. This concentrated solution was used as the starting sample for the GC/MS analyses.

Animal Maintenance

Male Wistar rats, weighing 100-120 g, were used as experimental animals. Rats used for all the experiments were procured from an authorized animal dealer (Ghosh Enterprise, Kolkata, India). Animals were kept in the Departmental Animal House of the Department of Zoology, University of North Bengal with water *ad libitum* and standard pellet food was given. The room temperature was kept between 25°C and 30°C. All the experiments were approved by the Departmental Animal Ethical Committee.

Chemicals

Freund's complete adjuvant (FCA) was purchased from Sigma-Aldrich, USA; biochemical assay kit for total protein, albumin, and creatinine was procured from Coral Clinical Systems, India; HPLC grade chemicals (ethanol and n-hexane) for GC/MS were purchased from SD Fine Chem Ltd., Mumbai. All the other chemicals required were purchased from Himedia, Mumbai, India.

Anti-inflammatory Effects of AU

Lethal dose 50% (LD50) test for LD determination

LD50 test for AU flower extract was done as per the OECD guidelines. The doses considered for the tests were 140 and 280 μl of homogenate taken from a stock of 1 g/3 ml solution. These doses corresponded to 50 g flower/60 kg body weight and 100 g flower/60 kg body weight. Three rats were taken in each group to observe the lethality induced by the sample.

Curing potential of AU in FCA-induced arthritic condition

The animals were divided into five groups, each containing five rats. Adjuvant-induced arthritis model was produced as per the method described by Bendele *et al.* [9], and all animals were administered subcutaneously a dose of 0.1 ml of FCA in the left hind paw interplanetary region with the help of a sterile single-use insulin syringe, excluding the positive control group animals. The animals were administered a dose of 0.1 ml FCA again on the 14th day in the same region to boost up the immune response. The first two groups were considered as the control groups. Among them, one group was considered

as a non-treated or positive control that received neither FCA nor the plant extracts. The other group was considered as a negative control (NC), in which only FCA injection was administered, but no treatment regime was initiated. The third and fourth groups were considered as experimental groups. These groups were fed daily doses of aqueous extract of AU flower to the tune of 70 μl (AU1) and 140 μl (AU2), respectively, corresponding to 25 and 50 g flower/60 kg body weight, from a stock solution of 1 g/3 ml extract. The fifth group (AVAU) was fed 1:1 (w:w) doses of *Aloe vera* (AV) flower and AU gel together to investigate the combined effect of the extracts, as AV has proven role as an anti-inflammatory product [10]. The doses were calculated on the basis of the daily possible consumable amount of the flower by an average healthy person. The animals were sacrificed on the 21st day to study the biochemical parameters such as total protein, total albumin, and serum creatinine [11]. The paw circumferences were measured with the help of a vernier caliper at regular interval of 3/4 days from the 1st day of the experiment, using the formula $2\pi\sqrt{(A^2+B^2)}/2$, where A and B are the measures of the paw diameters at two different planes [12]. Total protein and total albumin were measured using kits from Coral Clinical Systems, following manufacturer's instructions. Hemoglobin from the rat blood was estimated using Sahli's hemoglobinometer. Serum creatinine was measured biochemically by studying reactions between creatinine and alkaline picrate.

Instrumentation and Chromatographic Conditions

The GC/MS analyses of the ethanolic and n-hexane extracts of *Acmella uligonosa* flower were performed in Thermo Scientific Trace 1300 GC equipped with ISQ MS and an AI/AS 1310 auto-sampler. The instrument had a TG 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter, and 0.25 μm film thickness. The column oven temperature was kept at 80°C, with a gradual increase in steps of 5°C/min to 305°C; injection temperature was set at 250°C at a pressure of 5 kPa, with total flow and column flow of 10 ml/min and 1 ml/min, respectively. The rate of purge flow was 3.0 ml/min. The GC program ion source and interface temperatures were 220°C and 305°C, respectively, with a solvent cut time of 5 min. The MS program starting time was 5 min which ended at 51.00 min with event time of 0.50 s and mass range 50-650. Injection volumes of each ethanolic and n-hexane soluble *Acmella* fractions were 1 μl (split ratio 10:1). The samples were repeatedly used to find the best result. The interpretation of GC/MS mass spectrum was done using the Xcalibur software version 2.0.1.3 with the help of the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0g, built May 19, 2011.

Statistical Analysis

All the statistical analyses were done using MS-Excel 2007 and Kyplot version 2.0 beta. In the Kyplot analysis, the data represented mean ± standard deviation which was analyzed by one-way ANOVA. The results were considered significant when $P > 0.05$.

RESULTS

Anti-inflammatory Effects of AU

LD_{50} test for LD determination

Experimental groups fed with AU flower homogenates showed no toxicity, no mortality, or behavioral changes during the period of 7-day after the commencement of feeding (data not shown). It can be concluded that doses up to 100 g flower/60 kg body weight were non-toxic to the body of a model animal.

Curing potential of AU in FCA-induced arthritis

Rat paw circumference was reduced significantly in the experimental groups that were fed with AU flower aqueous extract. Interestingly, the AVAU group, which was fed with a combination of *Aloe* and *Acmella* aqueous extracts, showed the better result [Figure 2 and Table 1]. All the treated groups showed an increased level of hemoglobin which is reported to decrease in arthritic conditions. The combined doses (*Aloe* + *Acmella*) of the extract were followed by a high dose of *Acmella* that showed the best result in increasing the blood hemoglobin levels [Figure 3a]. Estimation of total protein showed that there was a significant role of the plant extract to bring the altered

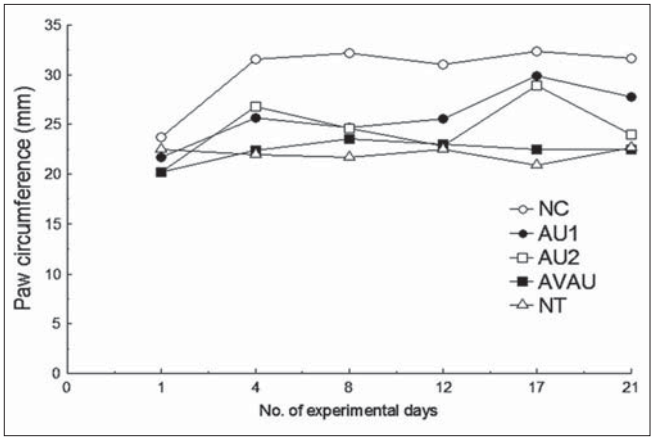

Figure 2: Change of paw circumference (mm) in different rat groups on different days after the initiation of treatment in arthritic rat models. Swelling is maximum in the diseased control (negative control [NC]) group, whereas combined dose of *Aloe vera* and *Acmella uliginosa* (AVAU) showed the best inhibition against arthritic paw swelling

Table 1: Mean paw circumference (mm) of rats of different experimental groups in different day intervals from the induction of arthritis

Groups	Day 1	Day 4	Day 8	Day 12	Day 17	Day 21
NC	23.74	31.56	32.17	31.01	32.31	31.62
AU1	21.64	25.65	24.64	25.51	29.85	27.75
AU2	20.16	26.82	24.62	22.80	28.87	23.97
AVAU	20.19	22.34	23.50	22.99	22.85	22.43
NT	22.57	22.46	22.24	22.56	22.14	21.99

NC: Negative control, AU1/AU2: *Acmella uliginosa* dose groups, AVAU: *Aloe vera-Acmella uliginosa* combination (1:1=w/w), NT: Non-treated

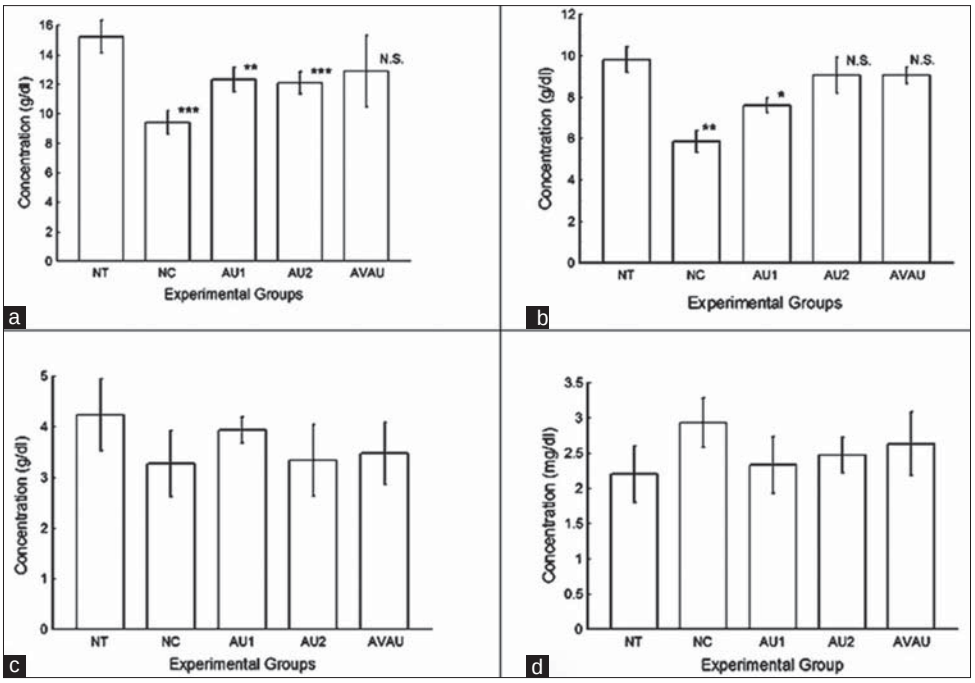

Figure 3: Measurement of different serum physiological parameters: (a) Hemoglobin in different rat groups: *Aloe vera-Acmella uliginosa* (AVAU) group showed the best efficacy in normalizing the altered (decreased) hemoglobin level of diseased animals, (b) estimation of total protein in different rat groups: AU2 and AVAU showed the best effect in the normalization of altered (lowered) total protein level, (c) estimation of total albumin level, negative control (NC) showing the most decreased value of albumin, AU1 shows the best restoration of altered albumin level, (d) serum creatinine values: AU1 followed by AU2 showed the best restoration property of serum creatinine compared to diseased condition

total protein levels toward normalcy. There has been a very significant elevation in the serum protein levels of experimental groups up to the normal levels. Both AU2 and AVAU groups showed normal protein levels after treatment, and there was no significant deviation from the normal value [Figure 3b]. Since total albumin level is reported to decrease with the arthritic severity, we measured total albumin level in all the experimental groups and have recorded an elevation of total albumin in all the experimental groups. The experimental groups showed 5.91%, 21.74%, and 17.07% decrease in the albumin levels in AU1, AU2, and AVAU groups, respectively, compared to 21.74% decrease in the disease control (NC) rats [Figure 3c]. Creatinine level generally increases in arthritic condition. In our experiment, it is clearly visible that AU flower extract can normalize the increased serum creatinine level. The NC group showed 31% increase in the creatinine level, whereas the experimental groups AU1, AU2, and AVAU showed only 9.09%, 13.63%, and 18.18% increase, respectively, in serum creatinine levels [Figure 3d]. This clearly indicates that the plant has a protective role against altered creatinine levels.

GC/MS Analyses

The GC/MS analyses of both ethanolic and n-hexane fractions of AU flower homogenate supports the presence of 96 compounds from the ethanolic fraction and 80 compounds from the n-hexane fraction, of which some anti-inflammatory compounds were identified through the literature search that related to the anti-arthritic properties of the plant. We have identified some key compounds which are already documented as potent inflammation inhibitors by other researchers. The principle anti-inflammatory and/or anti-arthritic compounds from the plant are listed in Table 2, and the structures of these compounds are depicted in Figure 4.

DISCUSSION

The FCA-induced arthritis model in rats is the most commonly used method of simulating a human disease condition. FCA-induced arthritis has been used as a model of chronic inflammation in rats and of considerable relevance for the study of pathophysiology and pharmacological control of inflammatory processes [17]. In this study, a dramatic cessation of rat paw edema was indicated in the treated experimental groups of animals from the 1st week of study, similar to others' findings [17-19]. Paw swelling is a visual cue to the inflammation, and hence, it can be concluded that AU flower aqueous crude extract can significantly reduce paw swelling in FCA-induced arthritic rats when fed orally. As seen in other studies, total protein and hemoglobin generally decrease in arthritic conditions, which is similar to our results. In our study, we also found that experimental group treated with aqueous extracts of AV and AU, combined in equal proportion showed the best result in increasing serum protein and blood hemoglobin levels, which is followed by a similar effective activity of the higher doses of *Acmella* [Figure 3a and b] [17]. The total albumin level also decreased along with the arthritic severity [18]. We estimated total albumin levels in all experimental groups and recorded elevations of total albumin in all experimental groups except that in the NC group. Creatinine level generally increases

Table 2: List of phytocompounds with anti-inflammatory activities identified from *A. uliginosa* with details; references confirming their anti-inflammatory potentials as pure or conjugated compounds

Peak RT	Peak area	Peak area (%)	Compound name	SI	RSI	Molecular formula	Molecular weight	*P*	CAS	References
n-Hexane fraction										
5.46	88,207.11	0.09	9-Octadecenoic acid (Z)-, phenylmethyl ester	605	735	$C_{25}H_{40}O_2$	372	32.51	55130-16-0	[13]
34.20	153,335.35	0.16	à-N-Normethadol	352	456	$C_{20}H_{27}NO$	297	4.15	38455-85-5	[14]
40.09	267,475.99	0.28	Astaxanthin	460	520	$C_{40}H_{52}O_4$	596	51.05	472-61-7	[15]
Ethanol fraction										
19.14	1,222,149.73	1.38	Caryophyllene oxide	669	793	$C_{15}H_{24}O$	220	15.04	1139-30-6	[16]
19.46	85,133.22	0.10	Fenretinide	355	434	$C_{26}H_{33}NO_2$	391	7.15	65646-68-6	[17]
20.74	161,146.11	0.18	Astaxanthin	460	520	$C_{40}H_{52}O_4$	596	51.05	472-61-7	[15]

RT: Retention time, SI: Strength index, RSI: Relative strength index, CAS: Chemical abstracts service number, P: Probability, *A. uliginosa: Acmella uliginosa*

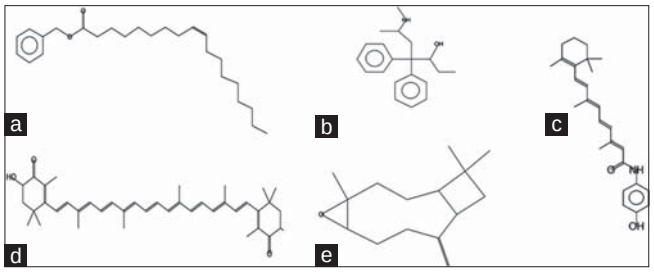

Figure 4: Structures of identified anti-inflammatory compounds of *Acmella uliginosa*, (a) 9-Octadecenoic acid (Z)-, phenylmethyl ester, (b) à-N-Normethadol, (c) astaxanthin, (d) caryophyllene oxide, (e) fenretinide

in arthritic condition as mentioned in previous studies [18]. In our experiment, we also found similar results.

AU is reported to have local anesthetizing properties often used by ethnic people as a pain-relieving agent. Considering the studies of different other workers on different other potent anti-arthritic plants such as *Nyctanthes arbor-tristis* [12] and *Aristolochia bracteata* [19], it can be said that AU flower possesses promising arthritis inhibitory active principles which can significantly bring altered parameters toward normalcy in disease models. Moreover, the combined effect of AU flower extract and AV gel in disease amelioration suggested a synergistic role of the plant extracts. Low hemoglobin level clearly states that during arthritis, the bone marrow loses its normal functioning property and low red blood cell (RBC) count results in low hemoglobin level. The rise in hemoglobin levels in experimental groups indicates the increased RBC concentration and bone marrow health. A rise in total protein and albumin in experimental groups clearly demonstrates the restoration of metabolic imbalances during arthritic condition. Creatinine formation in serum was decreased in the experimental groups that confirmed the stability in the physiology of metabolism [Figure 3d]. A similar study on *Strychnos potatorum* Linn. seeds in FCA-induced arthritic rat models by Ekambaram *et al.* [18] also showed similar changes in the above said parameters.

The animal experimental data were supported by the GC/MS analyses of the plant flower extracts. A good number of fatty acids, steroidal, and other products were profiled including different anti-inflammatory compounds. Ayurveda is a system which establishes the synergistic role in an herbal formulation of more than one plant for a better result against complex diseases. The synergistic role of these compounds may offer an amelioration of the disease condition. The group AVAU, which contains AV and AU in equal proportion showed the best inhibitory role against arthritis generatiozn and progression in experimental rat models that suggested a possible synergistic role of these plant-derived drugs. This work thus also established the efficacy of synergistic activity of the herbal formulations.

CONCLUSION

In this study, the role of AU flower homogenate in amelioration of arthritic paw swelling in rat models, as well as assessment of some biochemical parameters was established. All the experimental outcomes confirmed that this plant possessed significant curative properties against arthritis. Taking into consideration that different parameters are affected by a network of different complex biochemical pathways and need a different period to settle down, the preliminary 21 day long study promises that effective dose determination and further study would establish AU as a potent arthritis inhibitor. This study further confirmed the effect of crude AV gel in inflammatory conditions. The potential therapeutic values of the unprocessed crude flower homogenate of AU, as a remedy for inflammation as well as arthritic condition, were well documented in this work. The efficacy of the combined crude flower extracts of AU and AV gel further suggested of a possible synergistic activity leading to better remediation of disease conditions in the animal model.

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Original Research

Cryptolepine, an indoloquinoline alkaloid, in the management of diabetes mellitus and its associated complications

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ABSTRACT

Background: Effective long-term management is the key to treatment of diabetes mellitus (DM) and its complications. **Aim:** To ascertain the ability of cryptolepine (CRP) in managing DM and some associated complications. **Materials and Methods:** Changes in fasting blood sugar (FBS), body weight, response to thermally-induced pain, and semen quality were assessed in normal and alloxan-induced diabetic rats treated with CRP (10, 30, or 100 mg/kg), glibenclamide (10 mg/kg), or normal saline (2 ml/kg) *per os*. Hematological profile, liver and kidney function tests, lipid profile, as well as liver, kidney, and pancreas histopathological examinations were also conducted to establish possible effects of CRP treatment. **Results:** CRP treatment reduced ($P \leq 0.001$) FBS and body weight, inhibited ($P \leq 0.05$ - 0.001) the latency to tail flick or withdrawal from pain stimulus. It did not alter ($P > 0.05$): Hematological parameters, elevated ($P \leq 0.05$ - 0.001) plasma aspartate transaminase, alanine transaminase, and gamma-glutamyl transferase, reduced ($P \leq 0.01$) plasma urea, and elevated ($P \leq 0.001$) plasma creatinine associated with DM. CRP, however, reversed ($P \leq 0.05$ - 0.001) DM-associated elevation ($P \leq 0.05$ - 0.001) of plasma cholesterol, triglycerides, and low-density lipoproteins, and the reduction in high-density lipoproteins. CRP (10-30 mg/kg) showed dose-dependent regeneration of β-islet cells but could not repair degenerated liver and kidney tissue. CRP worsens dose-dependently ($P \leq 0.001$) reduced sperm quality associated with DM. **Conclusion:** CRP abolishes hyperglycemia, weight loss, cold allodynia, neuropathic pain, and hyperlipidemia as well as pancreatic β-islet cell damage associated with DM. It, however, does not improve liver and kidney damage and lowered semen quality.

KEY WORDS: Cold allodynia, cryptolepine, diabetes mellitus complications, glibenclamide, paw withdrawal, tail flick test

INTRODUCTION

Diabetes mellitus (DM) is a pathologic condition characterized by abnormally high blood glucose resulting from insufficient levels of insulin and/or insulin resistance [1]. This metabolic disorder results in the generation of reactive oxygen species (ROS), which cause oxidative damage, specifically in the visceral organs such as the heart, kidney, and liver [2]. DM is really a global health issue with about 285 million people having diabetes globally as of 2010; Type 2 making up about 90% of

the cases [3]. According to International Diabetes Federation, in 2013, an estimated 381 million people had DM; considering this prevalence, the number of DM individuals is estimated to almost double by 2030 with the greatest increase in prevalence expected in Asia and Africa [4]. DM is associated with several complications which result in damage to the eyes, kidneys, and nerves. Ocular complications, usually in the form of cataract and retinopathy (diabetic retinopathy), are the leading cause of visual impairment and blindness. Damage to the kidneys (diabetic nephropathy) could eventually lead to chronic kidney disease, whereas damage to the nerves of the body (diabetic neuropathy, DN), the most common complication of diabetes, manifests with symptoms such as numbness, tingling, pain, and altered pain sensation. In addition, proximal DN causes painful muscle wasting and weakness [5]. Other long-term complications of DM are related to damage of blood vessels, which is very much related to the impairment of fatty acid synthase and nitric oxide synthase that interact in the cells that line blood vessel walls [6]. Most of this excess risk is associated with an augmented prevalence of well-known risk factors such as hypertension, hyperlipidemia, obesity, and cardiovascular disease [7] as the majority (about 75%) of deaths in diabetics is due to coronary artery disease [8]. Other macrovascular diseases are stroke and peripheral vascular disease. The immunological and gastrointestinal systems are also not spared. Recent studies indicate that ROS plays a key intermediate role in the pathophysiology of DN [9]. Hyperglycemia, the main determinant of the initiation and progression of DN, not only generates more reactive oxygen metabolites but also attenuates antioxidative mechanisms through non-enzymatic glycosylation of antioxidant enzymes [1].

Cryptolepine (CRP), a naturally occurring indoloquinoline alkaloid (isolated from the roots of *Cryptolepis sanguinolenta* Family: Periplocaceae), is an antimalarial drug in Central and Western Africa [10]. It has, however, been perceived to be useful in the traditional management of DM. The aim of this study, therefore, was to ascertain the therapeutic usefulness of CRP in managing DM and some of its associated complication which includes weight loss, neuropathy, liver, kidney, and pancreatic tissue damage as well as decreased spermatocyte motility and viability.

MATERIALS AND METHODS

Experimental Animals

Laboratory-bred Sprague-Dawley rats (250-300 g; 8-10 weeks old) obtained from animal house of the Department of Biomedical and Forensic Sciences, School of Biological Sciences, University of Cape Coast, Ghana, were used in this study. The rats were kept under ambient conditions of temperature (23-26°C), relative humidity (60-70%), and 12 h light/dark cycle. Preceding the experimental session, water and rat chow were available *ad libitum*. All animals used were handled in accordance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals and were approved by the Departmental Ethics Committee.

Drugs and Chemicals Used

Alloxan monohydrate (BDH Chemicals, England) was used to induce diabetes. Glibenclamide (Denk Pharma GmbH & Co. KG., Germany) was the selected reference hypoglycemic agent.

Induction of DM

Healthy Sprague-Dawley rats weighing 170-200 g (fed for 14 h with 5% glucose solution to prevent hypoglycemic shock) were injected intraperitoneally with 140 mg/kg alloxan monohydrate in distilled water [11] after their fasting blood sugar (FBS) were ascertained by the enzymatic glucose oxidase method using a commercial OneTouch Select™ blood glucose meter (LifeScan Inc, Milpitas, CA, China). Drops of blood for the test were obtained from the tail vein of the rats. 5 days after, rats with FBS above 15.0 mmol/L were considered diabetic and were selected for this study.

Antihyperglycemic Activity of CRP

Diabetic rats were grouped into five ($n = 5$). Group 1, diabetic (negative) control, rats were treated with 2 ml/kg physiological saline. Group 2 rats were treated with 10 mg/kg glibenclamide. Group 3-5 received 10, 30, and 100 mg/kg, respectively, CRP *per os*. Group 6, the sham control had rats, in which diabetes was not induced but was treated with vehicle (i.e., 2 ml/kg normal saline) and kept under the same experimental conditions as all the other groups. Treatments started from day 5 post-induction. FBS was measured before induction of diabetes (day 0), and on day 5, 7, 10, and 13 post-induction, 1 h after drug treatment.

Effect of CRP Treatment on Body Weight

Rats were weighed initially before induction of diabetes and grouped after induction of diabetes as described above. The weights of rats in all groups were monitored individually on days 5, 7, 9, 11, 13, and 15 post-induction using a Sartorius top loading balance (CPA3202S, Goettingen, Germany)

CRP in Managing Neuropathic Pain in DM

Cold allodynia [12] and Tail flick [13] tests were thermally-induced pain models used to assess neuropathic pain in the diabetic rats.

