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Letter to the Editor

In vitro and *in vivo* neuroprotective effect of caffeic acid phenethyl ester

Dear Editor,

Propolis is a mixture produced by honeybee, which have hundreds of polyphenols. Caffeic acid phenethyl ester (CAPE) [Figure 1], an active component of honeybee propolis has been determined to have antioxidant, anti-inflammatory, antiviral, and anticancer activities [1,2]. It has been used to prevent oxidative stress-based deterioration in cells/tissues/organs in both cell culture and experimental animals. Although, CAPE was shown to protect animals and cells against ischemia reperfusion injuries or anoxia, its effects on neurotoxins and neurotoxic pharmacological agents were not investigated extensively. It has been evaluated the potential of CAPE to induce neuritogenesis in pheochromocytoma (PC12) in terms of the involvement of this mechanism in the protection against the cell death induced by the dopaminergic neurotoxin 1-methyl-4-phenylpyridinium (MPP) (+), as well as the effects on the expression of proteins associated with axonal growth and synaptogenesis [3]. It has been shown in the study that CAPE protects PC12 cells from the cellular death induced by the MPP (+) by increasing the network of neurites, also, CAPE induced the formation, elongation, and ramification of neurites induced by the dopaminergic neurotoxin.

We have provided additional evidences and data for the mechanisms of protective effect of CAPE on neurotoxicity induced by various factors. We have shown that CAPE has a considerable neuroprotective effect on pentylenetetrazol (PTZ)-induced seizures in mice [4]. Oxidative stress and resultant dysfunction in PTZ-induced seizure could contribute to increased generation of reactive oxygen species and support the hypothesis that CAPE may improve the epileptic seizures by its antioxidant effects. When we look at the molecular mechanism of protective effect of CAPE, we noticed that CAPE effectively depressed endogenous overproduction of nitric oxide (NO), which is induced by ischemia reperfusion injury of rabbit spinal cord [5]. NO has been produced by the action of nitric oxide synthase enzyme (NOS). Ischemia causes a surge in NOS1 activity in neurons, increases NOS3 activity in vascular endothelium and later an increase in NOS2 activity in a range of cells including infiltrating neutrophils and macrophages. The primary product of the interaction between NO and superoxide radical (O_2^-) is peroxynitrite ($^-$ONOO), which is capable of either oxidizing or nitrating various biological substrates, especially in neurons. There is abundant evidence in the literature that the cellular death, particularly neuronal, provoked by NO may be apoptotic [6]. At this point, CAPE was found to exhibit profound inhibition of NFκB, a critical molecule in the apoptosis pathway [7]. In another study [8], we applied CAPE to prevent the outcomes or the total clinical symptoms of experimental autoimmune encephalomyelitis (EAE). CAPE exerted its anti-inflammatory effect by inhibiting ROS production at the transcriptional level through the suppression of NFκB activation, and by directly inhibiting the catalytic activity of iNOS. Totally, it inhibited ROS production induced by EAE and ameliorated clinical symptoms in rats. CAPE is also able to block glutamate-induced excitotoxicity, which has an important role in ischemia, by inhibiting phosphorylation of p38 and caspase-3 activation [9].

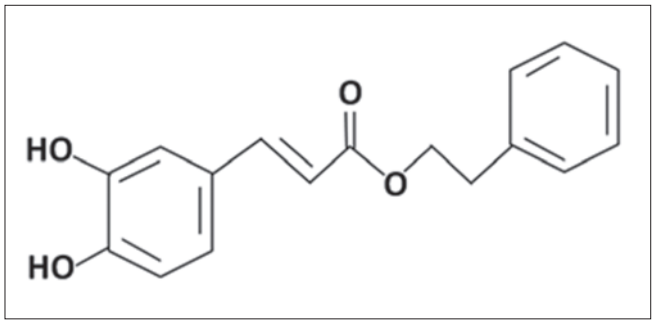

Figure 1: The structure of caffeic acid phenethyl ester

CONCLUSION

In order to emphasize the multi-faceted effects of CAPE, we would like the comments on the following: The clinical significance of CAPE arises not only from antioxidant, free radical scavenging, and direct neuroprotective activities, but also by strong NFκB, apoptosis, and NOS activity inhibitions, as well as inhibition of phosphorylation of p38, and caspase-3 activation.

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REFERENCES

1. Akyol S, Isik B, Altuntas A, Erden G, Cakmak O, Kursunlu SF, *et al.* Future opportunities in preventing ototoxicity: Caffeic acid phenethyl ester may be a candidate (Review). Mol Med Rep 2015 May 14. doi: 10.3892/mmr.2015.3785. [Epub ahead of print].
2. Akyol S, Akbas A, Butun I, Toktas M, Ozyurt H, Sahin S, *et al.* Caffeic acid phenethyl ester as a remedial agent for reproductive functions and oxidative stress-based pathologies of gonads. J Intercult Ethnopharmacol 2015;4:187-91.
3. dos Santos NA, Martins NM, Silva Rde B, Ferreira RS, Sisti FM, dos Santos AC. Caffeic acid phenethyl ester (CAPE) protects PC12 cells from MPP toxicity by inducing the expression of neuron-typical proteins. Neurotoxicology 2014;45:131-8.
4. Ilhan A, Iraz M, Gurel A, Armutcu F, Akyol O. Caffeic acid phenethyl ester exerts a neuroprotective effect on CNS against pentylenetetrazol-induced seizures in mice. Neurochem Res 2004;29:2287-92.
5. Sahin S, Sogut S, Ozyurt H, Uz E, Ilhan A, Akyol O. Tissue xanthine oxidase activity and nitric oxide levels after spinal cord ischemia/reperfusion injury in rabbits: Comparison of caffeic acid phenethyl ester (CAPE) and methylprednisolone. Neurosci Res Commun 2002;31:111-21.
6. Palluy O, Rigaud M. Nitric oxide induces cultured cortical neuron apoptosis. Neurosci Lett 1996;208:1-4.
7. Natarajan K, Singh S, Burke TR Jr, Grunberger D, Aggarwal BB. Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. Proc Natl Acad Sci U S A 1996;93:9090-5.
8. Ilhan A, Akyol O, Gurel A, Armutcu F, Iraz M, Oztas E. Protective effects of caffeic acid phenethyl ester against experimental allergic encephalomyelitis-induced oxidative stress in rats. Free Radic Biol Med 2004;37:386-94.
9. Wei X, Ma Z, Fontanilla CV, Zhao L, Xu ZC, Taggliabraci V, *et al.* Caffeic acid phenethyl ester prevents cerebellar granule neurons (CGNs) against glutamate-induced neurotoxicity. Neuroscience 2008;155:1098-105.

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Original Research

ScopeMed

Evaluation of wound healing potential of methanolic *Crinum jagus* bulb extract

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ABSTRACT

Objective: *Crinum jagus* (J. Thomps.) Dandy commonly called Harmattan or St. Christopher's lily belonging to the family Liliaceae is widely used traditionally in Southeastern Nigeria for treatment of skin sores. This study investigated the wound healing potentials of methanolic *C. jagus* bulb extract (MCJBE) using incision, excision, and dead space wound healing models. **Materials and Methods:** Phytochemical screening showed the presence