Cold allodynia

Diabetic rats were put into five groups, A-E ($n = 5$). Group A, the control, was treated orally with normal saline (2 ml/kg). Groups B, C, and D were treated orally with CRP at doses of 10, 30, and 100 mg/kg, respectively, whereas Group E was treated orally with 10 mg/kg Morphine. Group F, the sham control, were normal rats, which were treated with 2 ml/kg normal saline but were subjected to the same experimental conditions as the diabetic rats. The temperature of cold water was set at 4°C and allowed to stabilize for 5 min (temperature of testing room 21 ± 1°C). Each hind paw of the animals was then placed into the cold water and the time (in seconds) taken for the first brisk lift paw to occur was recorded; there was alternation between

right and left hind paws to prevent adaptation. The time to the brisk response is interpreted as the latency for cold pain withdrawal. Given that temperatures of 15°C and above are considered innocuous (based on human psychophysical data) and those rats explore their environment, 20 s was chosen as the upper time limit for a cold "pain" response. Any response with a latency >20 s was considered nonpainful [14]. A maximum cut-off time of 20 s was used to prevent tissue damage at the lower temperatures. Each rat was tested only once on any given test day to avoid any possible anesthetic or tissue damage effects that could be produced by repeated exposure to a cold environment. Test was done on day 0 (before induction of diabetes) as well as on day 5, 7, 9, 11, and 13 post-induction, 1 h after drug treatment.

Tail flick method

The tail flick test is a test of acute nociception, in which a moderately high-intensity thermal stimulus is directed to the tail of the rat. The time from onset of stimulation to a rapid flick/withdrawal of the tail from heat source is recorded. This experiment was done using hot water in a bath where temperature of the water was raised and maintained at 55°C. Diabetic rats were put into five groups, A-E ($n = 5$). Group A, the control, was treated orally with normal saline (2 ml/kg). Groups B, C, and D were treated orally with CRP at doses of 10, 30, and 100 mg/kg, respectively, whereas Group E was treated orally with 10 mg/kg Morphine. Group 6 was an sham control. The tail was immersed into the hot water 5 cm from the tip with the rats held vertically above the water bath. The cutoff time for the test was set to 10 s after which the subjects were withdrawn to prevent tissue damage. The time (in seconds) from the dipping of tail into the hot water and rapid flick/withdrawal from hot water (tail flicking latency) was recorded. Each animal was tested once at each day of pain assessment, to avoid voluntary adaptation to hot temperatures and also tissue damage. The test was conducted on day 0 (before induction of diabetes) and repeated on days 5, 7, 9, 11, 13, and 15 post-diabetic.

Collection of Blood Samples

By cardiac puncture [12] technique, samples of blood from animals from CRP treatment groups as well as the positive, negative, and sham control groups were collected at the end of treatment period and transferred into separate MediPlus vacutainer K3 EDTA tubes (Sunphoria Co. Ltd., Taiwan) for hematological studies or into serum separator tubes (SST) which containing silica particles and a serum separating gel (BD Vacutainer® Blood Collection Tube Product, USA) for chemical pathology studies.

Hematological Profile

Full blood count on samples was done using a hemato-analyzer (Sysmex XP - 300™ Automated Hematology Analyzer, USA).

Liver and Kidney Function Tests and Lipid Profile

Blood samples transferred into SSTs were allowed to clot and centrifuged at 3000 rpm for 10 min. The serum separated thereafter was collected into 5 ml sterile glass tubes for liver and kidney function tests as well as lipid profile using a clinical chemistry analyzer (Vital Scientific N. V, Netherlands) at Kasoa Government Hospital, in the Central Region of Ghana.

Histopathological Assessment

The pancreas, liver, and kidneys from treated and control animals were collected after sacrificing and dissection, re-sectioned and fixed in 10% phosphate-buffered formalin. The fixed organs were then processed using standard techniques [15]. Each of the organs was treated at all levels of tissue processing at Komfo Anokye Teaching Hospital in Kumasi, Ghana, and obtained slides observed under an Olympus trinocular light microscope (Jenoptik, Germany) at ×100 magnification power. Images were captured by a microscope camera with LC Micro software (Olympus Soft Imaging Solutions GmbH, Germany) connected to the third eyepiece of the microscope.

Effect of CRP Treatment on Semen Quality

Spermatocyte motility was determined according to the method described by Zemjanis [16]. Briefly, a drop of semen was collected from the caudal epididymis for the CRP -treated and the controls onto a glass slide. Sodium citrate buffer (2.9%) was then added to the semen and mixed until the desired dilution was obtained. Percentage motility was then evaluated microscopically within 2-4 min of semen collection. The total spermatozoa in the caudal epididymis sperm sample were counted from five large squares (volume: 0.5 mm^3) using the improved Neubauer hemocytometer (depth 0.1 mm, area: 1/400 mm^2; Yancheng Cordial Lab Glassware Co. Ltd., Jiangsu, China (Mainland). The sperm morphological abnormalities and percentage live/dead ratio was assessed using the method described by Wells and Awa [17]. These were obtained from a total count of 400 spermatozoa in smears obtained with Wells and Awa stains (0.2 g of Eosin and 0.6 g of fast green dissolved water and ethanol in ratio 2:1). Live/dead ratio was determined using 1% eosin and 5% nigrosin in 3% sodium citrate dehydration solution.

Statistical Analysis

GraphPad Prism Version 5 was used for data analyses. Data are presented as mean ± standard error of mean. Differences in data for sham, treatments, and the negative control (NC) were analyzed using one-way ANOVA. Further, comparisons were performed using Dunnett's *post-hoc* test. $P \leq 0.05$ was considered significant.

RESULTS

Effects of CRP on DM Hyperglycemia

Hyperglycemia was observed by day 3 post-induction with mean FBS of 4.56 ± 0.22 mmol/L elevating to 20.34 ± 1.29 mmol/L in diabetic rats. Treatment with CRP (10, 30, and 100 mg/kg)

and glibenclamide (reference hypoglycemic drug) induced a significant reduction ($P \leq 0.001$) in FBS to between 3.0 and 4.2 mmol/L. There were no significant changes in blood glucose levels in the sham control group [Figure 1].

Effects of DM and CRP Treatment on Body Weight

With the induction of DM, there was a significant reduction ($P \leq 0.001$) in body weight, i.e., 188.8 ± 2.7 g in normal rats to 152.9 ± 3.4 g in diabetic rats. Treatment with CRP (10 and 30 mg/kg) and glibenclamide caused significant increments ($P \leq 0.05$) in the weight of the diabetic animals. There was, however, a consistent decrease in the weights of the diabetic but untreated (NC) group and the 100 mg/kg CRP treatment group [Figure 2].

Effect of CRP on DM Neuropathic Pain

Cold allodynia

Cold allodynia reduced significantly ($P < 0.001$) between normal and diabetes, i.e., from 11.41 ± 0.53 s to 6.68 ± 0.60 s. The 100 mg/kg CRP dose produced a significant ($P \leq 0.05$) inhibition of cold allodynia in the diabetic rats; similar to morphine ($P \leq 0.01$), the reference analgesic [Figure 3].

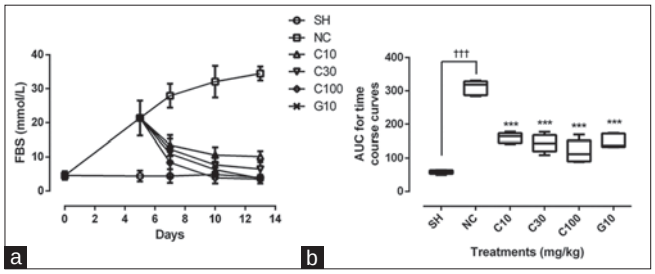

Figure 1: Cryptolepine (C) (10, 30, and 100 mg/kg) and glibenclamide (G) (10 mg/kg) treatments on, (a) The time course of fasting blood sugar and, (b) The area under the time-course curves (AUC) in alloxan-induced DM. values plotted are means±standard error of mean. †††$P \leq 0.01$ represents the significant difference between the normal and the diabetic state, ***$P \leq 0.001$; comparing drug treatments to the negative control (NC) (One-way ANOVA followed by Dunnett's *post-hoc* test)

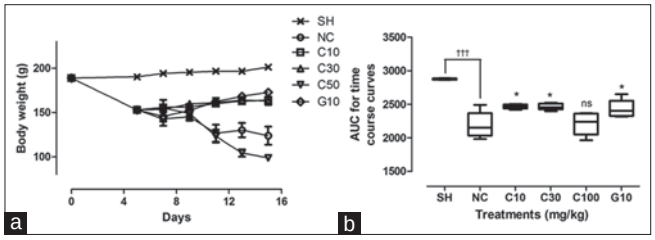

Figure 2: Cryptolepine (C) (10, 30, and 100 mg/kg) and glibenclamide (G) (10 mg/kg) treatments on, (a) The time course of cold allodynia and, (b) The area under the time-course curve (AUC), in alloxan-induced DM. Values plotted are means±standard error of mean, n=5. †††$P \leq 0.01$ represents the significant difference between the sham control (SH) and the diabetic state, *$P \leq 0.05$, ns P>0.05 comparing drug treatments to the negative control (NC) (One-way ANOVA followed by Dunnett's *post-hoc* test)

Tail flick test

Latency period to pain reduced significantly ($P \leq 0.001$) between normal and diabetic rats (i.e., from 3.52 ± 0.095 to 2.192 ± 0.093 s). Treatment with CRP (30 and 100 mg/kg) increased the latency to tail flicking (withdrawal) from hot water significantly ($P \leq 0.01$); an effect similar to morphine ($P \leq 0.01$), the reference analgesic [Figure 4].

Effect of CRP on Hematological Parameters in DM

Except platelet count which decreased significantly ($P \leq 0.001$) with DM, there were no significant ($P > 0.05$) decrements in hematological parameters associated with DM in rats. CRP and glibenclamide treatments did to affect hematological parameters significantly [Table 1].

Liver Function Test

Although there were no significant ($P > 0.05$) changes in plasma levels of alkaline phosphatase, total protein (PROT), albumin (ALB), globulin (GLOB), and total bilirubin (TBIL) associated with DM, and treatment of DM with glibenclamide and CRP, the aspartate transaminase (AST), alanine transaminase (ALT), and gamma-glutamyl transferase (GGT) levels were significantly elevated ($P \leq 0.05$ - 0.001) with DM. Treatment with glibenclamide

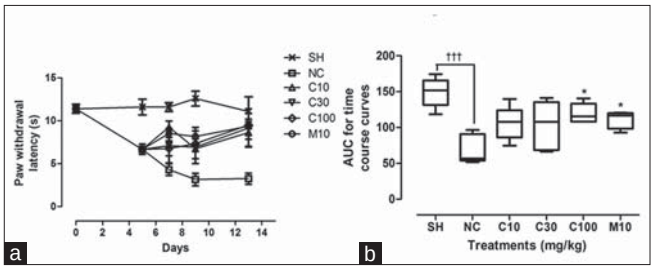

Figure 3: Cryptolepine (C) (10, 30, and 100 mg/kg) and morphine(M) (10 mg/kg) treatments on, (a) the time course of cold allodynia and, (b) area under the time course curves (AUC) in alloxan-induced DM. Values plotted are means±standard error of mean. †††$P \leq 0.01$ represents the significant difference between the sham control (SH) and the diabetic state; ns P>0.05, *$P \leq 0.05$; comparing treatments to the negative control (NC) (One-way ANOVA followed by Dunnett's *post-hoc* test)

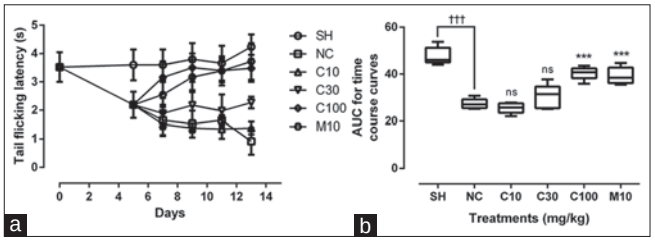

Figure 4: Cryptolepine (C) (10, 30, and 100 mg/kg) and morphine (M) (10 mg/kg) on, (a) the time course of neuropathic pain and, (b) Area under the treatment time course curves (AUC) in alloxan-induced DM. Values plotted are means±standard error of mean, (n=5). †††$P \leq 0.01$ represents the significant difference between sham control (SH) and the diabetic state; ns P>0.05, ***$P \leq 0.05$; comparing treatments to the negative control (NC) (One-way ANOVA followed by Dunnett's *post-hoc* test)

resulted in a significant ($P \leq 0.001$) reversal of these elevated parameters. CRP treatment, however, did not have any significant ($P > 0.05$) effect on the elevated parameters. In fact, AST was further elevated ($P < 0.01$) with CRP treatment [Table 2].

Kidney Function Test

Associated with DM was a significant reduction ($P \leq 0.01$) in plasma urea and a significant elevation ($P \leq 0.001$) in creatinine. Glibenclamide treatment significantly reversed ($P \leq 0.001$) these changes, however, CRP treatment could not alter significantly ($P > 0.05$) these changes [Table 2].

Lipid Profile

DM was also associated with a distorted lipid profile as there was significant elevation ($P \leq 0.05$ - 0.001) of cholesterol (CHO), triglycerides (TAG), and low-density lipoproteins (LDL) and a significant reduction in high-density lipoproteins (HDL). Treatment with glibenclamide and CRP significantly reversed ($P \leq 0.05$ - 0.001) the DM-associated distortion in lipid profile. Very LDL (VLDL) levels were, however, not significantly affected [Table 2].

Effect of CRP on Histopathology in DM

Pancreas

The normal control group showed normal histology of the islets of Langerhans and the exocrine acinar cells [Figure 5a]. Induction of DM resulted in necrosis of the pancreatic islets of Langerhans cells. Both cells also display degenerative changes within their nuclei [Figure 5b]. Glibenclamide treatment appeared to have normal islet cells [Figure 5c]. CRP treatment (10 and 30 mg/kg) showed a dose-dependent regeneration of islet cells [Figure 5d and e]. CRP (100 mg/kg) shows reduction

Table 1: Hematological parameters in alloxan-induced diabetic rats and that following a 15 days treatment with 10, 30, and 100 mg/kg cryptolepine or 10 mg/kg glibenclamide

Parameters	SH	NC	G10	C10	C30	C100
RBC ($\times 10^6/\mu L$)	6.8±0.7	6.0±0.9 ns	6.3±1.6 ns	7.7±0.6 ns	6.6±1.30 ns	6.5±1.04 ns
HCT (%)	0.5±0.1	0.4±0.1 ns	0.5±0.1 ns	0.6±0.2 ns	0.5±0.1 ns	0.4±0.3 ns
Hb (%)	13.9±1.2	12.3±1.6 ns	12.3±2.7 ns	14.4±0.9 ns	13.9±1.9 ns	12.1±1.3 ns
MCV (fL)	75.6±0.8	71.3±0.4 ns	81.3±4.1 ns	81.4±3.1 ns	83.8±3.7 ns	80.2±2.8 ns
MCH (Pg)	20.4±1.4	20.5±0.8 ns	19.5±0.9 ns	18.7±1.6 ns	21.1±1.4 ns	18.6±1.5 ns
MCHC (g/dl)	27.0±1.9	28.8±1.1 ns	24±2.3 ns	23.0±2.7 ns	25.2±1.6 ns	23.2±1.6 ns
WBC ($\times 10^3/\mu L$)	4.3±2.4	2.5±1.3 ns	3.8±0.2 ns	4.4±1.2 ns	4.3±0.29 ns	3.6±1.40ns
PLT ($\times 10^3/\mu L$)	869±50	460±43***	457±14***, ns	556±31***, ns	486±16***, ns	362±52***, ns

Values are means±standard error of mean; (n=5). Significant difference between sham control (SH) and other treatment groups: ns implies P>0.05, ***Implies $P \leq 0.01$ (One-way ANOVA followed by Dunnett's *post-hoc* test). Significant difference between negative control (NC) and other treatment groups ns implies $P > 0.05$ RBC: Red blood cell, HCT: Hematocrit, HB: Hemoglobin, MCV: Mean corpuscular volume, WBC: White blood cell count, PLT: Platelet count, MCH: Mean cell hemoglobin, MCHC: Mean cell hemoglobin concentration

Table 2: Blood biochemistry values of liver and kidney function tests and the lipid profile for normal and alloxan-induced diabetic rats and that following a 15 days treatment of diabetic rats with 10 mg/kg glibenclamide (G) or 10, 30, and 100 mg/kg cryptolepine (C)

Parameters	SH	NC	G10	C10	C30	C100
Liver function test						
AST	42.5±3.5†	57.0±2.8*	47.5±2.8 ns	73.5±2.5***,†	79.0±1.5***,†††	81.0±6.0***,†††
ALT	34.25±3.0†††	89.2±6.7***	38.5±2.7 ns,†††	82.5±5.8***, ns	85.0±3.0***, ns	86.0±8.6***, ns
GGT	17.7±1.0†††	45.3±1.6***	25.2±2.0 *,††	44.0±6.0***, ns	50.0±2.2***, ns	46.5±1.5***, ns
ALP	19.0±8.0	17.0±4.5 ns	16.0±5.0 ns	9.0±6.0 ns	12.5±6.5 ns	15.0±2.0 ns
PROT	71.5±4.5	64.0±4.3 ns	69.4±5.8 ns	65.5±1.5 ns	66.0±3.6 ns	70.0±2.7 ns
ALB	41.0±2.0	39.0±0.5 ns	39.6±0.8 ns	42.5±2.5 ns	40.0±2.0 ns	39.5±0.5 ns
GLOB	30.5±2.5	25.0±1.4 ns	29.8±1.2 ns	27.0±1.0 ns	26.0±1.0 ns	30.5±1.5 ns
TBIL	6.9±1.0	5.5±0.9 ns	7.2±1.1 ns	10.2±1.9 ns	11.2±1.4 ns	11.9±1.4 ns
Kidney function test						
Urea	7.5±0.3†††	3.8±0.3**	6.8±0.4 ns,††	3.1±0.5***, ns	3.2±0.3***, ns	3.0±1.0***, ns
CREA	1.4±0.3†††	2.7±0.1***	1.5±0.2 ns,†††	2.5±0.2**, ns	2.1±0.1*, ns	2.4±0.1**, ns
Lipid profile						
CHO	91.5±2.5†	117.5±4.7*	89.5±2.5 ns,††	108.5±3.9*, ns	123.5±6.8**, ns	90.0±9.0 ns,†
TGC	25.2±2.8†††	57.0±1.4***	30.4±2.3 ns†††	29.0±0.9 ns,†††	32.5±1.4 ns,†††	38.5±2.5*,†††
HDL	130±6.0†††	23.0±7.0***	135±8.0 ns,†††	75.0±2.2***,†††	85±6.5***,†††	94.5±4.5***,†††
LDL	25.0±4.0†††	65.0±5.6***	20.2±2.2 ns,†††	34.0±2.8 ns,†††	42.5±2.5 **,†††	45±3.5**,††
VLDL	10.9±2.7	11.0±1.5 ns	12.5±2.3 ns	6.0±2.0 ns	6.8±2.5 ns	7.5±1.5 ns

Values are as means±standard error of mean; (n=5). For significant difference between sham control (SH) and other groups: ns implies $P > 0.05$, *Implies $P \leq 0.05$, **Implies $P \leq 0.01$, ***Implies $P \leq 0.001$. For significant difference between negative control (NC) and other groups: NS implies $P > 0.05$, †Implies $P \leq 0.05$, ††Implies $P \leq 0.01$, †††Implies $P \leq 0.001$ (One-way ANOVA followed by Dunnett's *post-hoc* test). AST: Aspartate transaminase, ALT: Alanine transaminase, GGT: Gamma glutamyl transferase, ALP: Alkaline phosphatase, PROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, CREA: Creatinine, CHO: Cholesterol, TGC: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

of cells in the middle portion of the islets of Langerhans, which shows a sign of no or little regeneration, with degenerated and disorganized acinar cells [Figure 5f].

Liver

The normal histology of the liver has polygonal, tightly parked hepatocytes, containing basophilic central rounded nuclei separated by the hepatic sinusoids radiating from the central vein (CV) and with the presence of non-activated spindle-shaped Kupffer cells within the sinusoids [Figure 6a]. Induction of DM resulted in tissue appearing edematous, with the presence of activated Kupffer cells with some highly basophilic nuclei of some hepatocytes [Figure 6b]. Glibenclamide-treated diabetic animals appears to have normal liver histology with a very few highly basophilic hepatocytes [Figure 6c]. The 10 mg/kg CRP treatment showed disorganized hepatocytes with distorted margins of the CV. Some hepatocytes appeared degenerated with eosinophilic cytoplasm and little infiltration of mononuclear cells (Figure 6d). In the 30 mg/kg CRP-treated group, the histology showed mononuclear cells within sinusoids with normal nuclei in hepatocyte, and well-defined margins of the CV. The architecture of the hepatocyte appears normal (Figure 6e). There was, however, a loss of hepatocyte architecture in the 100 mg/kg CRP-treated group. Liver appears edematous with infiltration of lymphocytes within sinusoids but with well-defined CV. There is also demonstration of activation of Kupffer cells and the presence of few degenerative nuclei (necrosis) of the hepatocytes at the pyknotic stage (Figure 6f).

Kidney

The histology the kidney showed tubules and renal corpuscles, with well-defined capsular space (Figure 7a). The NC however had no capsular space around the glomerulus, distorted renal corpuscles, and localized coagulative necrosis of the tubules around the corpuscles [Figure 7b]. This was also seen in the 10 mg/kg CRP treated group [Figure 7d]. With glibenclamide treatment, there is capsular space (but not well-defined) and normal renal corpuscles, with little distortion in the renal

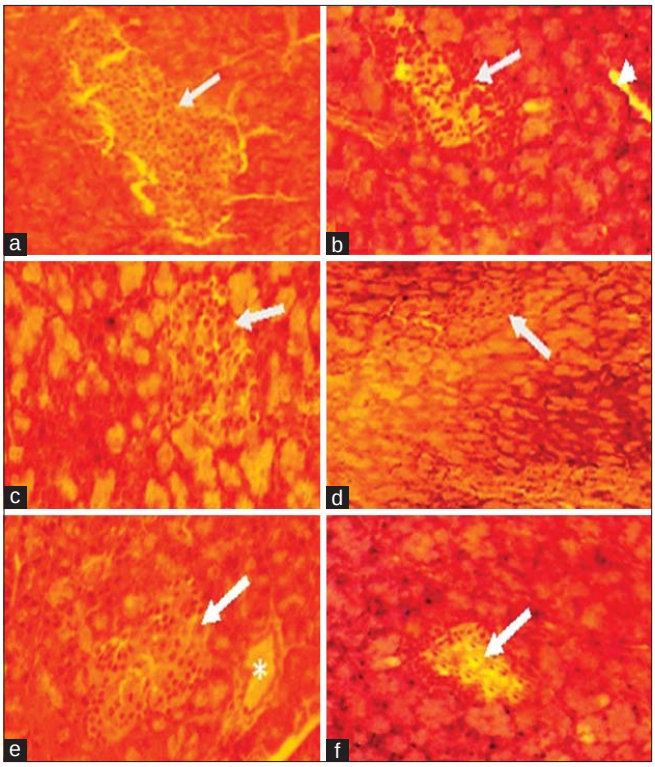

Figure 5: Photomicrographs of the pancreas showing, (a) normal pancreatic islets of Langerhans (white arrows), (b) diabetic pancreas showing loss of cells mostly from the middle portion of the endocrine islets of Langerhans (arrow) with highly basophilic nuclei of surrounding secretory exocrine acinar cells, and the presence of connective tissue septum (arrow head), (c) glibenclamide treatment: Normal histology (white arrow) - sign of total regeneration, (d) 10 mg/kg cryptolepine (CRP) treatment: Reduction of cells in the middle portion of the Islets of Langerhans (arrows); a sign of little regeneration, (e) 30 mg/kg CRP treatment: Total regeneration of cells in the middle portion of the Islets of Langerhans (arrows) and the presence of an intralobular duct (asterisks) of the exocrine acinar, and (f) 100 mg/kg CRP treatment: Reduction of cells in the middle portion of the Islets of Langerhans (arrows) (H and E stain, ×100)

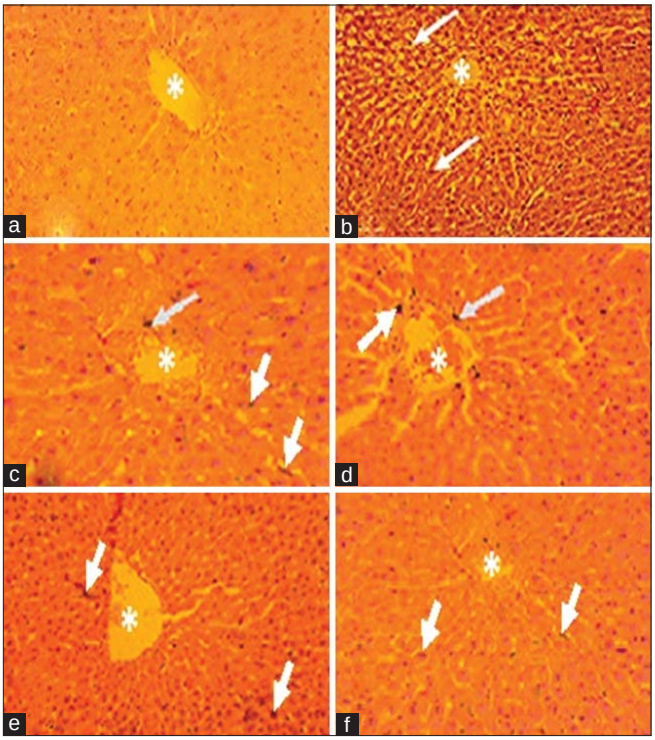

Figure 6: Photomicrographs of liver showing: (a) Normal histology with non-activate Kupffer cells and a normal hepatocytes and CV, (b) Diabetic liver showing edematous tissue with the presence of activated Kupffer cells (arrows), and highly basophilic nuclei of some hepatocytes, (c) glibenclamide treatment showing normal histology with very few highly basophilic hepatocytes, (d) 10 mg/kg cryptolepine (CRP) treatment showing slightly edematous tissue with few highly basophilic nuclei and the presence of activated Kupffer cells, (e) 30 mg/kg CRP treatment showing very mild edematous appearance with very few highly basophilic nuclei of hepatocytes and activated Kupffer cells (arrows), and (f) 100 mg/kg cryptolepine treatment showing the presence of numerous basophilic nuclei (H and E stain, ×100)

tubules (Figure 7c). The 30 mg/kg CRP-treated animals have well-defined capsular space with normal renal corpuscles and normal renal tubules [Figures 7e]. With 100 mg/kg CRP treatment, however, there was edematous kidney with displaced glomeruli with empty Bowman's capsule and also with distorted renal corpuscles with absence of capsular space [Figure 7f].

Semen Examination

Following the induction of diabetes, there were significant decrements ($P \leq 0.001$) in sperm count, sperm motility, and percentage live/dead ratio. CRP treatment did not improve these parameters but further reduced these dose-dependently ($P \leq 0.001$) indicating no ameliorative effects of CRP on sperm abnormalities associated with DM [Table 3].

DISCUSSIONS

This study was conducted to ascertain the therapeutic usefulness of CRP in managing DM and some of its associated complications. Alloxan was used to induce experimental diabetes in Sprague-Dawley rats. This chemical selectively destroys insulin-producing pancreatic β-islet cells with a corresponding inverse changes in the plasma insulin concentration, and hence persistent hyperglycemia [18]. Persistently, high blood glucose directly increases hydrogen peroxide production by murine mesangial cells and lipid peroxidation of glomeruli and glomerular mesangial cells [19]. Hyperglycemia promotes glycosylation of circulating and cellular protein and may initiate a series of auto-oxidative reactions that culminate in the formation and accumulation of advanced glycosylation end-products (AGE) in tissue proteins [20]. AGE has oxidizing potential and can promote tissue damage by free radicals. In addition, increased lipid peroxidation impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors. Its products (lipid radicals and lipid peroxides) are harmful to the cells in the body and associated with atherosclerosis and damage to brain, kidney, liver, and other tissue [1,2]. In addition, diabetes and it associated hyperglycemia can be sources of DNA damage via the oxidation of DNA bases and sugar-phosphate binding sites [21,22]. The occurrence of these alterations can result in mutagenic effects and/or DNA replication arrest and could be associated with risks for developing DN in DM patients [23,24].

CRP demonstrated an antihyperglycemic effect similar to glibenclamide, a sulfonylurea hypoglycemic agent. This may suggest that CRP could work via a similar mechanism as glibenclamide, which binds to and inhibits the ATP-sensitive potassium channels inhibitory regulatory subunit sulfonylurea receptor 1 [25] in pancreatic beta cells. This inhibition causes cell membrane depolarization, opening voltage-dependent calcium channels, resulting in an increase in intracellular calcium in the beta cell. This subsequently stimulates insulin release which enhances the utilization of glucose, and hence a fall in plasma glucose concentration [26].

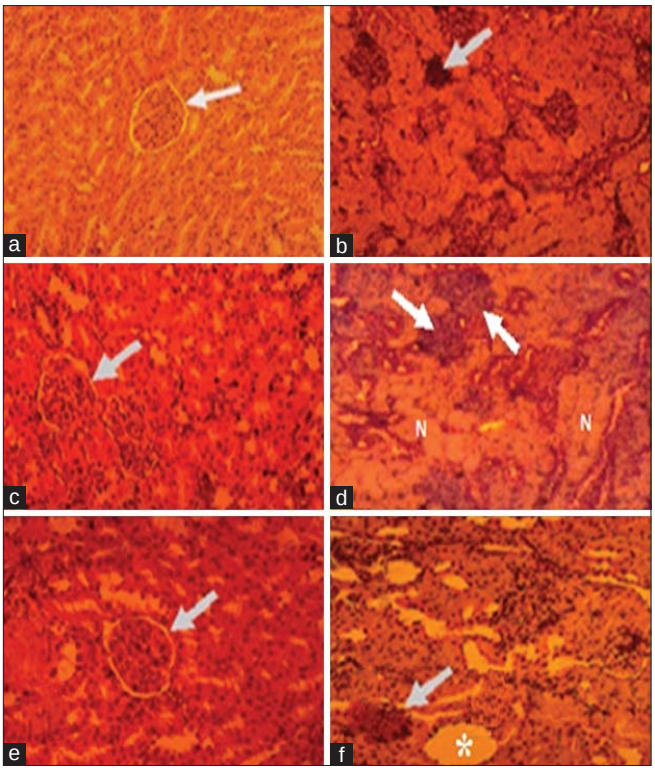

Figure 7: Photomicrograph of kidney of cryptolepine (CRP)-treated alloxan-induced diabetic rats. (a) Sham: Normal histology of tubules, renal corpuscles, and a well-defined capsular space (white arrows), (b) negative control: Diminished outline of renal corpuscles and absence of capsular space (arrows), (c) glibenclamide treatment: Atrophy of tubules and corpuscles with intact capsular space (white arrow), (d) 10 mg/kg CRP treatment: Diminished outline of renal corpuscles (arrow) and absence of capsular space with localized degenerative areas (N) of the cortex of the kidney, (e) 30 mg/kg CRP treatment: Normal histology of tubules, renal corpuscles, and well-defined capsular space, (f) 100 mg/kg CRP treatment: Section reveals semi-capsular space (white arrows) around the glomerulus of the corpuscles and loss of glomerulus represented by an empty space (asterisk) in corpuscle (H and E stain, ×100)

Table 3: Effects of CRP treatment on sperm motility, viability, and sperm count

Treatment groups	% Motility	% Viability	Cell count (/μL)
Sham	96±0.12	98±0.05	$23.2\pm2.98\times10^{9}$
NC	60±6.10***	70±5.5***	$3.9\pm0.5\times10^{9}$***
CRP 10 mg/kg	20±0.12***	5±0.5***	$1.90\pm0.24\times10^{6}$***
CRP 30 mg/kg	5±0.12***	1±0.19***	1±1.27***
CRP 100 mg/kg	0.1±0.12***	0.1±0.15***	0.2±0.025***

Values are means±standard error of mean, (n=5) ***P≤0.001 indicating significant difference from sham control (One-way ANOVA followed by Dunnett's *post-hoc* test, CRP: Cryptolepine, NC: Negative control

DM, in this study, was associated with weight loss. In DM, because there is a lack of insulin, the processes of glucose transfer from the blood into cells, where it can be used the generate energy, is impaired. The body therefore, begins to utilize lipids and protein from muscles as an alternate source of fuel; causing a reduction in overall body weight. Induction of the release of insulin by

glibenclamide and possibly by CRP would, therefore, reverse this weight loss as portrayed in this study.

As DM progresses, complications set in. DN is one such complication which results in allodynia and hyperalgesia (neuropathic pain) [27]. Allodynia refers largely to pain evoked by Aβ-fibers or low-threshold Aδ- and C- nociceptive fibers in response to a non-nociceptive stimulus while hyperalgesia is increased pain sensitivity [28], which could be mediated by bradykinin B(1) receptors [29]. CRP, just like morphine, inhibited cold allodynia and thermal hyperalgesia. Morphine interacts with κ opioid receptorsand produces analgesia by causing hyperpolarization of interneurons within the dorsal spinal cord and depressing the release of transmitters such as enkephalin, serotonin, or norepinephrine, and specific receptors located on the nociceptive fibers that transmit pain sensation to the higher centers. In addition, morphine can interact with μ opioid receptors located in the supraspinal structures and activate the supraspinal system with the release of the endorphins inhibit the transmission of pain signals by these fibers [30,31]. The thermal antihyperalgesic properties of CRP may also be due to downregulation of bradykinin B(1) receptors, which have been found to be upregulated alongside the development of Type 1 diabetes [29], or possibly could be involved in raising the nociceptive threshold, or desensitization of nerve endings of A-delta-fiber and A-beta-fiber that are lowered with DM.

Although there were reductions of hematological parameters associated with DM in this study, these were statistically not significant, except for platelet count, which decreased significantly. DM has been reported to be associated with significant reduction in red blood cells (RBCs), Hemoglobin (Hb), mean corpuscular volume, mean corpuscular Hb concentration, mean corpuscular Hb, hematocrit, and red cell distribution width [32-34]. The occurrence of anemia in DM has been reported due to the increased non-enzymatic glycosylation of RBC membrane proteins by ROS [35]. Oxidation of these proteins and hyperglycemia in DM causes an increase in the production of lipid peroxides that lead to hemolysis of RBC [36]. A significant reduction in PLT has been reported in liver cirrhosis and other advanced liver diseases which may be associated with DM [37,38]. Thrombopoietin (TPO) is predominantly produced by the liver and constitutively expressed by hepatocytes. With the damage of hepatocytes, TPO production is reduced. This leads to reduced thrombopoiesis in the bone marrow and consequently to thrombocytopenia [39].

Among the parameters measured in the liver function test, AST, ALT, and GGT were elevated with DM. AST and ALT are enzymes present in hepatocytes and are associated with liver parenchymal cells. These enzymes are, therefore, significantly elevated in acute hepatocyte damage. The mutual rise in serum AST and ALT is a sure indication of hepatocellular damage since ALT is a more sensitive marker of hepatocellular damage [40]. An elevation of AST only would not have been an indication of hepatocyte damage since is not a specific indicator [41]. Cirrhosis results in significant elevation of AST and ALT [42,43] and is common in diabetic patients as more than 80% of patients with cirrhosis have been found to suffer glucose intolerance [44]. This clearly indicates that animals developed cirrhosis, and this assertion is further supported by the thrombocytopenia recorded in this study. GGT levels are elevated in biliary disease, cholelithiasis, and cholecystitis associated with DM [45]. It is conclusive, therefore, that DM is associated with hepatocellular damage. Unfortunately, neither glibenclamide nor CRP treatment could reverse this liver injury. The further elevation of AST in the CRP-treated groups, however, may not be further damage to hepatocytes as AST is not only from hepatocytes but could also be from RBC as well as cardiac and skeletal muscles [41]. Serum total proteins (ALB and GLOB) and TBIL (conjugated and unconjugated) were within normal ranges in DM indicating that diabetes may not cause chronic active hepatitis, liver cirrhosis, immune system over activity, and chronic inflammatory disorders [46] and does not have any deleterious effects on the liver's synthetic function, hepatic metabolism, or biliary excretion.

Associated with induced DM was a significant reduction in plasma urea and a significant elevation in creatinine. Although a low level of urea is not usually a cause for concern in kidney function, it may be seen in severe liver disease [43]. Because urea is synthesized by the liver, severe liver failure causes a reduction of urea in the blood, with liver insufficiency the ammonia is not detoxified to urea. Persistent hyperglycemia is known to be a significant risk factor for diabetic nephropathy. Hyperglycemia may directly result in mesangial expansion and injury by an increase in the mesangial cell glucose concentration. The glomerular mesangium expands initially by cell proliferation and then by cell hypertrophy. Transforming growth factor β is particularly important in the mediation of expansion and later fibrosis via the stimulation of collagen and fibronectin [47]. This affects urea and creatinine clearance from blood. Creatinine is a more specific indicator of kidney function. It indicates, therefore, that the kidney was impaired in function. Clinical findings indicate that uncontrolled DM is the most common cause of kidney failure, accounting for nearly 44% of new cases [48], in the complication known as diabetic nephropathy [49]. Renal disease in diabetes is found to be caused by abnormalities of vasodilatation and generates ROS-mediated by endothelial-derived nitric oxide [50,51]. Glibenclamide treatment significantly reversed the changes in kidney function associated with induced DM; however, CRP treatment could not alter significantly these changes. A study aimed at elucidating the role of glibenclamide in the prevention of diabetic nephropathy indicated that glibenclamide attenuates some biochemical and histological changes produced by diabetic nephropathy [52]. This could be due to the tight regulation of DM offered by glibenclamide.

DM was also associated with a negative lipid profile as CHO, TAG, and LDL were significant elevated, and HDL was significant reduced [34,53]. Established DM results in an increased efflux of free fatty acids from adipose tissue and impaired insulin-mediated skeletal muscle uptake of free

fatty acids increase fatty acid flux to the liver [54], with the subsequent increase in VLDL production and/or retarded clearance from the plasma of large VLDL. TAG enrichment of the lipolytic products through the action of cholesteryl ester transfer protein, together with hydrolysis of TAG and phospholipids by hepatic lipase, leads to results in increased production of IDLs and LDLs [55]. Plasma residence time of LDLs may be prolonged because of their relatively reduced affinity for LDL receptors [56]. Reduction in HDL associated with DM appears to be due to increased transfer of CHO from HDL to TAG-rich lipoproteins, with reciprocal transfer of TAG to HDL. TAG-rich HDL particles are hydrolyzed by hepatic lipase and, as a result, are rapidly catabolized and cleared from plasma [53]. Treatment with glibenclamide and CRP significantly reversed the DM-associated alteration in lipid profile due to controlled hyperglycemia [57].

Histological studies reveal destruction of islet of Langerhans cells of the pancreas by Alloxan used in inducing diabetes. Alloxan is rapidly taken into the β-islet of Langerhans cells where it is reduced, by SH-containing cellular compounds, reduced glutathione, cysteine and protein-bound sulfhydryl groups (including SH containing enzymes), to dialuric acid [58]. Dialuric acid is then re-oxidized back to alloxan establishing a redox cycle for the generation of superoxide radicals. The superoxide radicals undergo dismutation to hydrogen peroxide (reaction is catalyzed by superoxide dismutase). The ROS produced now cause damage to the DNA and a subsequent damage of pancreatic islets [58]. Hyperglycemic control (as seen with glibenclamide and CRP) results in significant reduction in the production of ROS, and hence, tissue susceptible to damage by ROS is protected. This could account for the regeneration/restoration of pancreatic β-islet cells accompanying treatment. Oxygen radicals generated in DM has tremendous damaging effect on the liver and kidney [59-61], as revealed by the blood biochemical and histopathological studies, and hence hyperglycemic control could be very helpful in restoring these organs. Glibenclamide and 30 mg/kg CRP treatment were moderately able to ameliorate the hepatocellular and kidney damage associated with DM due to their significant diabetes control. Drugs and chemicals rendering antioxidative properties can attenuate alloxan toxicity.

Similar to previous findings, there was reduction in the sperm motility and count as well as the percentage live/dead ratio while the number of abnormal spermatozoa also increased [62,63]. Observations from this study showed no ameliorative but further deterioration effect in semen quality by CRP. Decreased viability and spermatocyte cell count in DM could be attributed to damage of the secretory epithelial cells of the seminiferous tubules probably due to oxidative damage from glucose auto-oxidation and excessive production of superoxide radicals and formation of advanced glycation end products associated with DM [64]. Spermatozoa are highly susceptible to damage by excess concentration of ROS due to the high content of poly unsaturated fatty acid within their plasma membrane [65]. CRP could not reverse the oxidative stress on the spermatozoa caused by alloxan-induced diabetes because *in vitro* antioxidant activities of the compound are very minimal. CRP has been found to produce a variety of pharmacological effects including antimuscarinic activity [66], α-adrenoceptor antagonism [67], and cytotoxicity [68] which could adversely affect male fertility.

CONCLUSION

CRP treatment abolishes hyperglycemia, weight loss, cold allodynia, neuropathic pain, and undesirable lipid metabolism associated with DM as it has exhibited interesting antihyperglycemic, analgesic, and cytoprotective effect on the pancreas. However, it does not have a significant ameliorative effect on DM-induced disorders of the liver, the kidney histopathology, and quality of semen production.

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AUTHORS CONTRIBUTION

EOA, GAK, SK, and RNAOM designed the experiment, analyzed the data, and prepared the final manuscript. DA, KKA, DK, and ADF contributed to the study design, performed the experiments, collected all data, and drafted the initial manuscript. All authors approved of the final manuscript.

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Short Communication

Low dose effects of a *Withania somnifera* extract on altered marble burying behavior in stressed mice

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ABSTRACT

Aim: *Withania somnifera* root (WSR) extracts are often used in traditionally known Indian systems of medicine for prevention and cure of psychosomatic disorders. The reported experiment was designed to test whether low daily oral doses of such extracts are also effective in suppressing marble burying behavior in stressed mice or not. **Materials and Methods:** Groups of mice treated with 10, 20, or 40 mg/kg daily oral doses of WSR were subjected to a foot shock stress-induced hyperthermia test on the 1st, 5th, 7th, and 10th day of the experiment. On the 11th and 12th treatment days, they were subjected to marble burying tests. Stress response suppressing effects of low dose WSR were estimated by its effects on body weight and basal core temperature of animals during the course of the experiment. **Results:** Alterations in bodyweight and basal core temperature triggered by repeated exposures to foot shock stress were absent even in the 10 mg/kg/day WSR treated group, whereas the effectiveness of the extract in foot shock stress-induced hyperthermia and marble burying tests increased with its increasing daily dose. **Conclusion:** Marble burying test in stressed mice is well suited for identifying bioactive constituents of *W. somnifera* like medicinal plants with adaptogenic, anxiolytic and antidepressant activities, or for quantifying pharmacological interactions between them.

KEY WORDS: Bioassay, foot shock stress, marble burying test, thermoregulation, treatment regimen, *Withania somnifera*

INTRODUCTION

Withania somnifera is a psychoactive medicinal plant often used in traditionally known systems of medicine in India and other countries [1-4]. Modern herbal researchers and practitioners considered it to be an adaptogenic plant useful for treatments of chronic diseases caused by, or associated with, exaggerated anxiety and other nervous system disorders [5-8]. However, as yet the questions concerning bioactive constituents of its medicinally used extracts, or on their therapeutically interesting doses and dosing regimen, still remain open [4,9].

Recent observations made during our exploratory dose-finding studies have revealed that fairly low daily oral dose of *W. somnifera* extracts increases stress resistance in laboratory rodents, and indicated that their anxiolytics or antidepressants like effectiveness in stressed mice increases with increasing number of treatment days [10,11]. Similar was also the observations made with several plant metabolites encountered in *W. somnifera* and other adaptogenic herbs [12-15]. In this communication, the results of an experiment suggesting that marble burying test in stressed mice could be helpful for better understanding of low dose pharmacology of *W. somnifera* like adaptogenic herbs and of their bioactive constituents are summarized and briefly discussed in light of our current knowledge on medicinal phytochemistry and pharmacology of the plant.

MATERIALS AND METHODS

Animals and the Extract Used

Experimentally inexperienced Wistar Albino male mice (25 ± 5 g) were procured from Central Animal House of Institute of Medical Sciences, Banaras Hindu University,

Varanasi (Registration number: 542/AB/CPCSEA). They were group-housed at an ambient condition (25°C ± 1°C and relative humidity 50% ± 10%) and in 12:12 h light/dark cycle with free accesses to food and water. Ethical clearance for animal experimental work was obtained from the Central Animal Ethical Committee of the University (Dean/2014/CAECI/604, dated 30/05/2014) before the commencement of experiments.

The medicinally used and pharmaceutically well standardized *W. somnifera* root (WSR) extract together with its analytical details was generously supplied by Natural Remedies Private Limited, Bengaluru, Karnataka, India. A total withanolides content of the extract was 2.7% (w/w).

Animal Grouping and Drug Treatments

A total of 30 randomly selected mice were allotted to five experimental groups consisting of six animals each. One of them serving as a non-stressed control group was not subjected to foot shock stress-induced hyperthermia tests while all others were subjected to the test on the 1st, 5th, 7th, and 10th days of the experiment. The non-stressed and stressed control groups were treated once daily with 10 ml/kg of 0.3% aqueous carboxymethyl cellulose (CMC, Central Drug House, New Delhi, India) for 12 consecutive days, and the three stressed ones were similarly treated with 10, or 20 or 40 mg/kg daily oral doses of WSR suspended in the vehicle. On all test days, body weights and basal core temperatures of all animals were recorded before the day's treatment, and all tests were performed 1 h thereafter.

Foot Shock Stress Induced Hyperthermia Test

The test procedure described elsewhere in details [10,12] were strictly followed. In short, five consecutive electric foot shocks (2 mA, 50 Hz of 2 ms duration) were delivered to each individual animal of a stressed group, and its core temperature was recorded again 10 min after it was placed back to its home cage. The difference between this temperature and its basal core temperatures recorded on the test day was used as an index for stress-triggered hyperthermia. On all test days, the animals of the non-stressed control group were also placed individually for 1 min in the stress chamber, but no foot shocks were given them. Otherwise, all animals of all groups were handled similarly.

Marble Burying Test

On the 11th day, an individual animal of a group was placed in a test cage (30 cm × 23 cm) where 12 glass marbles (color and size of marbles were kept constant) were evenly spaced for standard marble burying condition. On the 12th day, they were tested similarly, in the two-zone marble burying condition, whereupon 8 glass marbles were evenly spaced only on one-half of the box. After 15 min (standard condition) or 30 min (two-zone condition) of exposure, the number of marbles, at least, two-thirds covers by husk was counted [16].

Statistical Analysis

Mean ± standard error of mean was calculated for the observed values in each experimental group. A statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student - Newman–Keuls multiple comparison tests and two-way ANOVA followed by Bonferroni *post hoc* test unless otherwise stated. GraphPad Prism - 5 (GraphPad software Inc., La Jolla, California, USA) was used for statistical analysis. A $P < 0.05$ was considered as statistically significant.

RESULTS

As expected from the observation made during our earlier studies with *W. somnifera* and other herbal extracts [10-13], mean body weight of the stressed control group from the 5th day onward of the experiment decreased continuously, whereas that of the unstressed control group continued to increase. On all observational days, no statistically significant differences between the mean body weights of none of the foot shock stressed and WSR treated groups and the non-stressed control group were observed (results not shown). Moderate but significant elevation in the mean basal core temperature of the stressed control group observed during the course of the experiment was also completely suppressed by repeated daily 10 mg/kg and the higher daily oral doses of WSR [Figure 1a].

Results of the foot shock stress triggered hyperthermia test summarized in Figure 1b revealed that even a single oral 40 mg/kg WSR dose is effective in suppressing stress induced hyperthermic response. The effectiveness of all tested doses of the extract in this test increased not only with its increasing dose but also with increasing number of treatment days. These observations reaffirm that biological processes and mechanisms regulating stress triggered hyperthermic responses are involved in the modes of action of WSR.

Results of the marble burying tests are summarized in Figure 2a and b. The mean number of marbles buried by the stressed control group in both version of the test were higher than the corresponding values of the non-stressed group, but statistically significant differences between the two groups were observed in

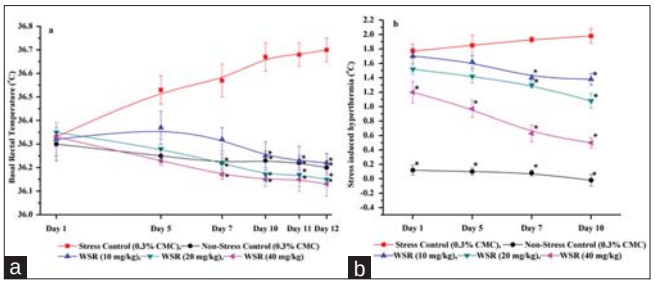

Figure 1: Effect of WSR extract on (a) basal rectal temperature and (b) stress induced hyperthermia in male mice. WSR: *Withania somnifera* root extract, CMC: Carboxymethyl cellulose, SEM: Standard error of the mean . Values are mean±SEM ($n = 6$). *denotes statistically significant difference (two-way analysis of variance followed by Bonferroni *post hoc* test) relative to the corresponding values of the stress control group (*$P < 0.05$)

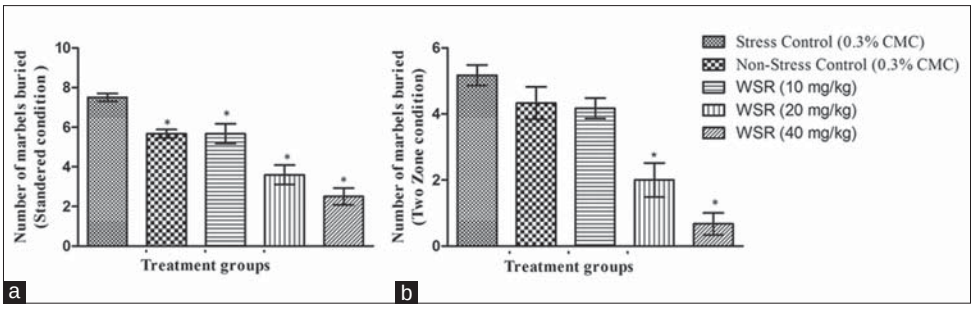

Figure 2: Effect of WSR extract in mice marble burying test: (a) Standard condition on day 11 and (b) Two-zone condition on day 12. WSR: *Withania somnifera* root extract, CMC: Carboxymethyl cellulose, SEM: Standard error of the mean. Values are mean±SEM (n = 6). *denotes statistically significant difference (analysis of variance followed by Student - Newman-Keuls multiple comparison test) relative to the values of the corresponding stress control group (*P < 0.05)

the standard version of the test only [Figure 2a]. These numbers for the 10 mg/kg/day WSR treated group in both versions of the test were almost identical to those of corresponding ones for the non-stressed control group, and its dose dependent effects in decreasing these numbers were observed in both version of the test.

DISCUSSION

Reported results reveal that 10 mg/kg daily oral dose of WSR is high enough for completely suppressing the foot shock stress-triggered alterations in body weight, basal core temperature, and marble burying behavior of mice, and that its effectiveness in the foot shock stress-induced hyperthermia and in both versions of the marble burying test increases with its increasing daily dose. It has recently been reported that a single 25 mg/kg intraperitoneally administered doses of WSR possess anxiolytics or antidepressants like activities in marble burying test [17], and that delayed anxiogenic effects of stress in mice are also detectable in this test [18]. This test is often used for assessing behavioral effects of genetic manipulations and drugs in rodents, and predictive validity of stress-induced hyperthermia tests for identifying drug leads against anxiety disorders have also been well established [14-19].

Our observations strongly suggest that standard version of marble burying test is more sensitive than its two zone version for quantifying anxiogenic state in mice triggered by prior exposures to unpredictable foot shock stress, and that this version of the test is well suited for stress response suppressing as well as antidepressants or anxiolytics like effects of fairly low daily oral doses of WSR like herbal extracts. Since, *W. somnifera* extracts devoid of withanolides also possess adaptogenic and other therapeutically interesting bioactivities [20-22], appropriate uses of the bioassay procedure used in the described experiment for identifying their bioactive constituents or for clarifying possible pharmacological interactions between them can be warranted. Such efforts will not only be useful for more rational medicinal uses of *W. somnifera*, but also could lead to novel therapeutic principles and drug leads urgently needed for prevention and cure of mental health problems accompanying, or caused by, stress triggered chronic diseases including cancer and Alzheimer's disease [23-27].

In any case, it remains certain that fairly low daily oral doses of the tested extract is effective in increasing stress resistance and that its slightly higher ones could be useful for treatments of co-morbid depression and anxiety in stressed patients. Since total contents if withanolides in WSR is only 2.7%, it seems reasonable also to assume that phytochemicals other than withanolides are also involved in its observed low dose effects. This inference is well supported by our current knowledge on medicinal phytochemistry and experimental pharmacology of the plant [2,28,29].

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Review Article

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Anti-fibro-hepatocarcinogenic Chinese herbal medicines: A mechanistic overview

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ABSTRACT

Chinese herbal medicine (CHM) is an integral component of complementary/alternative medicine and it is increasingly becoming the preferred therapeutic modality for the treatment of liver fibrosis and hepatocellular carcinoma (HCC) worldwide. Accordingly, the World Health Organization (WHO) has attested to the popularity and efficacy of indigenous herbal therapies including CHM as a first line of treatment for some diseases including liver disorders. However, the WHO and drug discovery experts have always recommended that use of indigenous herbal remedies must go hand-in-hand with the requisite mechanistic elucidation so as to constitute a system of verification of efficacy within the ethnobotanical context of use. Although many CHM experts have advanced knowledge on CHM, nonetheless, more enlightenment is needed, particularly mechanisms of action of CHMs on fibro-hepato-carcinogenesis. We, herein, provide in-depth mechanisms of the action of CHMs which have demonstrated anti-fibro-hepatocarcinogenic effects, in pre-clinical and clinical studies as published in PubMed and other major scientific databases. Specifically, the review brings out the important signaling pathways, and their downstream targets which are modulated at multi-level by various anti-fibro-hepatocarcinogenic CHMs.

KEY WORDS: Chinese herbal medicine, fibro-hepato-carcinogenesis, immunomodulation, inflammation, mechanistic elucidation

INTRODUCTION

Chinese herbal medicine (CHM) that forms an integral component of traditional Chinese medicine (TCM) keeps growing popular worldwide [1,2]. The past few decades have seen increased scientific investigations on commonly used CHMs often reflective of the ethnobotanical context, in which they are used. As a result, it is common to find scientific studies on whole Chinese herbal formulas, whereby the pharmacological and therapeutic effects more often are attributed to the entire components of the herbal formula. This has always been the source of criticism of CHM therapy just like other indigenous herbal remedies, especially from strict adherents of western medicine. There has been a paradigm shift in recent years with regards to research on CHMs which have seen an incredible focus on mechanistic elucidation as well as structural and functional characterization of individual components of CHMs. Many scientific efforts have been made to highlight the mechanisms of the action of CHMs [3], but there is still more work to be done. Hepatitis B virus (HBV) endemicity correlates with the incidence of liver fibrosis and its attendant complications including cirrhosis and hepatocellular carcinoma (HCC). China is noted for a high incidence of HBV infections and alcohol abuse [4]. Coincidentally, these two factors are crucial risk factors for HCC [4]. Almost 80-90% of HBV-related HCC in Asia and Africa occur in China [5,6]. Although, many scientific efforts have been made to highlight the mechanism of action of CHMs [3] used in the treatment of hepatocarcinogenic disorders, nonetheless the mechanistic elucidation of CHMs remains incomplete. This review provides a mechanistic overview of CHMs which have demonstrated *in vitro* and *in vivo* anti-liver fibrosis, anti-cirrhosis, and anti-HCC effects featured

in published scientific articles from PubMed and other major scientific databases. Specifically, it highlights the mechanisms of the action of CHMs in the light of specific therapeutic targets that can be explored in future studies.

CHM

CHM is an integral component of TCM. CHM has multi-compound composition, multi-modulatory, and multi-target action [1] [Figure 1]. It produces less adverse effects in the treatment of liver diseases [7-9]. In CHM practice, liver disease is assumed to be caused by a number of factors including poor blood circulation and dysregulated metabolism [10]. Thus, CHM therapy against liver disease is sorely to reduce blood stagnation, eliminate toxins and improve the immune system. CHM practice involves the use of either one herb/plant extract or a mixture of two or more herbal extracts based on a time-tested system of herbology. According to the principles and theories governing CHM practice, one pharmacologically active compound from one of the constituent herbs is normally regarded as "King herb" [11]. The "King herb" is the main medicine which exerts the expected therapeutic action. To enhance the therapeutic action of the "King herb," the other component herbs play auxiliary functions, such as enhancing delivery of the "King herb" to target site, reduce toxicity/side effects of the "King herb," and most importantly, provide synergistic effect to the "King herb."

PATHOGENESIS OF FIBRO-HEPATOCARCINOGENESIS

Fibro-hepato-carcinogenesis epitomizes a spectrum of pathological events in the liver manifesting as liver fibrosis, cirrhosis and HCC if not treated at the initial stages. The whole pathological process begins as a result of dysregulated wound healing process secondary to chronic hepatic inflammation. Hepatic stellate cell (HSC) is the key hepatic cell implicated in liver fibrosis. Under normal physiological conditions, quiescent ito cells store retinoids (vitamin A) and play crucial homeostatic roles in the liver. However, in response to chronic inflammatory and fibrogenic stimuli, quiescent ito cells do not only transform into a fibrogenic phenotype (myofibroblasts) but also proliferate and increase the synthesis and the accumulation of extracellular matrix (ECM) in liver sinusoidal space. HSC morphological transformation represents the crucial pathological event for the initiation of fibrogenesis and its progression to fibrotic liver disease. As a result, increased output of fibrogenic and inflammatory genes mainly precede secretion of fibrogenic (transforming growth factor beta 1 [TGF-β_1]), and inflammatory (tumor necrosis factor-alpha [TNF-α], interleukin 1 beta [IL-1β], IL-6) cytokines to sustain fibrogenesis. Furthermore, there is ECM accumulation, the proliferation of myofibroblasts, and recruitment and activation of other hepatic and non-hepatic cells in an autocrine and paracrine manner. If left untreated, liver fibrosis progresses to cirrhosis, but this transition can be hastened by comorbidity factors including HBV and hepatitis C viral (HCV) infections and alcohol abuse [Figure 2]. Cirrhosis is a manifestation of advanced liver fibrosis and it is characterized by hepatic nodules that progressively distort normal hepatic architecture and function resulting in increased resistance to portal blood flow. These pathological events elevate sinusoidal pressure leading to portal hypertension and the risk of HCC

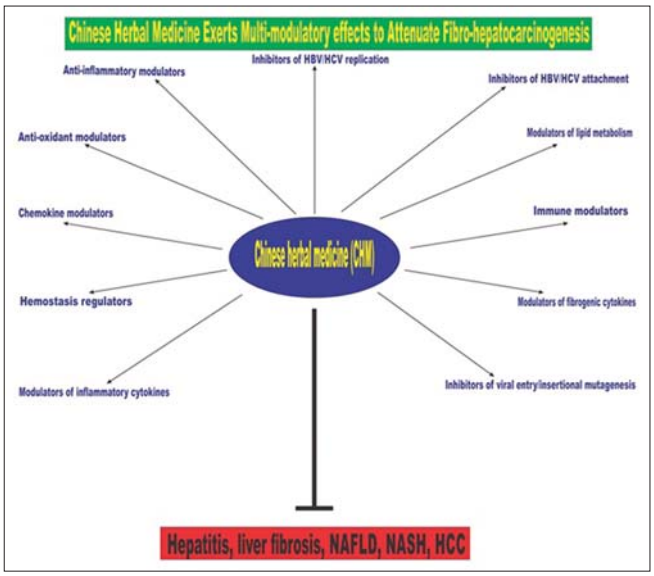

Figure 1: A diagrammatic depiction of the multi-modulatory and multi-target pharmacological effects of Chinese herbal medicine (CHM) which underpin the promising efficacy of CHM against liver disease in general

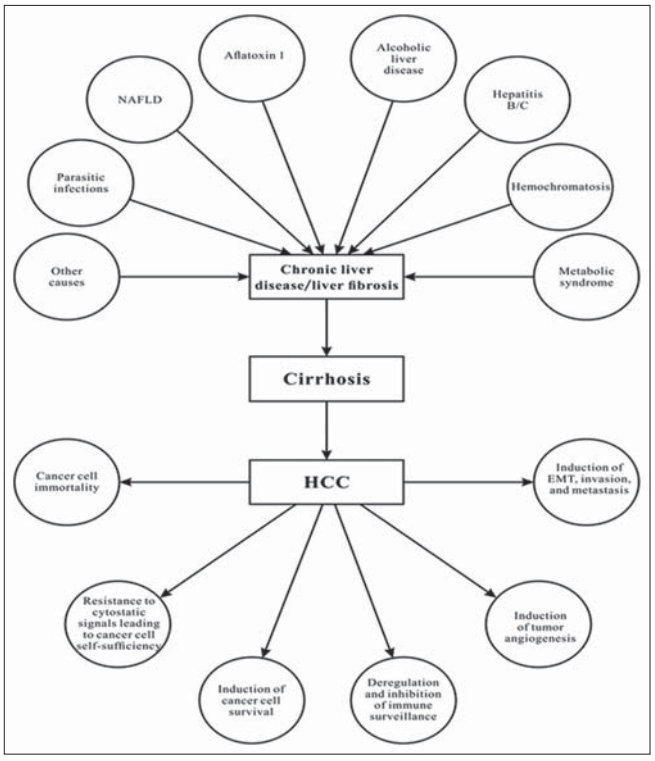

Figure 2: An illustration of the multi-etiology of fibro-hepato-carcinogenesis. Many etiological factors may act synergistically to promote progression of chronic liver injury to liver fibrosis and cirrhosis if left untreated, and this ultimately increases the risk of HCC and the manifestation of the six phenotypic hallmarks of HCC

and death [12]. Underpinning fibro-hepatocarcinogenesis is a constellation of dysregulated cell signaling pathways mainly mediated by growth factors, cytokines, chemokines, transcriptional factors, and their resultant target genes. Thus, molecular underpinnings of fibro-hepatocarcinogenesis are not only diverse but also play crucial roles in homeostasis.

The progression of liver fibrosis to cirrhosis through to HCC usually takes many years or decades. In view of this, most infected persons are asymptomatic [13]. However, this progression can be hastened within few months by factors such as neonatal liver disease, HCV infections, human immunodeficiency virus/HBV/HCV co-infections, severe delta hepatitis, and drug-induced liver disease [13]. HCC accounts for most liver-related mortality [14] and tumor progression is implicated as the main cause of death in HCC patients [15]. Furthermore, a significant percentage of patients may die from other complications arising from liver fibrosis and cirrhosis such as ascites, spontaneous peritonitis, hepatic encephalopathy, hepato-pulmonary syndrome, porto-pulmonary hypertension, and pain. The early detection and treatment of liver fibrosis and cirrhosis are crucial for overall management of HCC risk. However, the treatment of HCC is a major problem partly because of its complex nature such as high degree of cancer clonal heterogeneity, intra-tumor genetic heterogeneity, and emerging compensatory pathways in response to therapy-related inhibition of some pathways in cancer [5,16]. Among primary liver cancers, HCC represents the major histological subtype, accounting for 70-85% of the total liver cancer burden worldwide [4].

EPIDEMIOLOGY OF LIVER DISEASE IN CHINA

China has the largest population (1.3 billion people) in the world comprising 56 different ethnic groupings [17]. With the establishment of Central Cancer Registries by the Health Ministry of China in 2002 to take records of cancer cases and deaths, there has been a consistent increase in both the incidence and mortality of cancer [18-20]. For example, in 2006 the 3rd National Death Survey report showed that cancer is the second leading cause of death in China, before then a report from 2004 to 2005 had placed the national mortality rate of cancer at 135.88/100,000, with 170.17/100,000 in males and 99.97/100,000 in females (Ministry of Health, National Death Survey Report 2004-2005, Beijing). Liver cancer is the third reported cancer case in both rural and urban China and among the top 10 cancers in recent years [21]. HBV and alcohol abuse which account for the most reported cases of liver fibrosis and cirrhosis were reported to have changed, with the former decreasing while the latter increases [21]. Hospitalization due to the alcohol-related and non-viral-related cirrhosis was shown to have increased [21]. Viral related liver disease burden has increased quiet significantly. For example, in Guangdong province (the most populous province in China), the predicted annual cost of HBV-related liver disease was purged at RMB 10.8 billion [22], speculatively, more than twice the annual budget of a developing country. Meanwhile, novel therapeutic agents locked up in CHMs remain untapped or poorly explored. If this rich readily available ethnobotanical heritage is properly harnessed through cutting edge scientific approaches, it can save the increasing disease burden.

CHMS EXERT ANTI-INFLAMMATORY EFFECTS

Chronic hepatic inflammation has been widely implicated in the initiation of liver fibrosis [23-25]. Many CHMs produce their effects by modulating pro-inflammatory factors including IL-1β, IL-2, IL-6, IL-8, IL-12, TNF-α, nuclear factor kappa B (NF-κB), prostaglandin E_2 (PGE_2), interferon gamma (IFN-γ), nitric oxide (NO), cyclooxygenase-2 (COX-2), intercellular adhesion molecule 1 (ICAM-1), and activator protein 1 (AP-1). The modulation of inflammatory mediators and their downstream protein scaffolds have become crucial targets for the treatment of liver fibrosis, cirrhosis, and HCC. Below are some specific inflammatory mediators modulated by some CHMs to cause attenuation of fibro-hepato-carcinogenesis.

NF-κB

NF-κB is an important target for therapy against liver fibrosis, in view of its role in inflammation. Lu *et al*. had demonstrated that myeloid differentiation protein 88 (MyD88) inactivated phosphorylation of IκBα in an NF-κB/IκBα trimmer complex leading to activation of IκBα and toll-like receptors (TLRs) [26], and this cascade led to the release of pro-inflammatory cytokines [27]. The role of NF-κB activation in inflammation, particularly how it induces the expression of pro-inflammatory cytokines, cell cycle regulatory molecules, and angiogenic factors have been elaborated [28]. By using a direct kinase assay and immunoblot analyses, it was shown that a seed extract of *Phaseolus angularis* markedly inhibited NF-κB expression and effectively ameliorated hepatic inflammation [29]. An extract of *Cinnamomum cassia* inhibited mRNA expression of induced nitric oxide synthase (iNOS), COX-2, and TNF-α through suppression of NF-κB activation [30]. Furthermore, an extract from the roots of *Polygala tenuifolia* inhibited the translocation of NF-κB by blocking TLR4 and MyD88 expression in lipopolysaccharide (LPS)-stimulated BV2 cell lines [31] indicating that TLRs and adaptor proteins such as MyD88 could be important targets of some CHMs.

TNF-α

A number of CHMs inhibited TNF-α expression to modulate TNF-α in experimentally induced liver fibrosis. CHMs with specific negative modulatory effects on TNF-α include extracts from *Zanthoxylum schinifolium* [32], *P. tenuifolia* [31], *Clematis chinensis* [33], and *Angelica sinensis*, and *Sophora flavescens* [34].

IL

Interleukins play crucial roles in the inflammatory reactions including cell adhesion, neutrophil aggregation, inflammatory gene expression, and release of neurotoxic substances to exacerbate inflammatory response. Inhibition of interleukins by CHMs may significantly account for the anti-inflammatory

effects of most CHMs. Extracts from *Glossogyne tenuifolia* [35], *Vitex trifolia* [36], *Glycyrrhiza uralensis* [37], *Scutellaria baicalensis* and *Andrographis paniculata* [38], *Caesalpinia sappan* [39,40], and *Phellodendron chinense* [41] were shown to have down-regulated expression of IL-1β, IL-2, IL-6, and IL-12. IL-10 has severally been reported as a negative regulator of inflammation [42]. The mechanism of IL-10-dependent anti-inflammatory effect is linked to suppression of inflammatory cytokines [43]. Some CHMs were reported to have up-regulated IL-10 expression. Example, the root extracts of *Astragalus membranaceus* reversed down-regulation of IL-10 expression under colitis-inducing conditions [44].

IFN-γ

The role of IFN-γ in inflammation has been well-elaborated [45]. IFN-γ activates macrophages to release IL-1, TNF-α, IL-6, IL-8 and several other pro-inflammatory mediators, playing major roles in inflammation. A root extract of *A. membranaceus* markedly reduced the expression of IFN-γ [44].

PGE_2

PGE_2 causes vasodilation of peripheral blood capillaries at inflammatory site, thereby increasing vascular permeability, plasma exudation, edema, and inflammation and these effects can potentiate inflammatory reaction. Therefore, cessation of PGE_2 activity may enhance anti-inflammation. An extract of *Houttuynia cordata* successfully inhibited PGE_2 release in LPS-induced activation of mouse peritoneal macrophages [46]. Similarly, extracts of the flowers of *Carthamus tinctorius* markedly reduced the release of PGE_2 [47].

iNOS and NO Production

Vascular dilatation, vascular permeability, cell infiltration, and release of pain mediators are all orchestrated by NO production under the regulation of iNOS in smooth muscle cells. Inhibition of this pathway may significantly halt inflammation and its associated complications. Lim *et al*. had reported inhibition of iNOS-dependent NO synthesis from RAW 264.7 cells by the action of phylligenin, a compound isolated from *Forsythia koreana* and it led to abrogation of the inflammatory response in the studied cells [48].

COX-2

COX-2 is a member of the cyclooxygenase family. It is constitutively expressed on inflammatory cells [49]. It is only expressed on tissues secondary to stimulation by inflammatory stimuli or tissue injury. A number of CHMs have been shown to markedly decrease iNOS and COX-2 expressions in experimental models of liver fibrosis. For example, extracts of the Ramulus of *Taxillus liquidambaricola* [50], and the aerial parts of *Pogostemon cablin* [51]. Further, some CHMs were shown to down-regulate a panel of pro-inflammatory mediators including IL-1β, TNF-α, iNOS, ICAM-1, and COX-2. A typical example is the extracts of *A. sinensis* and *S. flavescens* which significantly inhibited IL-1β, TNF-α, iNOS, ICAM-1, and COX-2 [34]. Extracts of *P. angularis* inhibited NF-κB and AP-1 [3].

Mitogen-activated Protein Kinases (MAPKs)

MAPK pathway is crucial in inflammatory responses, particularly its downstream mediators such as p38. Essentially, p38 enhances assembly and activation of leukocytes, regulates transcription factors and cytokine biosynthesis [3]. MCP-1 regulates many cells in the inflammatory process such as mononuclear cells, B cells, and T cells causing cell migration and aggregation at the site of inflammation. p38 and MCP-1 represent major targets for anti-inflammatory agents. Many CHMs exert their effects by inhibiting p38, and MCP-1 mRNA expression. For instance, extract of *Z. schinifolium* suppressed p38 and TNF-α-induced MCP-1 expression [32]. It was practically impossible to include in this review all CHMs with anti-inflammatory effects, and we seldom tried it, nonetheless those captured in this review comprehensively reflect the general picture. We wish to state that there are many other CHMs with anti-inflammatory effects, which readers can source elsewhere.

CHMS DEMONSTRATE INHIBITION OF HSC ROLE IN FIBROGENESIS

Many reports have conclusively implicated HSCs as the main hepatic cell responsible for liver fibrosis [52,53]. CHMs may interrupt one or more stages of HSC transformation, to attenuate liver fibrosis. For example, genipin, an isolate from one of the herbal components of *Yinchenhao Tang*, suppressed wound-induced HSC migration and proliferation to ameliorate liver fibrosis [54].

HSC Activation

HSC activation was also inhibited by Fuzheng Huayu, Chinese herbal formula, through blockade of fibronectin/integrin-5β1 signaling pathway [55,56]. Xiao Chaihu Tang inhibited HSC proliferation by suppressing cell secretion [57]. Root extracts of two Chinese herbs (*A. membranaceus* and *Salvia miltiorrhizae*) inhibited HSC activation and proliferation in keloid fibroblasts [58].

HSC Proliferation

Gypenosides inhibited platelet-derived growth factor (PDGF)-induced HSC proliferation via suppression of PDGF-Akt-$p70^{S6k}$ and inhibition of cyclin D1 and D2 expression [59]. Ganoderic and ganodenic acids derived from *Ganoderma lucidum* (“*Lingzhi*”) significantly inhibited HSC proliferation via suppression of platelet-derived growth factor β receptor (PDGFβR) phosphorylation [60].

HSC Apoptosis

Some CHMs selectively inhibited hepatocyte apoptosis but enhanced apoptosis of HSCs. Genipin, the pharmacologically active agent isolated from one of the herbal components

of Yinchenhao Tang inhibited *in vitro* TGF-β_1-induced hepatocyte apoptosis [61]. Subsequently, it was confirmed that genipin suppressed hepatocyte apoptosis in primary cultured murine hepatocytes via Fas-mediation [62]. Further genipin-treated mice resisted Ca^{2+}-induced mitochondrial permeability transition (MPT) compared to control and model [63]. Tetrandrine, an isolate from the roots of *Stephaniae tetrandrae* potently induced apoptosis of T-HSC/Cl-6 cells by activating caspase-3 protease and cleavage of poly (ADP-ribose) polymerase [64].

CHMS EXERT ANTI-OXIDANT AND ANTI-LIPID PEROXIDATIVE EFFECTS

Many CHMs inhibit oxidant and lipid peroxidation whiles at the same time enhance in-built hepatic antioxidant machinery to attenuate reactive oxygen species (ROS)-mediated inflammation and fibrogenesis. The production of ROS in hepatocytes as well as perisinusoidal cells has been attributed to many factors including oxidant activity, lipid peroxidation, mitochondrion electron transport chain, damaged mitochondria, cytochrome P450 isoforms, e.g., P450 2E1 (CYP2E1), xanthine oxidases, nicotinamide adenine dinucleotide phosphate oxidases, and altered metabolism [65]. ROS-dependent oxidative stress causes increase in MPT leading to hepatocyte necrosis and apoptosis [24]. Moreover, ROS (e.g. hydrogen peroxide, superoxide radical, and nitrosative species) increases the expression of specific genes linked to fibrogenesis, among which are pro-collagen type 1, monocyte chemoattractant protein 1 (MCP-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) through activation of many signal transduction pathways and transcription factors such as c-jun N-terminal kinases, AP-1, and NF-κB [66]. ROS generated by activated Kupffer cells and damaged hepatocytes activate HSCs by increasing their fibrogenic potential. It is therefore of enormous significance in the treatment of liver disease to arrest or suppress oxidative stress and lipid peroxidation. A typical CHM shown to produce suppression of oxidant and lipid peroxidation activities is extracts from *S. miltiorrhizae*. Extracts from *S. miltiorrhizae* enhanced superoxide dismutase (SOD) activity whiles reducing malondialdehyde (MDA) levels in experimentally induced liver fibrosis [67]. Furthermore, extracts from *S. miltiorrhizae* up-regulated glutathione levels whilst at the same time reduced lipid peroxidation in a dose-dependent manner [68]. Other CHMs have shown significant anti-oxidant and anti-lipid peroxidation effects both *in vitro* and *in vivo* by reducing oxidant biomarkers (MDA, alanine aminotransferase, aspartate aminotransferase, total bilirubin, and alkaline phosphatase), fibrogenic biomarkers (hyaluronic acid, laminin, type III procollagen, and type IV collagen) but increased anti-oxidant activity (increased glutathione S-transferase and SOD activities). CHMs in this group (anti-oxidant and anti-lipid peroxidation promoters) worth mentioning include *Panax notoginseng* (Tianqi) extract [69], *Gingko biloba* (Yinxing) extract [70], berberine [71,72], Yichenhao Tang extract [72], extract of *Solanum nigrum* [73], Xiao Chaihu Tang [74], Handan Ganle, taurine [75], and several other CHMs [Table 1].

CHMS EXERT ANTI-VIRAL REPLICATIVE EFFECTS

Cessation and inhibition of virus-derived ROS are important in the treatment of hepatitis-related liver fibrosis. Worldwide HBV and HCV have been acclaimed as the most common causes of chronic liver disease [4]. Pathologically, HBV can integrate into host genome (insertional mutagenesis) to induce chromosomal instability leading to liver disease progression. Unlike HBV, various HCV proteins such as core protein, the envelope, and non-structural proteins have been shown to exert oncogenic potential [24]. Several CHMs were shown to exert antiviral effects in both pre-clinical and clinical studies. *In vitro* berberine, artemisinin and artesunate inhibited viral reproduction [143]. Other CHMs including aucubin [144], nobiletin an isolate from the peelings of *Citrus unshiu* [145], and oxymatrine [146] inhibited viral reproduction and replication. *Handan Ganle* inhibited viral replication in patients with decompensated cirrhosis [147]. Moreover, *Xiao Chai Hu Tang* enhanced IFN-γ and antibody production against hepatitis B core and antigens in chronic HBV patients [148].

CHMS PRODUCE IMMUNOMODULATORY EFFECTS

TGF-β and PDGF are the two most potent fibrogenic cytokines [24] and have classically been considered to provide fibrogenic and proliferative stimuli to HSC.

TGF-β

The specific role of TGF-β in liver fibrosis has severally been elucidated [149,150]. Yang *et al.* have shown effective modulation of TFG-β/Smad signaling by a synergized root extract derived from *A. membranaceus* and *S. miltiorrhiza*, which led to decreased fibrogenic biomarkers and liver fibrosis [9]. Subsequently, the synergized root extract inhibited TGF-β_1-induced HepG2 cell proliferation and invasion by modulating TGF-β/Smad signaling [151]. The synergized root extract suppressed DEN-induced HCC, decreased pro-neoplastic markers (GGT and GST-P) and down-regulated PAI-1 mRNA expression in TGF-β_1-stimulated HepG2 cells [152]. To further elaborate the mechanism of action of the synergized root extract, it was observed that it switched pSmad3L-dependent signaling (oncogenic) to that of pSmad3C (tumor suppression) [153]. *S. miltiorrhiza* extracts A&B down-regulated TGF-β_1, and TIMP-1 gene expressions and blocked MAPK activity [154]. Rehin and emodin, isolated from *Rheum palmatum* inhibited TGF-β_1 expression [155]. *Buzhong Yiqi Tang* and *Renshen Yangrong Tang* produced significant immunomodulatory effects to reduce liver fibrosis [156]. Put together, this observation with specific regard to the synergized root extract needs further investigations, in view of the fact that DEN-induced HCC model is highly sensitive and accurately mimic the pathological features of human liver fibrosis and HCC [157]. Many other Chinese herbal formulae modulate several signaling pathways at multi-level to produce anti-fibro-

Table 1: A list of some extracts and isolated phyto-compounds from CHMs and their mechanisms of action against fibro-hepato-carcinogenesis

Phyto-compound	Botanical source	Pharmacological activity	Putative mechanism of action	Target	References
Phenyl ethanol glycosides (glycosides)	*Cistanche tubulosa*	Anti-fibrotic, hepatoprotective	Restores ECM metabolism by modulating TGF-β_1-dependent signaling	TGF-β, NF-kB	[76]
Berberine (quaternary ammonium salt)	*Coptis chinensis*	Anti-lipogenic, hepatoprotective	Represses expression of lipogenic genes; general restoration of hepatic lipid metabolism	IRS-1, SREBP1c, CPT1, SCD1, FAS	[77-80]
Ombuine (flavonoid)	*Gynostemma pentaphyllum*	Anti-lipogenic, hepatoprotective	Repression of lipogenic genes to restore hepatic lipid metabolism	NO, AST, ALP, TMAO, insulin pathway	[81-83]
Glycyrrhizin[b]	*Glycyrrhiza uralensis*	Anti-hepatocarcinogenic	Inhibits HBV replication; modulates PLA2; activates IL-10 activity	PLA2, IL-10	[84-89]
Silymarin[a] (silibinin, isosilibinin, silicristin, silidianin) (flavonolignans)	*Silybum marianum*	Anti-viral	Blocks integration of virus DNA into host cells; inhibits absorption and translocation of transferrin	Apolipoprotein B, IL-10, AST	[90-94]
Quercetin rhamnoside, gallic acid, geraniin, quercetin glycoside	*Phyllanthus niruri*	Anti-viral, anti-hepatotoxic	Clears viral proteins (HBsAg, HBeAg, HBV DNA)	Annexin A7 protein	[95-97]
Resveratrol, polydatin (anthraquinones)	*Polygonum cuspidatum*	Anti-viral, hepatoprotective	Represses expression of HBeAg, HBV DNA	HBeAg, HBV DNA	[98-100]
Saikosaponins C[b] and B2 (terpenoids)	*Bupleurum chinense*	Anti-oxidant, hepatoprotective, anti-viral	Free radical scavenging of reactive chemical species; suppresses viral attachment, entry and fusion	Viral homing factors	[101-106]
Astragaloside[a], astragalus polysaccharide, salvianolic acid (flavonoids and saponins)	*Salvia miltiorrhiza, Astragalus membranaceus*	Anti-viral, anti-fibrosis, anti-HCC	Attenuate fibrosis and HCC by modulating fibrogenic factors	MAPKs, TGF-β, Smad proteins, Imp7/8, PAI-1, GSH, SOD, MMP9, NF-KB, TNF-α	[107-112]
Matrine, oxymatrine (alkaloids)	*Sophora flavescens*	Anti-viral, anti-inflammatory	Improves liver vasomotion in NO-dependent manner	TLR9, IL-8, IL-6, sICAM-1, eNOS	[113-115]
Periplocoside A (pregnane glycosides)	*Periploca sepium*	Hepatoprotective	Reverses liver damage by modulating inflammatory cytokines and hepatic enzymes	IL-4, IFN-γ	[116,117]
Baicalein[b]	*Scutellaria baicalensis*	Anti-inflammatory, anti-oxidant, hepatoprotective	Attenuates liver injury via chelation and anti-oxidant activity	SOD, GSH, NF-kB, JNK, ERK, IL-6, TNF-α	[118-122]
Lignans, schischinone, schisandrin B (lignans)	*Schisandra chinensis*	Anti-viral, anti-inflammatory	Inhibits viral replication; increases HO-1 expression	HO-1, NF-kB, Nrf2	[123,124]
Extracts of Panax	*Panax notoginseng*	Anti-oxidant, hepatoprotective, anti-inflammatory	Attenuates NAFLD in rats by modulation both inflammation and lipid accumulation	MDA, GSH, CYP2E1, SOD, TNF-α, IL-6	[125-127]
Penta-oligogalacturonides (glucuronides)	*Crataegus pinnatifida*	Anti-oxidant, anti-lipidemic	Negatively regulate triglycerides, PAP, and GPAT	Acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, carnithine palmitoyltransferase I, PAP	[128,129]
Kernels of Prunus	*Prunus armeniaca*	Anti-steatosis, anti-oxidant, free radical scavenging activity	Attenuates experimental liver steatosis via regulation of lipid metabolism and hepatic enzymes	ALT, AST	[130,131]
Saucerneol G (lignans)	*Saururus chinensis*	Anti-fibrotic, hepatoprotective, anti-inflammatory	Attenuates liver fibrosis in rats by regulating hepatic enzyme and anti-oxidant activity	MDA, ALT, AST, HA, SOD, NF-kB, MAPKs	[132,133]
Salvianolic acid[a] B	*Salvia miltiorrhiza*	Anti-fibrotic, hepatoprotective	Inhibits HSC activation, ECM accumulation and HSC proliferation by modulating TGF-β_1	TGF-β1, TIMP1	[134,135]
Extract of Brucea	*Brucea javanica*	Anti-cancer, pro-HCC-specific apoptosis	Selectively induces HCC apoptosis by activating mitochondria-dependent apoptotic pathways	Cytochrome c, caspase3	[136,137]

(Contd...)

Table 1: (*Continued*)

Phyto-compound	Botanical source	Pharmacological activity	Putative mechanism of action	Target	References
Dioscin (steroidal saponins)	*Dioscorea opposita*	Anti-oxidant, anti-inflammatory, hepatoprotective	Attenuates NAFLD by modulating lipogenic and fibrogenic signaling	MAPK pathway, AST, ALT, NF-kB, TLR4, MyD88	[84,138,139]
Gallic acid (trihydroxybenzoic acid)	*Punica granatum*	Anti-oxidant, hepatoprotective	Attenuates NAFLD in rats by modulating choline metabolism and enhancd anti-oxidant activity	TAG, choline	[140,141]
Camptothecin (alkaloidal quinoline)	*Camptotheca acuminata*	Anti-HCC	Promotes HCC-specific apoptosis via TRAIL-mediated apoptosis	TRAIL, ERK, p38	[142]

ECM: Extracellular matrix, TGF-β: Transforming growth factor beta, NF-kB: Nuclear factor kappa B, FAS: Fatty acid synthase, SCD1: Stearoyl-CoA Desaturase1, IRS1: Insulin receptor substrate, CPT1: Carnitine palmitoyltransferase 1, SREBP: Sterol regulatory element-binding protein, CHM: Chinese herbal medicine, NO: Nitric oxide, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, TAMO: Trimethylamine N-oxide, PLA2: Phospholipase A2, HBV: Hepatitis B virus, IL: Interleukin, HBsAg: Hepatitis B surface antigen, HBeAg: Hepatitis B e antigen, HCC: Hepatocellular carcinoma, MAPKs: Mitogen-activated protein kinases, GSH: Glutathione, SOD: Superoxide dismutase, TNF-α: Tumor necrosis factor-alpha, PAI-1: Plasminogen activator inhibitor-1, MMP9: Matrix metallopeptidase 9, TLR: Toll-like receptors, ICAM-1: Intercellular adhesion molecule 1, eNOS: Endothelial NOS, IFN-β: Interferon gamma, JNKs: c-jun N-terminal kinases, MDA: Malondialdehyde, ALT: Alanine aminotransferase, HA: Hyaluronic acid, TIMP1: Tissue inhibitor of metalloproteinase-1, NAFLD: Nonalcoholic fatty liver disease, PAP: Phosphatidic acid phosphohydrolase, MyD88: Myeloid differentiation protein 88. [a]: (Have shown efficacy against hepatitis, liver fibrosis, fatty liver disease, cirrhosis and HCC); [b]: (Have shown efficacy against hepatitis, fatty liver disease, and cirrhosis); names in parenthesis '()' represent chemical classes

Table 2: Some CHM formulas, their composition and putative mechanisms of action against fibro-hepato-carcinogenesis

Name of Chinese herbal formula	Constituent herbs	Traditional Chinese indication	Current indication	Mechanism of action	References
Biejia* Ruangan tablets	*Placenta hominis, Radix notoginseng, Radix codonopsis, Astragalus membranaceus, Radix isatidis, Fructus forsythia, Rhizoma curcumae, Radix Paeoniae rubra, Radix angelica sinensis*	Enhances portal circulation and dissolves hepatic lumps	Anti-fibrotic, hepatoprotective	It produces hepatoprotection by regulating ECM metabolism mainly downregulating the expression of matrix proteins. TIMP-1 suppression in TGF-β/Smad3-dependent manner	[158,159]
Huanglian* Jiedu decoction	*Fructus gardeniae, Cortex phellodendri, Rhizoma coptidis, Scutellariae baicalensis*	Dissipates heat from qi	Anti-HCC, anti-cancer	Inhibits tumor growth, proliferation, angiogenesis via suppression of eEF2	[160]
Fuzheng* Huayu tablet	*Gynostemma pentaphyllammak, Salvia miltiorrhiza, Semen persicae, Fructus Schisandrae chinensis, Pollen pini, Radix fermentation mycelium*	To enhance blood flow, blood thinner, invigorates the liver	Treatment of HBV/HCV-related liver fibrosis	Its anti-fibrotic activity is attributed to repression of oxidant genes and modulation of TGF-β/Smad, VEGF and BM-7 signaling	[1,161,162]
Xiao Chai Hu Tang	*Scutellaria baicalensis, Bupleurum chinensis, Ginseng, Jujube, Licorice, Ginger, Pinellia*	To treat shaoyang diseases	To improve liver function; anti-HCC, anti-viral	Decreases fibrotic and oncogenic factors and genes by regulating TIMP-1, TNF-α, TGF-β/Smad signaling pathways	[57,163]
Huangqi decoction	*Astragalus membranaceus, Rhizoma glycyrrhizae*	Used to improve liver function	Anti-fibrotic, anti-cirrhosis	Attenuates experimental liver fibrosis via modulation of TGF-β and PDGF signaling pathways	[164]

*Contain other non-herbal components including fungi and minerals. HCC: Hepatocellular carcinoma, CHM: Chinese herbal medicine, HBV: Hepatitis B virus, ECM: Extracellular matrix, TIMP-1: Tissue inhibitor of metalloproteinase-1, TGF-β: Transforming growth factor beta, VEGF: Vascular endothelial growth factor, TNF-α: Tumor necrosis factor-alpha, PDGF: Platelet-derived growth factor

carcinogenic effects [Table 2].

PDGF

Many other CHMs in like manner have modulated cytokines involved in fibrogenesis. Example, *Ganoderma lucidum* extract and *Ganoderma* polysaccharide inhibited HSC proliferation through blockade of PDGFβR phosphorylation [165]. *Berberis anistata* fruit extract down-regulated expression of NF-κB, α-SMA, and TGF-β_1 [166,167]. *Ginkgo biloba* extract down-regulated expression of NF-κB, TGF-β_1, and collagen genes [70]. Cordyceps polysaccharide inhibited PDGF expression [168].

CHM TARGETS SPECIFIC GENES

Some CHMs exert specific effects on some genes, especially genes implicated in fibro-hepato-carcinogenesis.

c-fos and c-jun

Tetrandrine down-regulated c-fos and c-jun gene expressions, while they up-regulated expression of Smad7 [169].

Smurf2

Glycyrrhizin an isolate from *G. uralensis* decreased NF-κB binding activity and also down-regulated smurf2 gene expression [63]. *Buzhong Yiqi Tang* and *Renshen Yangrong Tang* produced significant immunomodulatory effects to reduce liver fibrosis [156].

CONCLUSION

Admittedly, pathogenesis of liver disease is complex, normally enjoying the participation of many cell types, cytokines, chemokines, adhesion molecules and genes, notwithstanding, efforts should be made to tailor scientific investigations to specific targets which are crucial for treatment of liver diseases. It is heartwarming the array of isolated phyto-compounds from CHMs which have demonstrated efficacy against various pathological manifestations of fibro-hepato-carcinogenesis such as liver fibrosis, steatohepatitis, cirrhosis, and HCC. Indeed, there has been an increased effort to characterize these phyto-compounds in the light of their reported indigenous uses but more still needed to be done. For instance, efforts should be focused on structure activity relations of these compounds to help advance understanding on their specific effects at the molecular level. It is long held that Chinese herbal formulas are the historical antecedents of modern-day combination therapy, valid as it may be, it is important that future studies thoroughly investigate individual compounds as single chemical entities, then their combined effects can be predicted with certainty. As it is now, it is difficult to tell which compound or extract from which component herb is producing which effect and to what extent. Although the "one fits all" leaning of westernized medicine is without challenges, it is also important that "many fits all" characteristic of CHMs is subjected to thorough component analysis. We agree with others who are in support of combinatorial approach since it taps into the enhanced synergistic actions of many compounds with varying pharmacological activities. However, the question of herb-herb and herb-drug interactions remains outstanding just as toxicity details. It is worth notice that future studies should address these concerns. CHMs exert multi-modulatory and multi-target effects against pathological manifestations (liver fibrosis, cirrhosis, and HCC) of fibro-hepato-carcinogenesis but future research efforts must focus on structural and functional elucidation of single compounds isolated from herbal components of Chinese herbal formulae.

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Review Article

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Therapeutic significance and pharmacological activities of antidiarrheal medicinal plants mention in Ayurveda: A review

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ABSTRACT

Diarrhea is a serious problem affecting 3-5 billion people per year around the world, especially children of below 5 years. 70% of the world population uses traditional and indigenous medicine for their primary health care. The facts of these indigenous remedies are passed verbally and sometimes as documents. Since ancient time, Ayurveda is the main system of healing in South East Asian countries. Indian literature from ayurvedic texts and other books claim the potency of several plants in the treatment of diarrhea. As the global prospective of ayurvedic medicine is increasing, interest regarding the scientific basis of their action is parallely increasing. Researchers are doing experiments to establish the relation between the claimed action and observed pharmacological activities. In the present article, an attempt was made to compile the scientific basis of medicinal plants used to cure diarrhea in Ayurveda. Literature was collected via electronic search (PubMed, ScienceDirect, Medline, and Google Scholar) from published articles that reports antidiarrheal activity of plants that were mentioned in Ayurveda classics. A total of 109 plant species belonging to 58 families were reported for their antidiarrheal activity. Several Indian medicinal plants have demonstrated promising antidiarrheal effects, but the studies on the antidiarrheal potentials of these plants are not taken beyond proof of concept stage. It is hoped that the article would stimulate future clinical studies because of the paucity of knowledge in this area.

KEY WORDS: Ayurveda, diarrhea, medicinal plant, traditional medicine

INTRODUCTION

Gastroenteritis is a clinico-pathological term that refers to inflammation and oxidative stress of the intestines which leads to disturbance in the balance of secretory and absorptive function of the intestines resulting in diarrhea [1,2]. Hence, diarrhea can be defined as a gastrointestinal disorder in which there is a rapid transit of gastric contents through the intestine, which is characterized by abnormal fluidity and high frequency of fecal evacuation, usually semisolid or watery fecal matter, three or more times/day [1-3]. There is an increase in flow rate of feces with or without the presence of blood and mucus, accompanied by increased secretion and decreased absorption of fluid, leading to loss of water and electrolytes [2,4]. The major causative agents of diarrhea in human beings include a variety of enteric pathogenic bacteria such as *Salmonella typhi*, *Shigella flexneri*, *Escherichia coli*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Candida albicans* [4,5]. Viruses, protozoans, helminths, intestinal disorders, immunological factor, and medications can also cause diarrhea in human being [6-8]. Etiological factors for diarrhea include the food intolerances, contaminated drinking water, undercooked meat and eggs, inadequate kitchen hygiene, poor sanitation [9], bile salts, hormones, irritable bowel syndrome, and intoxication [10]. According to the World Health Organization (WHO), diarrhea affects 3-5 billion people/year worldwide and causes 5 million deaths per annum [11]. Children, however, are more susceptible to the disease, which is the one of the leading causes of death in infants and children below 5 years of age [12].

Due to high mortality and morbidity, especially in children, the WHO together with the United Nations Children's Fund has initiated Diarrhea Disease Control Program to control diarrhea in developing countries. Oral rehydration solution [13], zinc solution [14], probiotics [15], and specific antibiotics have reduced mortality rate in diarrheal disease. However, chronic diarrhea is still a life challenging problem in some regions of the world. Unfortunately, the program does not reach to the needy, and the disease is still a major challenge in front of primary health practitioner as well as researcher. Therefore, the different traditional systems of medicines such as Chinese medicine [16], Japanese medicine [17], acupuncture therapy [18], and ayurvedic medicine [19] are included in this program.

Since ancient time's medicinal plants have been used to treat different ailments due to their accessibility, availability, inherited practice, economic feasibility, and perceived efficacy [20]. Nowadays, use of medicines from plant source increases significantly with conventional therapies. Hence, the plants are gaining more attention by the researchers to find out new and effective agents for different diseases. Several medicinal plants in the different regions of the world have been used to cure diarrhea [19,21].

The knowledge of indigenous medicines is passing from generation to generation orally worldwide [22]. It is, therefore, documentation of such knowledge as well as reported the scientific basis of their pharmacological potential is necessary since they are usually consider as free from adverse effects. A range of medicinal plants were reported for their effectiveness in diarrhea [23-27]. The protective role of these plants is probably due to their anti-inflammatory, antioxidant, and astringent properties [28]. India has a rich plant resources providing valuable medicine, which are conveniently used in Ayurveda, Unani, and other system of medicines for the treatment of various diseases [29]. Keeping this in view, the present article was initiated, with an aim to compile the scientific basis of medicinal plants used to cure diarrhea. A variety of curative agents from these indigenous plants has been isolated. These isolated compounds are belonging to different phytochemical classes such as flavonoids, saponins, terpenoids, steroids, phenolic compounds, and alkaloids [30-32]. Flavonoids and saponins inhibit the release of prostaglandins, autocoids, and contractions caused by spasmogens as well as motility and hydroelectrolytic secretions [33,34] while saponins may prevent release of histamine [35]. Polyphenols and tannins provide strength to intestinal mucosa, decrease intestinal secretion, intestinal transit and promotes balance in water transport across the mucosal cells [36].

Previously, we enumerated a large number of plants, which are used in the ayurvedic system as antidiarrheal [19]. A majority of these plants have been investigated pharmacologically with respect to the potential antidiarrheal activity. In this review, we present ethnopharmacological data of 109 plant species belonging to 58 families mentioned in ayurvedic texts for controlling diarrhea with their possible mechanism of action [Table 1 and Figure 1]. Mostly, leaf (23%), root (14%), barks (11%), fruit (9%), and seed (8%) of the plants are used for antidiarrheal activity [Figure 2].

DISCUSSION

Since ages, human beings have relied on plants as a resource of the therapeutic arsenal in the fight against certain human diseases. Plant-based drugs have formed the basis of traditional medicine systems, i.e., Ayurveda, Siddha, Unani, Homeopathy, and Chinese. Herbal-based therapy is one of the popular and effective practices to overcome the illness. The WHO also promotes utilization of local knowledge of plant-based medicines in health care. It has been reported by the WHO that about 70-80% of the population in developing countries relies on traditional/ethno medicines/for their primary health care. Since ancient time ayurvedic system of medicine is indigenous to and widely practiced in India. Nature has bestowed India with an enormous wealth of medicinal plants. Therefore, their rational uses for combating diseases are described traditionally.

Acharya charaka has mentioned a group of antidiarrheal plants named as *Purish-Samgarahaniya Mahakashaya*, which includes priyangu (*Callicarpa macrophylla*), ananta (*Hemidesmus indicus* R.B.), seed of amra (*Mangifera indica*), katvanga (*Ailanthus excelsa* Roxb.), lodhra (*Symplocos racemosa*), mocharasa (*Salmalia malabarica* Schott and Endl.), samanga – *Rubia cordifolia*, flower of dhataki – *Woodfordia fruticosa*, padma – lotus (*Nelumbo nucifera*), and filaments of padma – lotus (*N. nucifera*). Moreover, he also listed some most useful antidiarrheal plants such as katavanga (*A. excelsa* Roxb.), *mustaka* (*Cyperus rotundus* Linn.), amrita (*Tinospora cordifolia* [Willd.] Miers ex Hook. f. & Thoms.), ativisha (*Aconitum heterophyllum* Wall. ex. Royle.), bilva (*Aegle marmelos* Correa), kumuda (*N.nucifera* Gaertn.), utpala, padma, kutaja bark (*Holarrhena antidysenterica* [Linn.] Wall.), gambhari fruit (*Gmelina arborea* Roxb.), prishniparni (*Uraria picta* [Jacq.] Desv. Ex DC.), and bala (*Sida cordifolia*) [187]. In addition, Acharya Susuruta mentioned that the vacha (*Acorus calamus* Linn.) and haridra (*Curcuma longa* Linn.), etc., are best for *amatisara* (diarrhea where undigested food matter pass in stool) while ambastha (*Cissampelos pareira* Linn.) and priyangu (*C. macrophylla*) are best for pakwatisara (diarrhea where only digested food matter pass in stool) [188].

The ayurvedic Pharmacopoeia mentioned more than 1200 species of plants, nearly 100 minerals and over 100 animal products officially. Although there is no record of pharmacological testing during the period when ayurvedic texts were written. However, nowadays, extensive researches are carried out concerning the phytopharmacological basis of their therapeutic principles. Public, academic as well as government organizations are showing interest in the scientific mechanism of action exerted by these plants. Similar to modern and other traditional medicines, ayurvedic medicines have been also evaluated for their phytopharmacology with the help of advances in science and technology. Scientific screening on laboratory animal and *in vitro* evaluations supports traditional uses of medicinal plants.

In the present scenario, modern pharmaceuticals offer a number of medicines for diarrhea, but diarrhea still remains a major health threat to the people in tropical and subtropical countries. It is one of the leading causes of mortality in children especially under the age of 5 years [12]. Different factors such as infections, malnutrition, food intolerances, intestinal disorders, and some medications may trigger diarrhea [6-8]. Currently, available pharmacological treatments are seem to be insufficient in diarrhea control. It is because of lack of admittance, high cost, and adverse effects of modern pharmaceuticals as well as therapeutic approaches. Therefore, investigations on drugs from different alternative and complementary medicines along with traditional system of medicines were going on.

Table 1: Antidiarrheal medicinal plants

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Atibala	*Abutilon indicum* (Linn.) Sweet.	Malvaceae	Leaf	Methanolic and aqueous extract	Loperamide (1 mg/kg)	Gastrointestinal motility test, castor oil-induced diarrhea model, and PGE_2-induced enteropooling model		[37]
Khadir	*Acacia catechu* Willd.	Fabaceae	Heartwood	Ethyl acetate extract (250 mg/kg)	Diphenoxylate (10 mg/kg) and atropine (1 ml/200 g, p.o.)	Castor oil-induced diarrhea model		[38]
Babool	*Acacia nilotica* Delile & Ssp. *indica* (Benth.) Brenan.	Caesalpiniaceae	Bark	Petroleum ether, methanolic and aqueous extract		Castor oil- and magnesium sulfate-induced diarrhea and barium chloride-induced gastrointestinal motility test		[39]
Ativisha	*Aconitum heterophyllum* Wall. ex. Royle.	Ranunculaceae	Root	Ethanolic extract (50, 100, and 200 mg/kg) and isolated aconitine	Loperamide (2 mg/kg, p.o.) and atropine (0.1 mg/kg, s.c.)	Castor oil-induced diarrhea model, Small intestinal transit time, PGE_2-induced enteropooling, and gastric emptying test	Prevented Na^+ and K^+ loss	[40]
Vacha	*Acorus calamus* Linn.	Araceae	Root and essential oil	Methanolic extract and n-hexane fraction		Castor oil-induced diarrhea model, spasmolytic activity	Inhibition of spontaneous and High K^+-induced contractions and antispasmodic action	[41,42]
			Rhizome	Aqueous and methanolic extract (3, 7.5, and 15 mg)		Castor oil-induced diarrhea model		
Bilva	*Aegle marmelos* Correa.	Rutaceae	Unripe fruit pulp	Aqueous extract	-	Antimicrobial activity	Through reduced bacterial adherence to intestinal wall and Invasion of Hep-2 cells	[43-50]
			Leaf	Aqueous extract (50, 100, and 200 mg/kg)	Loperamide (3 mg/kg orally)	Castor oil-induced diarrhea, magnesium sulfate-induced diarrhea, and gastric transit time		
			Fruit	Polyherbal formulation (25, 50, and 100 mg/kg)	Mebarid (10 ml/kg, p.o.)	Castor oil-induced diarrhea model, intestinal secretion model, and antispasmodic effect	Inhibition of intestinal transit of food material and inhibition of intestinal secretion	
			Unripe fruit	Aqueous and methanolic extract		Castor oil-induced diarrhea model		
			Fruit	Aqueous extract	Diphenoxylate and yohimbine	Castor oil-induced diarrhea model		
			Dried fruit pulp	Ethanolic extract	-	*In vitro* antibacterial activity		
			Root	Chloroform extract	-	Castor oil-induced diarrhea model		

Contd...

Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
			Unripe fruit	(50, 100 mg/kg)	-	Gastrointestinal motility test and castor oil-induced diarrhea model		
Gorakhganja	*Aerva lanata*	Amaranthaceae	Whole plant	Alcoholic and aqueous extract (400 and 800 mg/kg)	Loperamide (3 mg/kg, i.p.)	Castor oil-induced diarrhea		[51]
Aralu	*Ailanthus excelsa* Roxb.	Simaroubaceae	Bark	Chloroform, aqueous and ethanolic extract	Atropine (0.1 mg/kg, i.p.)	Castor oil-induced diarrhea model		[52]
Shirish	*Albizia lebbeck* Benth.	Caesalpiniaceae	Seed	Crude extract	Loperamide (1 mg/kg, i.p.)			[53]
Saptaparna	*Alstonia scholaris* R.Br.	Apocynaceae	Bark	Methanolic extract	Loperamide	Castor oil-induced diarrhea model	Spasmolytic activity mediated possibly through CCB	[54,55]
				Ethanolic extract (250 mg/kg)	Loperamide (50 mg/kg)	Castor oil-induced diarrhea model		
Tanduliya	*Amaranthus spinosus* Linn.	Amaranthaceae	Whole plant	Ethanolic extract (100, 200, and 400 mg/kg)	Yohimbine	Castor oil-induced diarrhea model, charcoal-induced gastrointestinal motility test		[56]
Eshwari	*Aristolochia indica* Linn.	Aristolochiaceae	Root	Aqueous extract and ethanolic extract	Diphenoxylate (5 mg/kg, p.o.)	Castor oil-induced diarrhea model, charcoal-induced gastrointestinal motility test		[57]
Shatavari	*Asparagus racemosus* Willd.	Liliaceae	Root	Ethanolic and aqueous extract	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model, gastrointestinal tract motility, PGE_2-induced enteropooling		[58]
Hijjala	*Barringtonia acutangula* (Linn.) Gaertn.	Lecythidaceae	Root	Ethanolic extract (200 and 400 mg/kg)	Diphenoxylate (5 ml/kg, p.o.)	Castor oil-induced diarrhea model		[59,60]
			Leaf and seed	Methanolic extract (200 and 400 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil and magnesium sulfate-induced diarrheal models		
Kovidara	*Bauhinia purpurea* Linn.	Caesalpiniaceae	Leaf	Ethanolic extract		Castor oil and magnesium sulfate-induced diarrheal models		[61]
Kanchnar	*Bauhinia variegata* Linn.	Caesalpiniaceae	Stem bark	(250, 500, and 1000 mg/kg, p.o.)		Castor oil-induced diarrhea, castor oil magnesium sulfate-induced enteropooling, and gastrointestinal motility test using charcoal meal test		[62]
Daruharidra	*Berberis aristata* DC.	Berberaceae	Stem	Aqueous extract	Loperamide (2 mg/kg, po)	Castor oil-induced diarrhea model	Inhibit the intestinal secretary response	[63-65]
			Bark	Ethanolic, aqueous extract and isolated berberine	Loperamide (25 mg/kg)	Castor oil-induced diarrhea model		
			Stem	Ethyl alcohol extract (250, 500 mg/kg)	Loperamide (3 mg/kg orally)	Magnesium sulfate-induced diarrhea, castor oil-induced intestinal secretions		
Sinduri	*Bixa orellana* Linn.	Bixaceae	Leaf	Methanolic extract (125, 250, and 500 mg/kg)	Loperamide (3 mg/kg orally)	Castor oil-induced diarrhea model		[66]

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Sallaki	*Boswellia serrata* Roxb. Ex Coleb.	Burseraceae	Gum resin	Hydroalcoholic extract and 3–acetyl–11–keto–b–boswellic acid	Atropine (1 mg/kg)	Upper gastrointestinal transit in croton oil-treated animal, castor oil-induced diarrhea model	Inhibition of acetylcholine-induced contractions by the L-type Ca^{2+}channel blockers	[67]
Parnabija	*Bryophyllum pinnatum* (Lam.) Kurz.	Crassulaceae	Leaf	Aqueous extract (100, 200, and 300 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model, castor oil-induced enteropooling, small intestinal transit time		[68]
Priyala	*Buchanania lanzan* Spreng.	Fagaceae	Leaf	(200 and 400 mg/kg)	Loperamide (1 mg/kg)	Castor oil-induced diarrhea model, charcoal meal test	Inhibition Na^{+}-K^{+}ATPase activity	[69]
Palash	*Butea monosperma* Lam. Kuntze.	Fabaceae	Stem bark	Ethanolic extract	Loperamide (3 mg/kg orally)	Castor oil-induced diarrhea model and PGE_2-induced enteropooling		[70]
Latakaranja	*Caesalpinia bonducella* Flem.	Caesalpiniaceae	Leaf	Methanolic extract and its ethyl acetate, chloroform, and petroleum ether fractions (200 and 400 mg/kg)	Loperamide (5 mg/kg, p.o.)	Castor oil-induced diarrhea model	Antibacterial activity	[71]
Gumohar	*Caesalpinia pulcherrima* L.	Caesalpiniaceae	Bark	Ethanolic extract (500 mg/kg)	Loperamide (50 mg/kg orally)	Castor oil-induced diarrhea model		[72]
Arka	*Calotropis gigantea* R.Br.	Asclepiadaceae	Aerial part	Hydroalcoholic extract (200 and 400 mg/kg)	Atropine (3 mg/kg, i.p.)	Castor oil-induced diarrhea model		[73]
Arka	*Calotropis procera* (Ait.) R.Br.	Asclepiadaceae	Dry latex	(500 mg/kg)	Atropine (0.1 mg/kg, i.p.)	Castor oil-induced enteropooling, electrolyte concentration in the intestinal fluid and intestinal transit		[74-77]
			Latex	Ethanolic extract (250, 500 mg/kg)	Loperamide (4 mg/kg)	Castor oil-induced diarrhea model		
			Leaf	Methanolic extract	Loperamide (3 mg/kg orally)	Castor oil-induced diarrhea model		
				Aqueous and alcoholic extract (100, 200 mg/kg)		Castor oil-induced diarrhea model, charcoal meal test, enteropooling method		
Tea	*Camellia sinensis* (Linn.) O. Kuntze.	Theaceae	Leaf	Aqueous extract		Castor oil-induced diarrhea model and PGE_2-induced enteropooling		[78]
Hinsra	*Capparis zeylanica* Linn.	Capparidaceae	Leaf	Methanolic extract (100, 150, and 200 mg/kg)	Loperamide (3 mg/kg orally)	Castor oil-induced diarrhea model and small intestine transit method		[79]
Erand karkati	*Carica papaya* Linn.	Caricaceae	Fruit	Alcoholic and aqueous extract (100, 200 and 400 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model and magnesium sulfate-induced diarrhea		[80]
Shitiwar	*Celosia argentea* Linn.	Amaranthaceae	Leaf	Alcoholic extract (100, 200 mg/kg)	Atropine (0.1 mg/kg, s c)	Castor oil-induced diarrhea model, charcoal meal test, PGE_2-induced diarrhea		[81]
Patra	*Cinnamomum tamala* Buch.–Ham.	Lauraceae	Bark	Ethanolic extract (25, 50, and 100 mg/kg)		Castor oil-induced diarrhea model		[82]
Twaka	*Cinnamomum zeylanicum* Linn.	Lauraceae	Bark	Aqueous extract (100 and 200 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model and magnesium sulfate-induced diarrhea		[83]

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Patha	*Cissampelos pareira* Linn.	Menispermaceae	Root	Ethanolic extract (25-100 mg/kg)		Castor oil-induced diarrhea model	Inhibitory effect on the concentration of Na^+ and K^+, reduction in the lipid peroxidation and Prevention from oxidative stress	[84]
Hulhul	*Cleome viscosa* L.	Capparidaceae	Whole plant	Methanolic extract	Diphenoxylate (5 mg/kg orally)	Castor oil-induced diarrhea model and PGE_2-induced enteropooling gastrointestinal motility		[85]
Vaamana–haati	*Clerodendrum indicum*	Verbenaceae	Leaf	Methanolic extract and chloroform fraction	-	Castor oil-induced diarrhea model		[86]
Aparajita	*Clitoria ternatea* L.	Fabaceae	Leaf	Methanolic extract (100, 200, and 300 mg/kg)	Loperamide (3 mg/kg)	Castor oil-induced diarrhea model and small intestine transit method		[87,88]
			Root	Alcoholic extract (100, 200, and 400 mg/kg)	Atropine (5 mg/kg, i.p.)	Castor oil-induced diarrhea model, intestinal transit and castor oil-induced enteropooling		
Dhanyaka	*Coriandrum sativum* Linn.	Apiaceae	Leaf	Aqueous extract (150 and 300 mg/kg)	Loperamide (3 mg/kg)	Castor oil-induced diarrhea model		[89]
Varuna	*Crataeva nurvala* Buch.–Ham.	Capparidaceae	Stem bark	Ethanolic extract (500 mg/kg)		Castor oil-induced diarrhea model, castor oil-induced enteropooling, and small intestine transit model		[90]
Jiraka	*Cuminum cyminum* Linn.	Apiaceae	Seed	Aqueous extract	Loperamide (3 mg/kg)	Castor oil induce diarrhea model, PGE2-induced enteropooling model, intestinal transit by charcoal		[91]
Haridra	*Curcuma longa* Linn.	Zingiberaceae	Rhizome	Aqueous extract (200 mg/kg)		Castor oil-induced diarrhea model		[92]
Durva	*Cynodon dactylon* Pers.	Poaceae	Whole plant	Methanolic extract (200 and 300 mg/kg)	Atropine (5 mg/kg orally)	Castor oil-induced diarrhea model, gastrointestinal charcoal meal test, and enteropooling model		[93]
Mustaka	*Cyperus rotundus* Linn.	Cyperaceae	Rhizome	Methanolic extract (250-500 mg/kg)		Castor oil-induced diarrhea model		[94,95]
				Aqueous extract	-	Antibacterial activity against EPEC and EIEC and Shigella flexneri	Antibacterial, antigiardial and antirotaviral activities	
Goraksha	*Dalbergia lanceolaria* Linn.f.	Fabaceae	Bark	Petroleum ether, ethanolic extract	Diphenoxylat (5 mg/kg, p.o.)	Castor oil and magnesium sulfate-induced diarrhea		[96]
Shimsapa	*Dalbergia sissoo* Roxb. ex DC.	Fabaceae	Leaf	Ethanolic extract		Castor oil-induced diarrhea model and magnesium sulfate $MgSO_4$-induced diarrhea		[97]
Kusha	*Desmostachya bipinnata* L.	Poaceae		Alcohól aqueous extract (200, 400 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model, gastrointestinal motility test with charcoal meal test		[98]
Virataru	*Dichrostachys cinerea* W. & A.	Mimosaceae	Leaf bark and root	Ethanolic extract (200 and 400 mg/kg)	Loperamide (5 mg/kg, p.o.)	Castor oil-induced model and small intestinal transit model		[99]
Tinduka	*Diospyros peregrina* Gruke.	Ebenaceae	Bark and seed	(250 and 500 mg/kg)		Castor oil-induced diarrhea model		[100]

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Amalaki	*Emblica officinalis* Gaertn.	Euphorbiaceae	Fruit	Ethanolic extract (500 mg/kg)	Loperamide (3 mg/kg)	Castor oil-induced diarrhea model	Inhibition of intestinal motility, antimicrobial action, and antisecretory effects	[101-103]
				Crude extract (500-700 mg/kg)	Loperamide (10 mg/kg)	Castor oil-induced diarrhea model and enteropooling model	Mediated possibly through dual blockade of muscarinic receptors and Ca^{2+} channels	
				Methanolic extract		Diarrhea-induced by castor oil and magnesium sulfate, gastrointestinal motility in charcoal meal tests, and PGE_2-induced enteropooling		
Paribhadra	*Erythrina indica* Lam.	Fabaceae	Leaf	Ethanolic and aqueous extract (500 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model, PGE_2-induced intestinal fluid enteropooling, and gastro intestinal tract motility model		[104]
Dugdhika Big	*Euphorbia hirta* Linn.	Euphorbiaceae	Whole plant	Ethanolic and water extract (2.0 mg/kg)		Castor oil-induced diarrhea model, PGE_2-induced enteropooling, gastrointestinal motility in both $BaSO_4$ and charcoal meal tests		[105]
Kapittha	*Feronia limonia* Linn. Swingle	Rutaceae	Leaf	Ethanolic extract (250, 500 mg/kg)	Loperamide (50 mg/kg)	Antibacterial activity, castor oil-induced diarrhea model		[106,107]
				Ethanolic extract (500 mg/kg)	Loperamide (25 mg/kg)	Castor oil-induced diarrhea model		
Vata	*Ficus benghalensis*	Moraceae	Leaf	Methanolic (3, 7.5 and 15 mg/kg)		Castor oil-induced diarrhea model	Increasing colonic water and electrolyte re-absorption or by inhibiting intestinal motility	[108,109]
			Root	Ethanolic extract (400 mg/kg)	Diphenoxylate (5 mg/kg, p.o.)	Gastrointestinal motility in charcoal meal test, castor oil-induced diarrhea model, and PGE_2-induced enteropooling		
Kakodumbara	*Ficus hispida* Linn.	Moraceae	Leaf	Methanolic extract	Diphenoxylate (5 mg/kg, p.o.)	Castor oil-induced and PGE_2-induced enteropooling model		[110]
Udumbara	*Ficus racemosa* Linn.	Moraceae	Bark	Ethanolic extract 400 mg/kg	Diphenoxylate (5 mg/kg, p.o.)	Castor oil-induced diarrhea model and PGE_2-induced enteropooling model		[109]
Ashvattha	*Ficus religiosa* Linn.	Moraceae	Stem bark	Hydroalcoholic, acetone extract	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model		[111]
Udumber	*Ficus glomerata* L.	Moraceae	Leaf	Methanolic extract (100 and 200 mg/kg)	Atropine (3 mg/kg)	Castor oil-induced diarrhea model, castor oil-induced enteropooling, and intestinal transit		[112]
Parpata	*Fumaria parviflora*	Papeveraceae	Aerial part	Aqueous and methanolic extract	Dicyclomine, (50 and 100 mg/kg) and loperamide (10 mg/kg, p.o.)	Castor oil-induced diarrhea model	CCB blockade of muscarinic receptors	[113]

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Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Kasmari	*Gmelina arborea* Roxb.	Verbenaceae	Root	Ethanolic and N–butanol (200, 400 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model		[114,115]
			Stem bark	Aqueous and methanolic extract (0.5, 1.0 mg/ml)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model		
Sariva	*Hemidesmus indicus* R.Br.	Apocynaceae	Root	Methanolic extract (500-1500 mg/kg)		Castor oil-induced diarrhea model	Inhibition of intestinal motility and bactericidal activity	[116,117]
				Aqueous and ethanolic extract (100 and 200 mg/kg)	Loperamide (3 mg/kg)	Charcoal meal test and enteropooling model		
Kutaja	*Holarrhena antidysenterica* (Linn.) Wall.	Asclepiadaceae	Seed	Ethanolic extract (200-800 mg/kg), Isolated alkaloid		Castor oil-induced diarrhea model, antibacterial activity against EPEC *in vitro*		[118]
Cirabilva	*Holoptelea integrifolia* Planch.	Urticaceae	Leaf	Ethanolic extract (250 and 500 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil and magnesium sulfate-induced diarrhea model		[119]
Bandhuka	*Ixora coccinea* Linn.	Rubiaceae	Flower	Aqueous extract (400 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model		[120,121]
			Leaf	Aqueous extract (400 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model		
Vyaghra errand	*Jatropha curcas* Linn.	Euphorbiaceae	Root	Methanolic extract (50 and 100 mg/kg)	Chlorpromazine (30 mg/kg, i.p.)	Castor oil or magnesium sulfate-induced diarrhea	Inhibition of prostaglandin biosynthesis and reduction of osmotic pressure, decreases in peristaltic activity, Castor oil-induced permeability changes in intestinal mucosal membrane to water and electrolyte	[122,123]
				Petroleum ether and methanolic extract	Chlorpromazine (30 mg/kg, i.p.)	Castor oil-induced diarrhea model, gastrointestinal motility after charcoal meal	Inhibition of prostaglandin biosynthesis and reduction propulsive movement of small intestine	
Madhuca	*Madhuca indica* J. F. Gmel.	Sapotaceae	Dried bark	Ethanolic extract (250 and 500 mg/kg)	Loperamide (50 mg/kg)	Castor oil-induced diarrhea model		[124]
Amra	*Mangifera indica* Linn.	Anacardiaceae	Stem bark and root bark	Methanolic extract (3, 7.5, and 15 mg/kg)		Castor oil-induced diarrhea model	By increasing colonic water and electrolyte reabsorption or by inhibiting intestinal motility	[108,125-127]
			Seed	Alcoholic and aqueous extract	Loperamide	Castor oil-induced diarrhea model		
			Leaf	Aqueous extract (25 and 50 mg/kg)	Loperamide (2 mg/kg)	Castor oil-induced diarrhea model	Enhancement of Na^+-K^+ ATPase activity	
			Seed	Methanolic and aqueous extract (250 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil- and magnesium sulfate-induced diarrhea model		

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Pudina	*Mentha longifolia* (Linn.) Huds.	Lamiaceae	Whole plant	Crude extract, petroleum spirit fraction, aqueous fraction (100-1000 mg/kg)	Loperamide	Castor oil-induced diarrhea model	Inhibition of spontaneous and high K^+induced contractions, spasmolytic activity, mediated possibly through CCB	[128,129]
			Essential oil	Essential oil (20-80 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model		
Lajjalu	*Mimosa pudica* Linn.	Mimosaceae	Leaf	Ethanolic extract (200 and 400 mg/kg)		Castor oil-induced diarrhea model and PGE_2-induced enteropooling, gastrointestinal motility in charcoal meal test		[130,131]
			Root	Ethanolic and aqueous extract (150 and 250 mg/kg)		Gastrointestinal motility in charcoal meal test		
Karvellaka	*Momordica charantia* Linn.	Cucurbitaceae	Leaf	Aqueous extract		Castor oil-induced diarrhea model, gastrointestinal transit, intestinal fluid accumulation and gastric emptying		[132]
Shobhanjana	*Moringa oleifera* Lam.	Moringaceae	Leaf	Hydroalcoholic extract (2500 mg/kg)	Loperamide (3 mg/kg)	Castor oil- and magnesium sulfate-induced gastrointestinal motility, castor oil, and PGE_2-induced Enteropooling, charcoal meal test		[133,134]
				Ethanolic extract (150 and 300 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model		
Surabhi–nimba	*Murraya koenigii* (Linn.) Spreng.	Rutaceae	Leaf	Aqueous extract (200 mg/kg) and alcoholic extract (400 mg/kg)	Loperamide (2 mg/kg)	Castor oil-induced diarrhea model, charcoal meal test, and PGE_2-induced diarrhea		[135,136]
			Seed	Hexane extract, carbazole alkaloids kurryam, koenimbine and koenine	Diphenoxylate (5 mg/kg orally) Atropine (0.1 mg/kg, i.p.)	Castor oil-induced diarrhea model and PGE_2-induced enteropooling, gastrointestinal motility in the charcoal meal test		
Kamini	*Murraya paniculata* (L.) Jack.	Rutaceae	Leaf	Ethanolic extract (300 and 600 mg/kg)	Loperamide (50 mg/kg)	Castor oil-induced diarrhea model		[137]
Kadali	*Musa paradisiaca* Linn.	Musaceae	Sap	0.25, 0.50, and 1.00 mL	Loperamide (2.5 mg/kg) Atropine (2.5 mg/kg)	Castor oil-induced diarrhea model, castor oil-induced enteropooling, and gastrointestinal motility	Inhibition Na^+-K^+ atpase activity	[138]
Jatiphala	*Myristica fragrans* Houtt.	Myristicaceae	Flower bud	Aqueous extract and petroleum ether extract	Atropine	Antispasmodic	Inhibited the contraction produced by acetylcholine, Histamine, and prostaglandin	[139]
Kamala	*Nelumbo nucifera* Gaertn.	Nymphaeaceae	Rhizome	(100, 200, 400, and 600 mg/kg)		Castor oil-induced diarrhea model and PGE_2-induced enteropooling and charcoal meal test		[140-142]

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
			Root bark Rhizome	Methanolic extract (100, 200, 400, and 600 mg/kg)	Atropine (0.1 mg/kg) Diphenoxylate (5 mg/kg)	Castor oil-induced diarrhea and PGE_2-induced enteropooling		
			Seed oil	Crude hexane extract	-	*In vitro* antibacterial activity against *Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhimurium, Escherichia coli*, and *Enterobacter aerogenes*		
Trivrita	*Operculina tuperthum*. (Linn.) Silva Manso.	Convolvulaceae	Root	Aqueous, ethanolic extract (300-1000 mg/kg)	Loperamide (10 mg/kg, p.o.)	Castor oil-induced diarrhea model	Possibly through the presence of Ca^{++} antagonist	[143]
Syonaka	*Oroxylum indicum* Vent.	Bignoniaceae	Stem bark	Methanolic extract (400 mg/kg)	Loperamide (66.67 μg/kg, p.o.)	Castor oil-induced diarrhea model		[144,145]
			Bark	Flavonoids rich fraction		Castor oil and magnesium sulfate-induced diarrheal models, barium chloride, and acetylcholine-induced intestinal contraction	Alteration of intestinal motility through modification in L^- type Ca^{2+} channels	
Cangeri	*Oxalis corniculata* Linn.	Gerniaceae	Whole plant	Aqueous and Methanolic extract (160, 320, and 640 mg/kg)	Loperamide (29.6 μg/kg, p.o.)	Castor oil-induced diarrhea model		[146]
Gandhaprasharni	*Paederia foetida* Linn.	Rubiaceae	Root	Ethanolic extract (100, 250, and 500 mg/kg)	Atropine (5 mg/kg)	Castor oil-induced diarrhea model, magnesium sulfate-induced diarrhea, gastrointestinal motility with barium sulfate milk, cisplatin-induced gastrointestinal motility, morphine-induced reduction of motility		[147]
Pind kharjura	*Phoenix dactylifera* Linn.	Palmaceae	Fruit	Aqueous extract (1000 and 1500 mg/kg)	Loperamide (5 mg/kg)	Castor oil-induced diarrhea model, enteropooling model, and gastrointestinal motility test		[148]
Maricha	*Piper nigrum* L.	Piperaceae	Fruit	Piperine			-	[149-153]
			Fruit	Aqueous extract (75, 150, and 300 mg/kg)	Loperamide (2 mg/kg, p.o.) Atropine (5 mg/kg, i.p.) Chlorpromazine (30 mg/kg, i.p.)	Castor oil and magnesium sulfate-induced diarrhea charcoal meal test and castor oil-induced intestinal secretions		
			Fruit	Aqueous extract (300 mg/kg)	Isosorbide dinitrate (150 mg/kg, p.o.) Glibenclmide (1 mg/kg, p.o.) Yohimbine (1 mg/kg, s.c.)	Castor oil-induced diarrhea model	On α_2 adrenergic receptors, potassium channels, and nitric oxide pathway	

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
				Piperine (8 and 32 mg/kg)		Castor oil, $MgSO_4$ and arachidonic acid-induced diarrhea, castor oil induced enteropooling	Inhibitory effect on prostaglandins	
				Piperine (10 mg/kg)		Castor oil-induced diarrhea model	Concentration-dependent inhibition of spontaneous contractions, CCB effect. Piperine (10-100 μm) caused a rightward shift in the Ca^{++} concentration–response curves in Ca^{++}-free medium	
Karkatasringi	*Pistacia integerrima* (J. L. Stewart ex Brandis)	Anacardiaceae	Gall	Methanolic extract (700 and 900 mg kg)	Loperamide (10 mg/kg)	Castor oil-induced diarrhea model, spontaneous contractions in isolated rabbit jejunum		[154]
Karanja	*Pongamia pinnata* (Linn.) Pierre.	Fabaceae	Leaf	Aqueous extract	-	Antibacterial, antigiardial and antirotaviral activity	Inhibits adherence of EPEC and invasion of EIEC and *Shigella flexneri* to epithelial cells	[155]
Peruka	*Psidium guajava* Linn.	Myrtaceae	Leaf	Aqueous extract	Loperamide (10 mg/kg, p.o.)			[156-158]
			Leaf	Aqueous extract (50-400 mg/kg)		Castor oil-induced diarrhea model		
			Bark	Methanolic and aqueous extract (100 mg/kg)	Loperamide (1 mg/kg, i.p.)	Gastrointestinal Motility, castor oil-induced diarrhea model, and PGE_2-induced enteropooling		
Bijaka	*Pterocarpus marsupium*	Fabaceae	Heartwood	Ethanolic extract (250 and 500 mg/kg,)	Loperamide (5 mg/kg, p.o.)	Castor oil and charcoal-induced gastrointestinal motility test, intestinal transit of charcoal meal		[159]
Dadima	*Punica granatum* Linn.	Punicaceae	Seed	Methanolic extract		Castrol oil-induced diarrhea and PGE_2-induced enteropooling	Antimotility and antisecretory activity	[160-162]
			Peels	Aqueous extract (100, 200, 300, and 400 mg/kg)		Castor oil-induced diarrhea model, spontaneous movement of the isolated rat ileum, acetylcholine-induced contractions test		
			Rinds of fruit	Polyherbal formulation Aqueous extract	Mebarid (10 ml/kg, po)	Castor oil-induced diarrhea model, intestinal secretion, and charcoal meal test		
Mayaphala	*Quercus infectoria*	Fagaceae	Gall	Ethanolic extract (250 and 500 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil and magnesium sulfate-induced diarrhea models		[163]
Sarpagandha	Rauvolfia serpentina Benth. ex Kurz.	Apocynaceae	Root	Methanolic extract (100, 200, and 400 mg/kg)	Diphenoxylate (5 mg/kg, p.o.)	Castor oil-induced diarrhea model		[164]
Manjistha	*Rubia cordifolia* L.	Rubiaceae	Root	Ethanolic extract (50, 100 mg/kg)		Castor oil-induced diarrhea model, gastrointestinal transit time	Decrease in both sodium and potassium excretion in the intestine	[165]

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Chandan	*Santalum album* Linn.	Santalaceae	Heartwood	Methanolic extract (200, 400, and 800 mg/kg)		Castor oil-induced diarrhea model	Spasmolytic role relaxed the acetylcholine-induced, 5-HT-induced and K^+-induced contractions	[166]
Asoka	*Saraca asoca* (Roxb.) De Wilde	Caesalpiniaceae	Stem bark	Hydroalcoholic, acetone extract (200 mg/kg)	Loperamide (3 mg/kg, p.o.)	Castor oil-induced diarrhea model		[167]
Kushtha	*Saussurea lappa* Clarke	Asteraceae	Essential oil	100, 300, and 500 mg/kg	Loperamide (5 mg/kg)			[168]
Raj Bala	*Sida rhombifolia*	Malvaceae	Root	Methanolic extract (200 and 400 mg/kg)	Diphenoxylate (5 mg/kg)	Castor oil-induced diarrhea model, intestinal transit, and castor oil-induced intestinal fluid accumulation (enteropooling)		[169]
Kupilu	*Strychnos nux–vomica* Linn. f.	Loganiaceae	Root bark	Aqueous and Methanolic extract (3, 7.5, and 15 mg)		Castor oil-induced diarrhea model		[42]
Kataka	*Strychnos potatorum* Linn.	Loganiaceae	Seed	Methanolic extract	Diphenoxylate (5 mg/kg)	Castor oil-induced diarrhea model, effects on gastrointestinal motility and PGE_2-induced gastric enteropooling		[170]
Lodhra	*Symplocos racemosa* Roxb.	Symplocaceae	Bark	Ethylacetate chloroform, n–butanol and aqueous fraction (300, 500 mg/kg)		Spontaneous movement of the isolated rabbit intestine		[171]
Jambu	*Syzygium cuminii* Linn. Skeels	Myrtaceae	Seed	Aqueous extract (125, 250, and 500 mg/kg)	Loperamide (2 mg/kg, p.o.)	Castor oil-induced diarrhea model, charcoal meal test, castor oil-induced intestinal secretions		[172]
Sharpunkha	*Tephrosia purpurea* (Linn.) Pers.	Fabaceae	Whole plant	Methanolic extract (300 mg/kg)	Verapamil (50 mg/kg)	Castor oil-induced diarrhea model		[173]
Arjuna	*Terminalia arjuna* (Roxb.) W. & A.	Combretaceae	Bark	Methanolic extract (100, 200, and 400 mg/kg)	Loperamide (3 mg/kg)	Castor oil and gastro intestinal motility test		[174]
Bibhitaki	*Terminalia bellirica* Roxb.	Combretaceae	Fruit	Aqueous and ethanolic extract (143, 200, and 334 mg/kg)	Loperamide (3 mg/kg)	Castor oil-induced diarrhea model, PGE_2-induced enteropooling and gastrointestinal motility test		[175]
Parisha	*Thespesia populnea Soland.* Ex. Correa	Malvaceae	Stem bark	Methanolic fraction (100 mg/kg) and residue fraction (10, 25, and 50 mg/kg) of aqueous extract	Loperamide (3 mg/kg) Atropine (3 mg/kg)	Castor oil-induced diarrhea model, PGE_2-induced diarrhea, charcoal meal test	Inhibition of elevated prostaglandin biosynthesis, reduced propulsive movement of the intestine	[176,177]
				Aqueous extract (100, 200, and 400 mg/kg) and alcoholic extract (50, 100, and 200 mg/kg)		Castor oil-induced diarrhea model; PGE_2-induced enteropooling, charcoal meal test		

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Table 1: *(Contd....)*

Sanskrit name	Botanical name	Family	Part used	Extract/dose	Standard drug and dose	Model	Mechanism	References
Guduchi	*Tinospora cordifolia* (Willd.) Miers ex Hook.f. & Thoms.	Menispermaceae	Stem	Ethanolic and aqueous extract	Loperamide (3 mg/kg, p.o.)	Castor oil and magnesium sulfate-induced diarrhea		[178]
Adhapushpi	*Trichodesma indicum* R.Br.	Boraginaceae	Root	Ethanolic extract		Castor oil-induced diarrhea model charcoal meal transit time, castor oil-induced enteropooling		[179]
Methika	*Trigonella foenum-graecum* Linn.	Fabaceae	Whole plant	Aqueous extract (100, 200 mg/kg)	Loperamide (1 mg/kg, i.p.)	Castor oil-induced diarrheal model		[180]
Pind tagar	*Valeriana hardwickii* Wall.	Valerianaceae	Rhizome	Aqueous-Methanolic extract	Loperamide (10 mg/kg)	Castor oil-induced diarrhea model	Inhibited K^+-induced contractions (0.01-0.3 mg/ml), CCB	[181]
Sampushpa	*Vinca major* L.	Apocynaceae	Aerial part	Ethanolic extract (250, 500, and 1000 mg/kg)	Loperamide (3 mg/kg, p.o.) Atropine (5 mg/kg, i.p.)	Castor oil-induced diarrhea model, castor oil and magnesium sulfate-induced enteropooling, gastrointestinal motility test using charcoal meal methods		[182]
Kutaja	*Wrightia tinctoria* Roxb. R.Br.	Apocynaceae	Bark	Ethanolic extract (500 and 1000- 189 mg/kg) and isolated steroidal alkaloid fraction (50 and 100 mg/kg)	Loperamide (0.5 mg/kg), atropine (0.1 mg/kg, i.p)	Castor oil-induced diarrhea model, charcoal meal, PGE_2-induced enteropooling		[183]
Adaraka	*Zingiber officinale* Rosc.	Zingiberaceae	Rhizome	Zingerone	Loperamide (5 mg/kg, i.p.)	Intraluminal pressure changes and expelled fluid volume from the colon	Inhibited spontaneous contractile movements in the isolated colonic segments, Inhibit colonic motility via direct action on smooth muscles	[184]
Badara	*Ziziphus jujuba* Mill.	Rhamnaceae	Leaf	Aqueous extract		Castor oil and magnesium sulfate-induced diarrhea models		[185]
Badara	*Ziziphus mauritiana*	Rhamnaceae	Root	Methanolic extract (25 and 50 mg/kg)	Diphenoxylate (2.5, 5 mg/kg) orally	Castor oil-induced diarrhea model and castor oil-induced fluid accumulation, spontaneous movement of the isolated rabbit jejunum, gastrointestinal transit time	An inhibition of the spontaneous pendular movement of the isolated rabbit jejunum and inhibited acetylcholine-induced contraction of rat ileum	[186]

PGE_2: Prostaglandin E2, CCB: Calcium channel blockade, EPEC: Enteropathogenic *Escherichia coli*, EIEC: Enteroinvasive *Escherichia coli*

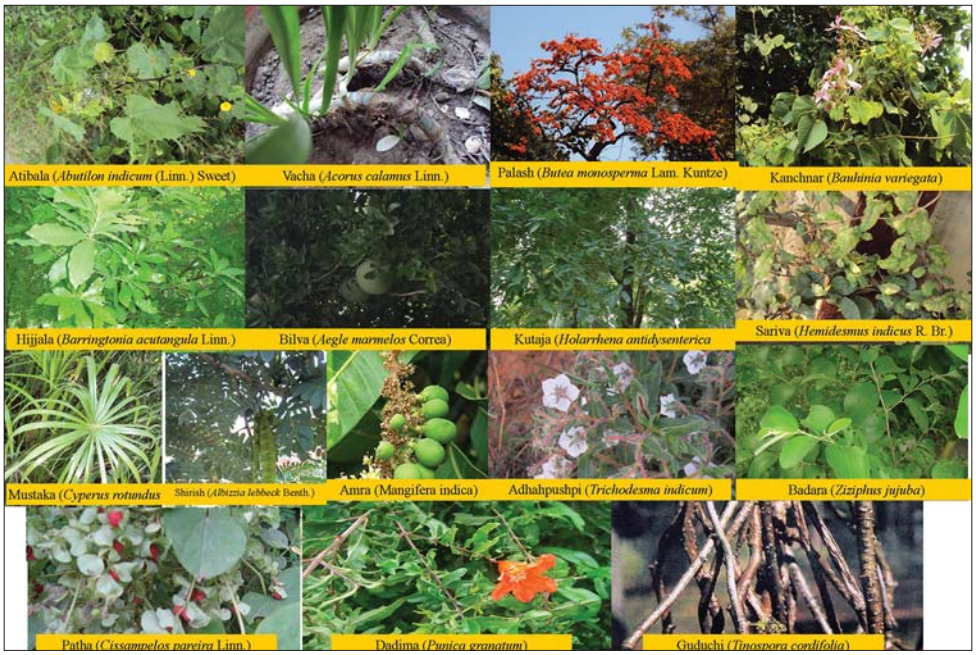

Figure 1: Antidiarrheal medicinal plants

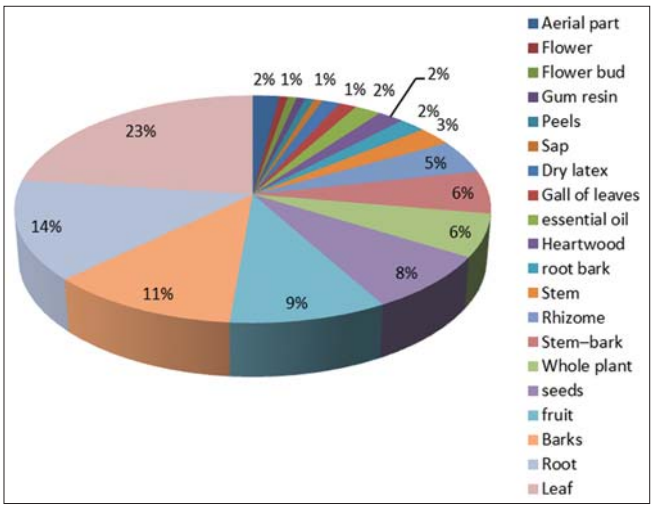

Figure 2: Distribution of plant parts investigated for antidiarrheal activity

Many phytoconstituents such as lupinifolin isolated from *Eriosema chinense*, -omoneukanrin B, dimethoxyflavone isolated from the stem bark of *Stereospermum kunthianum*, 6-(4-hydroxy-3-methoxyphenyl)-hexanonic acid, isovanillin, iso-acetovanillon from *Pycnocycla spinosa* Decne. Ex Boiss., have been evaluated for anti-diarrheal activity. However, in the mentioned list of ayurvedic plants limited isolation of the active constituents have been done which accounts for the numerous scope in this area for analytical, pharmacognostical as well as pharmacological screening of the active principles from these plants. Some of the constituents such as kurryam, koenimbine, koenine, piperine, and berberine are mentioned in the list with reported antidiarrheal activity [189-192].

Newer technologies such as in-silico, docking studies, interaction with enterotoxin from causative oraganism and nanotechnology were also employed in the antidiarrheal agent research works [193,194]. However, unfortunately, such advanced techniques were not used for the above listed ayurvedic plants. However, a few clinical trials reveal that the plants acts via a number of mechanisms, i.e., anti-inflammatory, antisecretory antimicrobial effect against *V. cholerae* and enterotoxigenic *E. coli*, rotavirus, detoxification of toxins and constipate, adsorbent, providing a rich source of calories; antimotility and antispasmodics effects [195].

CONCLUSION

The ethnomedicinal approach for diarrhea is a practical, cost-effective, and a logical for its treatment. Present data show that only a few isolated compounds from plants were investigated for antidiarrheal potential. Therefore, a significant research of chemical and biological properties of such less explored plants is still needed to determine their antidiarrheal efficacy which will possibly define their exact mechanism of actions.

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Mini Review

The consumption of propolis and royal jelly in preventing upper respiratory tract infections and as dietary supplementation in children

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ABSTRACT

Propolis and royal jelly (RJ), two important honeybee products, have been used commonly all over the world as traditional and ethnopharmacological nutrients since ancient times. Both of them have a lot of active ingredients which are known to be effective for several medical conditions. In this article, medical databases were searched for the usage of RJ and propolis in upper respiratory tract infections (URTI) and as a dietary supplementation, together and separately. 10-hydroxy-2-decenoic acid is the most prominent active compound showing antimicrobial effect within RJ. Caffeic acid phenethyl ester is the most famous one that shows antimicrobial and anti-inflammatory effect within propolis. When compared with propolis, RJ was found to have richer content for all three main nutrients; proteins, carbohydrates, and lipids. More clinical, experimental, and basic studies are needed to find out the best standardized mixture to cope with URTI in which RJ and propolis will be main ingredients in addition to the other secondary compounds that have health-beneficial effects.

KEY WORDS: 10-hydroxy-2-decenoic acid, caffeic acid phenethyl ester, infections, nutrition, propolis, respiratory tract, royal jelly

INTRODUCTION

The term "herbal medicine" has been used to identify the therapeutic plants or particular substances derived from the plants to support the body to fight against various diseases including infections or improving overall health. The utilization of complementary and alternative medicine (CAM) products in such type of infections is very popular. The similar things can be brought forward for nutritional usage of phytotherapeutic products as well. Two honeybee products, propolis and royal jelly (RJ), have been used commonly all over the world as traditional and ethnopharmacological nutrients since ancient times. Both of them have a lot of active ingredients which are known to be effective for several medical conditions. For example, caffeic acid phenethyl ester (CAPE) from propolis is thought to be responsible well-known effects of propolis including anticancer, antioxidant, immune-modulatory, antibacterial, antiviral, and anti-inflammatory [1]. As a lipid soluble antioxidant, CAPE has been used in a number of inflammatory and infectious diseases as traditional medicine [2]. 10-hydroxy-2-decenoic acid (10-HDA) from RJ has also several activities such as protective and therapeutic effects on infections and has nutritious properties [3].

REVIEW METHODOLOGY

Literature searches from PubMed, Medline, Scopus, ScopeMed, and Google Scholar were performed to find out the published articles about the topic to identify the nutritional value of propolis and RJ and the usage of them in upper respiratory tract infections (URTI). All articles written in English or having just an English abstracts were analyzed up to March 2016. Databases were searched for RJ in URTI, propolis in URTI, RJ in nutritional requirements, and propolis in nutritional requirements. As a second step, these parameters were combined for two mixtures and searched for new compounds or mixtures being used for the same purposes. If there is no actual contemporary review or original articles, older ones were considered to reach a reliable conclusion.

PROPOLIS

General Information

It is a mixture produced by honeybee after processes within his saliva. This product is used by the bees to protect their hives from infected single cells such as fungi and bacteria to moisture

and stabilize temperature within the hives, and to embalm the foreign materials and to repair all the cracks within the hives [4]. A study revealed the presence of 60 different important phytochemicals in methanolic extract of Nigerian bee propolis including phlobatannins, glycosides, tannins, anthraquinones, steroids, saponins, flavonoids, and alkaloids [5]. This greenish-brown and sticky product has different mixtures depending on what flowers and trees they accessed to and the place of the bees [6]. It has been claimed that every single molecule of propolis needs to be studied to show the source of the real effect and the underlying mechanism of these effects [6]. Akyol *et al*. suggested that the clinical importance of CAPE stems not only from free radical scavenging and antioxidant activities, but also by marked nuclear factor-κB (NF-κB), nitric oxide synthase activity, and apoptosis inhibition together with the suppression of caspase-3 activities and p38 phosphorylation [7].

The Antimicrobial Effects

Lately, antiviral features of CAPE have been reviewed by scientists suggesting CAPE and its targets may have been regarded as a new field to design new therapeutics [8]. The other goal of that study was to understand the molecular mechanism of virus-related diseases. Propolis was tested for experimental *Pseudomonas aeruginosa* keratitis in rabbits and found that it can be regarded as a useful supplemental compound but should not be considered as a substitute for a contemporary antibiotic cure for this type of keratitis [9]. A mixture of ethanolic extract of propolis (EEP) was found to inhibit viridans Streptococci and regarded as an antimicrobial compound [10]. On the other hand, several different propolis samples were found to have noteworthy antimicrobial activities against yeasts and Gram-positive bacteria [11]. It has been found to be effective on fungi as well [12]. All oral candidiasis patients administered to standardized propolis extract exhibited a significant lesion suppression comparable to those treated with nystatin [12].

The Value of Propolis as Dietary Supplement

Many investigations have been conducted to evaluate the effectiveness of propolis on experimentally induced diabetes including antidiabetic properties of Nigerian propolis [13]. The study indicated that EEP could benefit hypertriglyceridemia, hypercholesterolemia, and hyperglycemia along with keeping safe pancreas and liver against alloxan-induced diabetes in terms of biochemical and histological parameters [13].

The effectiveness of bee propolis on physical fitness and some other parameters were investigated after adding it to the food as a supplementation in rats [14]. Improved regeneration efficiency of hemoglobin, increased calcium and phosphorus absorption, improved utilization of iron, and increased weight gain were notified in the rats fed with propolis. In an experimental study, propolis and/or multifora pollen was found to be beneficial as co-adjuvants in the medical care of nutritional ferropenic anemia; supplemented the regular foods of rats with propolis and/or multifora pollen caused to a considerable amelioration in the digestive utilization of magnesium, iron calcium, and phosphorus [14].

RJ

General Information

RJ is a mix of yellow-white creamy and acidic secretion produced by the worker honeybees using their mandibular and hypopharingeal glands to supply a principal food for queen honeybee. It is a nutritive secretion rich in minerals, vitamins, carbohydrates, and proteins [15]. It has a lot of minerals (mainly calcium and iron), vitamins (mainly riboflavin, niacin, thiamin), fatty acids, sugars, proteins, and free amino acids. Many important activities of RJ have been attributed to its one of active contents, 10-HDA.

The Antimicrobial Effects

There seems to be some evidence of a possible role of RJ in infections, but the evidence even now inadequate to accept suggestions for medical care of children with respiratory tract infections. Four antimicrobial peptides were isolated and sequenced by quadrupole-time-of-flight tandem mass spectrometry: PFKLSLHL-NH_2 (Jelleine 1), TPFKLSLHL-NH_2 (Jelleine 2), EPFKLSLHL-NH_2 (Jelleine 3), and TPFKLSLH-NH_2 (Jelleine 4) from RJ and noticed that some of them have antimicrobial properties against yeast, Gram-negative and Gram-positive bacteria [16,17]. There are very short peptides presenting hydrophobic sequences. RJ exhibits bacteriostatic [18] and antimicrobial activities [19] just because of the acidic content, organic acids and proteins (mainly known as royalisin) it has. Sver *et al*. found that RJ exhibits immune-modulatory features by stimulating immune-co-potent cell proliferation and production of antibodies in mice or by reducing humoral immune functions in rats [20].

The Value of RJ as Dietary Supplement

RJ was investigated for its supplemental value that can enhance athletic performance which can also give an idea for its supporting role to human well-being without classifying those adults and children [21]. Most of the health-protecting properties of RJ have been attributed, at least in part, to actions of lipids found in RJ [22]. A possible beneficial effect of fresh RJ was reported in mice in terms of recovery from swimming to exhaustion [23]. No other effects of RJ were found in exercise but it was found to be effective on new bone formation and rapid maxillary expansion, which shows its valuable support to the general condition of the body [24]. Epidermal hydration, maintained by the epidermal lipid barrier, of which ceramide is one of the constituents, has been enhanced by dietary supplementation of RJ in mice [25]. The effects of RJ and CAPE on aggrecanases in chondrosarcoma cells were investigated to enlighten the molecular basis of these compounds in osteoarthritis [26]. interleukin-1 alpha (IL-1α) was used to induce the cells. 10-HDA has been well known to have an activity to promote collagen production. It was shown that these compounds could block the NF-κB cascade, which

is very important in osteoarthritis pathogenesis, which in turn might give estimation about the prevention of infections [27]. On the other hand, RJ has been suggested to support brain levels of dopamine in male bees, showing, in turn, the importance of tyrosine amino acids taken by nutritious supplements such as RJ [28].

MIXTURE OF PROPOLIS, RJ AND OTHER CAM PRODUCTS

Echinacea, a common immune-stimulant, is very well known as an anti-inflammatory and antioxidant product as well. Some clinical studies have shown that *Echinacea* could intensify the production of cytokines including tumor necrosis factor-α, IL-10, IL-6, and IL-1 by macrophages [29]. The effectiveness of a preparation containing propolis, vitamin C, and *Echinacea* extract for URTI in young children was investigated in a randomized, double-blind, and placebo-controlled study with a 3 months follow-up time interval [30]. The number of children who affected from URTI, the total number of disease episodes, and the mean number of episodes per child were found significantly lower. The total number illness days and duration of episodes were also lower compared to placebo group. According to this clinical study, the days affected by fever, the usage of antipyretics and antibiotics, the numbers of physician visits for URTI was significantly lower [30]. The positive effect of combination of *Echinacea*, propolis and vitamin C in URTI in children was reviewed by another study showing decrease the number of episodes, the duration of symptoms, and the number of days of illness [31].

There are several studies showing bee products to have antibacterial, antiviral and anti-inflammatory features [32], especially for the prevention of URTI [33]. Henatsch *et al*. overviewed of the effectiveness beehive products in otorhinolaryngology [34]. What they found is propolis might be used in safe in the medical care of acute otitis media, mouth ulcer, and stomatitis, while RJ could safely be used to reduce mucositis.

SELECTED THERAPEUTICAL EXPERIENCES

Due to the fact that foods ingested may influence attention, moods, emotion and cognition of children, there is growing evidence that parents are more inclined to use dietary supplements for their children [35]. Children more commonly take bee propolis as dietary supplement possibly due to the parental encouragement for children to prevent colds and flu and strengthen the immune system [36]. Propolis was found to be the fifth one within the top five supplements consumed among elementary school children in Taiwan [35].

CONCLUSION

Reviewing of the scientific reports on RJ and propolis could help us to reach two main conclusions in terms of nutritional properties and the usage of these mixtures in URTI as remedial agents: First, no published evidence reach a level that scientists and public accept the exact supportive role of these two CAM. Still there is a tremendous need for advanced good quality experimental and clinical studies. Second, placebo-controlled, randomized, double-blinded, and high-quality studies on the safety and efficacy of herbal therapy, especially for the mixture of propolis, RJ, and *Echinacea* in URTI are needed. There is obviously an urgent need to find out the best reliable and standardized mixture, which have been approved for health-beneficial effects such as propolis, RJ, *Echinacea* for children to cope with seasonal URTI as an alternative option in addition to classical treatment modalities.

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Mini Review

Trends and challenges toward integration of traditional medicine in formal health-care system: Historical perspectives and appraisal of education curricula in Sub-Sahara Africa

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ABSTRACT

The population residing Sub-Sahara Africa (SSA) continues to suffer from communicable health problems such as HIV/AIDS, malaria, tuberculosis, and various neglected tropical as well as non-communicable diseases. The disease burden is aggravated by shortage of medical personnel and medical supplies such as medical devices and minimal access to essential medicine. For long time, human beings through observation and practical experiences learned to use different plant species that led to the emergence of traditional medicine (TM) systems. The ancient Pharaonic Egyptian TM system is one of the oldest documented forms of TM practice in Africa and the pioneer of world's medical science. However, the medical practices diffused very fast to other continents being accelerated by advancement of technologies while leaving Africa lagging behind in the integration of the practice in formal health-care system. Challenging issues that drag back integration is the development of education curricula for training TM experts as the way of disseminating the traditional medical knowledge and practices imbedded in African culture . The few African countries such as Ghana managed to integrate TM products in the National Essential Medicine List while South Africa, Sierra Leone, and Tanzania have TM products being sold over the counters due to the availability of education training programs facilitated by research. This paper analyses the contribution of TM practice and products in modern medicine and gives recommendations that Africa should take in the integration process to safeguard the SSA population from disease burdens.

KEY WORDS: Bantu, curricula, formal system, integration, practice, products, Sub-Sahara Africa, traditional medicine

INTRODUCTION

The Contribution of Ancient African Traditional Medicine (TM) Practices in Modern Medicine

The Sub-Sahara Africa (SSA) continues suffering from the burden of diseases despite being a rich source of biodiversity from which many hospital medicines have been tapped, and a lot more are untapped. This untapped avenue has contributed minimally in solving health problems since TM has not yet been formally integrated into the existing conventional health-care delivery [1]. The reasons being partly because there are few TM curricula that are geared to trained human resources to undertake quality services and development of *materia medica* used in treatments in this region. However, different countries or continents elsewhere have their TM practices supported by documented *material medica* and the underlying philosophy for disease diagnosis and treatments such as Unani TM, Chinese TM, Ayurvedic medicine, Naturopathy, Homoeopathy, and Korean oriental medicine [2-4]. Therefore, the philosophy and theories of disease symptoms, diagnosis, and treatment used in African TM need to be established and learned because the surge for the use of TM is now not limited to countries of origin rather a trans-territory and a choice of many people even in developed countries [5-8]. African TM practices are imbedded in the indigenous knowledge of one's culture or society thus also serves as backups of what the local communities have maintained for centuries for their survival and prosper within their ecosystem.

In the written record, the ancient Pharaonic Egyptians medical practices are the oldest documented form of TM practice in Africa. From the beginning of the civilization in about 3300 BC until the Persian invasion in 525 BC, Egyptian medical practices were highly advanced for its time including simple non-invasive surgery, bones setting, and an extensive set of pharmacopeia in

the form of papyri. The Ebers Papyrus (c. 1500 BC) is among the oldest preserved medical documents and contains some 700 magical formulas and remedies. Records show that the diagnosis of diabetes disease was described in Ebers Papyrus as disease of "urine pass through" [9]. The Edwin Smith papyrus (c. 1600 BC) includes a description of simple non-invasive surgery whereby the position of diagnosis of breast cancer and its management is described as a "tumor do thou nothing there against" [10]. Several other papyri collected in Egypt influenced TM of other traditions, including the Greeks and Romans, but later other parts of the world [11].

No much is recorded about TM practices in Africa until the 19th century when Africa was partitioned and missionaries' works started. For example in by then Germany East Africa and British East Africa, which compose the current East Africa Community countries, one British traveller [12] witnessed cesarean section being performed by Ugandan people to serve the baby and mother. Similar reports of surgical practices were reported from Rwanda, whereby botanical preparations were used to anesthetize the patient to perform a cesarean and promote wound healing [12]. In Tanzania, the German ship's doctor Dr. Weck, Adolf Bastian (1826-1905), W. H. R. Rivers (1864-1922), and C. G. Seligman (1873-1940) observed and wrote a number of diseases being managed by TM in the Hehe community [13,14]. These few examples of ancient indigenous practices show the significant contribution to the modern practices of diagnosis and disease management since some still hold to date.

TM in SSA

In Africa, the Bantu-speaking peoples make up a major part of the population of nearly all African countries south of the Sahara. They belong to over 300 groups, each with its own language or dialect [15]. Despite the diverse culture and ethnic groups in SSA, still, most societies are dominated by the Bantu culture and believe [15]. Therefore, TM in the SSA region is rational in the context of Bantu cultures and is like theories in western medicine. The Bantu believes a human being is holistic yet corporate, in terms of the family, clan and whole ethnic group. Therefore, it is required never to do harm to the patient unless it is in his or her best interests or for the good of the community because if he suffers, he does not suffer alone but with his corporate group: When he rejoices, he is not alone but with his kinsmen, neighbors, and relatives. In the modern health-care system, this is a principle worth emulating; never to do harm to a patient unless the nurse or doctor, after serious consideration, believes that it is in the interests of the patient or it is necessary for the protection of other patients or the public [16-18]. The African Union Commission adopted the WHO/Afro (1976) definition of African TM as the total of all practices, measures, ingredients, and procedures of all kind whether material or not which guard against disease/illness to alleviate suffering and cure himself [19]. Thus, African TM does not regard man as a purely physical entity but also takes into consideration the sociological (family or other), whether living or dead (ancestors) and the "intangible forces" (God, gods,) of the universe [19,20]. Thus, disease is not merely a result of dysfunction of an organ caused by the invasion of microscopic organisms (Germ theory) but also may be due to intangible forces. Therefore, treatment in African TM is by use of both material substances and resources drawn from the cosmic world all together not separated (Holistic theory) in an attempts to restore a state of wholesomeness using various methods including plant remedies [19,20].

Interdependence of Traditional and Modern Medicine

The pharaonic pharmacopeia papyri described several plants including the bark of the willow tree, in which Hippocrates (~ 460-370 B.C.) who is acknowledged as a father of western medicine used to control headaches and other body pains [21]. It was through chemistry, the active molecule which is salicylic acid was identified, in 1889, and later aspirin, paracetamol, diclofenac, mefenamic acid, ibuprofen, etc., were synthesized based on the structure of salicylic acid [21,22]. Several other useful hospitals medicines and vaccination, such as quinine, ephedrine, amodiaquine, primaquine, chloroquine, mefloquine, atropine, reserpine, digoxin, tubocurarine, metformin, Scopolamine, taxol, and calanolide A, are now synthetically made from a structure of an initial naturally isolated compound from medicinal plants while others are semi-synthetically derived from the natural product precursors [22,23]. Several medicinal plants, such as Madagascar's rosy periwinkle *Catharansus roseus*, remain the basic source of anticancer drugs vincristine and vinblastine [23]. This indicates the potential of medicinal plants and its current contribution to hospital medicine, in which most of these are an essential medicine dispensed worldwide for treatment of different diseases. This confirms the contribution of both modern and TM in the advancement of the current health systems not only in SSA but worldwide.

Opportunities and Challenges to Promote TM Practices and Products in SSA

The contribution of TM and its practitioners was recognized, in 1978, by the Alma-Ata Declaration as important resources in achieving health for all by the year 2000 [24]. A number of resolutions and declarations have been adopted by the WHO governing bodies at regional and global levels including Resolution AFR/RC49/R5 on Essential Drugs in the WHO African Region. The resolution required the WHO to support Member States to carry out research on medicinal plants and to promote their use in health-care delivery systems [25]. The Regional Committee that adopted resolution AFR/RC49/R5 also called on the WHO to develop a comprehensive strategy on African TM with the focus on producing evidence [25-27]. Since then, African countries have been supporting these initiatives in different ways such as documenting ethnobiomedical information, scientific evidence/research, media promotion, implementing international and national plans and policies including the plan of action on the decade of TM for (2001-2010) that was extended to cover the period 2010-2020 [28,29]. Indeed, African Nations are aligning to these

international plans to pull efforts of promoting TM uses including developing robust policies and legislatives. Further, many African countries are signatories to the TRIPs 1994, CBD 1998, and Nagoya Protocol 2010, which require governments to put mechanisms for recognition and protection of the vast available local knowledge and associated used genetic materials including those in TM. This is the commendable direction taken in addressing the rights of traditional knowledge holders whom for centuries have transmitted this knowledge orally thus continued exposing the region in a risk of biopiracy and that some knowledge became forgotten or lost during oral transmission. The ethnobiomedical information originating from African culture could be appropriately coordinated and disseminated through formal training and research to bring about reliability and allow adoption for sustainability of the TM services that benefit the majority of the SSA population. Notably, only a few apprenticeships and formal training can be traced in SSA.

METHODOLOGY

The current review intends to appraise the trend and situation of African TM education training curricula in SSA as one element in the integration process in the formal health-care system. Analytical methodology used for this appraisal was through internet search from Google, Google Scholar, PubMed, HINARI, ISI, Global health training center, and Popline (K4 Health) database using the terms or key words: Curriculum or courses, or program in traditional or herbal medicine in Africa alone and combination. In additional terms such as degree, college, SSA, or country names were used in combination with the search titles. A manual evaluation of searched titles and reference lists of relevant studies and reviews was also conducted. Furthermore, all articles related to the subject were selected and web-link or the authors' affiliation to view the institutional webpage whether they offer any course or training program in TM. Basic courses or program in phytochemistry, pharmacognosy, natural products chemistry, and phytomedicine are not included in this appraisal because it is a science of the *materia medica* without necessarily having a reflection on two key characteristic aspects of TM, that is, practices accompanied with the use of *materia medica*.

RESULTS RETRIEVED

The analytical review of the information related to the subject on "curriculum or courses, or program in traditional or herbal medicine in Africa" revealed only a few institutions mostly universities or colleges in the countries residing SSA that undertake training in TM or complementary and alternative medicine [Table 1]. Many of the retrievable information indicated TM training to be tailor-made short courses geared to develop professional skills for a specific community of professionals. Furthermore, noted that, funds for most of the short courses were donor-based thus not sustainable beyond funding period, e.g., the Multi-disciplinary University Traditional Health Initiative project of South Africa. Some private owned colleges do conduct alternative and complementary medicine education training such as homeopathy as shown in Table 1. Most of the TM education programs in public universities are geared at analyzing the efficacy, safety, and quality of TM products while the clinical practices were being mostly left to private sector entities. Notably, several universities and research institutions in SSA countries are running some basic courses in phytochemistry, pharmacognosy, natural products chemistry, and phytomedicine. However, those universities/colleges are not listed as they are out of the scope of the present appraisal.

DISCUSSION

Previously Chitindingu *et al*., 2014 pointed out the training components in African TM that was offered in South Africa to have a theoretical approach rather than problem-solving approach [32]. Further, reports indicated difficulties in the initial stages of introducing TM curriculum in biomedical universities for undergraduates [4,30,32,33]. Nevertheless, the importance of TM in SSA call for setting priorities of developing medicines from *materia medica* while streamlining TM practices alongside with other health professional training and services. There only only limited huddles on TM products that are used in treatments as many are crude extracts or are in the form of raw materials containing the therapeutic active ingredients [34]. Some may have been used for centuries by individuals within their environment in the communities, and their efficacy and safety is well-known by the entire community and may be acceptable. These can be a good start-up that may proceed to be essential medicines to be streamlined in formal health system delivery services if they satisfy the health care needs of the majority of the population and the prescribers are made aware of their efficacy and safety. The few African countries that have managed to integrate TM products in the National Essential Medicine List such as Ghana is because it was able to develop curriculum which is used to train TM experts as the way of disseminating the indigenous medical knowledge and practices to several health professionals [35]. Several African countries such as South Africa, Sierra Leone, and Tanzania have TM products being sold over the counters facilitated by the research and training programs undertaken in these countries [30,31,32,36-39].

FUTURE PROSPECTS AND RECOMMENDATIONS

Notably, few on-going attempt to integrate TM in formal health care can be spotted in some SSA countries whereby collaborative initiatives of some Traditional Health Practitioners Organization such THETA-Uganda; TAWG-Tanzania; and ZINATHA-Zimbabwe work closely with the Ministry of Health in addressing the prevention and care of HIV/AIDS patients [40,41]. These attempts are good model toward integration of TM to the formal system if embraced by formal training of practitioners that participate in such collaboration. The training program will instill skills and confidence to Traditional Health Practitioners to work in partnership with modern doctors in the existing formal health system. Other areas that need improvement in the integration process are modernization of TM to allow

Table 1: Some universities and colleges in Sub-Sahara Africa that offer formal education training in traditional medicine

*Country	*Institution	Academic program or course offered	Citation
Ghana	The Kwame Nkrumah University of Science and Technology, Department of Herbal Medicine	Bachelor of Science Degree in Herbal Medicine	https://www.knust.edu.gh/admissions/prospective/ugprogrammes
	University of Ghana, Department of Pharmacognosy and Herbal Medicine	A Course for Bachelor of Pharmacy	http://pharmacy.ug.edu.gh/overview
	Endpoint homeopathic training institute	Diploma and Degree in Alternative Medicine	http://www.endpointcollege.com/
	The college of integrated medicine	Program in Complementary Health Care at Certificate Level	https://www.villagevolunteers.org/village-news/ghana-college-of-integrated-medicine/
	University of Ibadan, College of Medicine	Training Curricula for Undergraduate	http://www.wahooas.org/spip.php?article1017
Kenya	Kenyatta University, Department of Pharmacy and Complementary/Alternative Medicine	Courses for Undergraduate and Graduate Pharmacy	http://medicine.ku.ac.ke/index.php/department/pharmacy-and-complementary-medicine
	University of Nairobi, Department of Pharmacology and Pharmacognosy	Master of Science in Pharmacognosy and Complementary Medicines Course	http://pharmacology.uonbi.ac.ke//uon_degrees_details/733
Sierra Leone	University of Sierra Leone, Department of Pharmacognosy and Phytochemistry	Masters Degree Program in Traditional Medicine Pharmacy Courses in Traditional Medicine For Pharmacy Medical Undergraduate	[30,31]
South Africa	University of KwaZulu-Natal, The Department of Science and Technology-National Research Foundation Centre	IKS Research and Postgraduate Training in Traditional Medicine	http://aiks.ukzn.ac.za/about-dst-nrf-ciks
	University of the Western Cape South African, Herbal Science and Medicine Institute	Postgraduate Programs in Herbal Medicine Tailor-made short courses geared to develop professional skills for a specific health professionals community, e.g., courses for Clinical trials in herbal medicine	https://www.uwc.ac.za/Faculties/NS/SAHSMI/Pages/Programmes.aspx#.UMBzYqyxhP4
	College of Natural Therapies	Postgraduate and Co-graduate Educational Programs	http://www.collegeofnaturalhealth.co.za/
	Blackford Centre for Herbal Medicine	Diploma in Medical Herbalist	http://www.studyonline.co.za/herbal/enrol.php
Tanzania	Muhimbili University of Health and Allied Sciences Institute of Traditional Medicine	Postgraduate (MSc and Ph.D.) Program in Traditional Medicine Development Module for Undergraduate and Graduate Medical Students	http://www.muhas.ac.tz/index.php/academics/muhas-institutes/110-itm http://itm.muhas.ac.tz/index.php/training-programmes
	Department of Veterinary Medicine, Sokoine University of Agriculture	Master of Science in Natural Products Technology and value addition	http://www.suanet.ac.tz/index.php/education/programmes-offered-at-sua

*Listed universities/colleges are not exhaustive rather most retrievable list from SSA countries with curricula in traditional medicine. SSA: Sub-Sahara Africa

easy keeping, dispensing, and transportation in bulk; Clinical studies of herbal formulas need to be advocated to overcome the fear of being poisoned; Biodiversity depletion due to use of herbal material from wild source has to be addressed by engaging into extensive cultivation while adhering to Good Agricultural Practices. It is equally important that traditional practices and the philosophy for disease diagnosis and treatment be disseminated through education curricula, and the relevant research should be promoted sustainably.

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Letter to the Editor

On the use of carnosine and antioxidants: A letter from Russia

Sir,

Carnosine, an endogenously synthesized dipeptide found in muscular and other tissues, was reported to possess antioxidant properties. Favorable actions of carnosine were explained by its effects against reactive oxygen species (ROS), against peroxynitrite damage and different types of viral injury [1-6]. Moreover, one of the mechanisms of detoxification from aldehydes, accumulating in inflammation, ischemia, and other pathological conditions was reported to be conjugation with carnosine [7-10]. Among other potential applications, carnosine-containing eye drops have been recommended for the treatment, dissolution and prevention of cataracts, in particular, senile cataracts [5,11]. From the viewpoint of biochemistry, it is difficult to envisage how carnosine can contribute to the dissolution of cataracts, regarded to be the end stage of metabolic and structural transformations including conformational changes and aggregation of proteins [12]. To decide whether a local or general supplementation of a substance is indicated, the question should be answered whether there is a local or general deficiency. Such deficiency is prima facie improbable for substances that are supplied with food and can be synthesized in different cells and tissues of the body. Tissue carnosine concentrations are influenced by the diet being lower in vegetarians [1,4,13]. If the presence of carnosine in body fluids is important for the preservation of the lens transparency [5,11] or some physiological functions [4,14], the incidence of cataracts or other pathological conditions among vegetarians/vegans would be higher than in the general population. On the contrary, vegetarians have been reported to have a lower risk of cataract than meat eaters: There was a strong relation between the cataract risk and a diet group, with a progressive decrease in risk of cataracts in high meat eaters to low meat eaters, fish eaters, vegetarians, and vegans [15]. Admittedly, the topic is not without controversy [16], probably confounded by geographical (exposure to sunlight) and other factors. Furthermore, it has been suggested that carnosine is an anti-aging substance with a beneficial effect on the cardiovascular system [3,17,18]. There is, however, evidence that vegetarians have lower rates of coronary heart disease, hypertension, etc. Overall, their cancer rates appear to be moderately lower than in other people living in the same communities, and life expectancy appears to be greater [19]. A very low meat intake was reported to be associated with greater longevity [20]. However, benefits from the vegetarian versus carnivorous diet [9] are beyond the scope of this letter.

Carnosine has been used in sports nutrition to increase performance and was reported to have benefits in obesity and diabetes mellitus [2,21]. The latter is understandable as peptides are non-carbohydrate nutrients favorable for diabetics. Nutritive value of carnosine can explain for higher performance in sports. Dietary supplementation of carnosine has been shown to suppress stress in animals, to improve cognition and well-being in humans [22], which can be also explained by the nutritive value. Modulation of glycolysis and inhibition of the glycolysis-induced protein dysfunction have been attributed to carnosine [3]; however, modulation or "inhibition of dysfunction" is a regulatory function that cannot be ascribed to a simple substance as it presupposes a feedback mechanism. The following logical fallacy can be encountered in some papers on substances participating in metabolism such as carnosine. First, the important biochemical role of the substance is stressed, which is natural for a metabolite. After that, benefits from supplementation are discussed, even though it remains unproven, whether a deficiency of the substance occurs, and if it does, whether it can be compensated by a diet or some natural products. Purified preparations might be both expensive and unnecessary. Diet modification, e.g., consumption of more meat as a source of carnosine may suffice if supplementation is indeed indicated.

As for antioxidants affecting ROS, their increased levels may produce unpredictable outcomes since ROS can have both harmful and beneficial effects [23]. The same is true particularly for carnosine [24]. Generation of ROS is usually considered to be a side effect of aerobic metabolism [23], which is a ubiquitous process in living organisms. The redox status is maintained in dynamic equilibrium of oxidative and reductive processes under the impact of numerous factors [25-27]. Some experts suggest that boosting of antioxidant status has no benefits [25]. Certain reviews on antioxidants discuss vitamins and other substances with complex action mechanisms [28,29]. In general, antioxidants are regarded to be far from any scientifically founded clinical application [23]. It has largely remained unclear whether, when, and how much antioxidants should be taken in [23,30,31], the more so as antioxidants at higher doses may act as pro-oxidants [25].

Some generalizations regarding the oxidants/antioxidants balance, attempting to present it as universal biological concept, seem to be oversimplifications. The problem consists of several partly interrelated topics: Antioxidants and cancer [23], wine, ethanol and cardiovascular risk [28,32], radiation protection [33], lens transparency and cataract [5,11], flavonoids, healthy aging, menopause, atherosclerosis prevention [34], antioxidants, carnosine and diseases of the nervous system [22,35-37], etc. Indications to the use of particular substances are questionable.

Several large randomized clinical trials found that antioxidant supplementation does not reduce the risk of cardiovascular, mental disease and cancer, or the evidence was found to be not relevant to clinicians or consumers [23,30,35]. For example, numerous reports on inverse relationship between regular intake of low to moderate amounts of alcoholic beverages and cardiovascular risk have inspired some people to drink more alcohol in spite of possible psychosocial and other complications. While the association between alcohol consumption and decreased cardiovascular risks is regarded to be proven, it remains uncertain whether this implies causation. Although a number of points do suggest that the association between moderate alcohol consumption and decreased cardiovascular risk may indeed represent a cause-effect relationship, it is unclear to what extent it is related to antioxidants in wine and to ethanol per se [32]. The question can be clarified by large-scale experiments with different diets and supplements. However, human studies and animal experiments may be planned only in the presence of integrity, adequate equipment and ability to objectively evaluate scientific data.

The topic of antioxidants, in particular, is complicated by conflicts of interests. Some antioxidants seem to be propagated as inexpensive substitutes for evidence-based medications, i.e., in support for placebo marketing. There are many examples of marketed substances without scientifically demonstrated effectiveness [38-41]. Publications of questionable reliability are sometimes indirectly used in Russia for advertising of drugs and food supplements, for their official registration and obtaining permissions for practical use. As a result, substances with unproven effects can be offered to the elderly and other patients misinformed not only by advertising but also by some publications supposed to be scientific. The carnosine eye drops sold in Russia are relatively expensive; they are prescribed to aged patients. Theoretically, at least for patients with low income, carnosine preparations could be replaced by a meat-rich diet and/or by isotonic defatted meat broth or extract applied locally to eyes. It can be reasonably assumed that useful properties, e.g., chicken broth [6,42] are related not specifically to carnosine but to the whole mixture of peptides and other substances. A similar suggestion was made, e.g., in regard to glycosaminoglycan-containing chondroprotective agents versus natural glycosaminoglycans for osteoarthritis [41]. To support the placebo effect, the patients may be advised that the natural products can be sources of carnosine similarly to pharmaceuticals, although an effect is guaranteed neither from the drugs nor from meat products. However, considering uncertainties about antioxidant effects discussed above, we would rather abstain from such recommendations. Even more precarious, because of complication risks [43], are recommendations of peribulbar injections of carcinine (the analog of carnosine) as an antioxidant for prophylactic purposes [44]. Until recently, peribulbar injections of amino acid taurine were used in the former Soviet Union for the treatment of eye conditions associated with atherosclerosis and aging [45] or inflammatory conditions [46], against payment in elderly patients, while hematomas were observed as complications. Analogously to carnosine, taurine is widely distributed in tissues, synthesized within the body and supplied with animal-derived food products [47]. In conclusion, practical recommendations should be based on research of high quality shielded from conflicts of interest. Only such research should be included into reviews and meta-analyses.

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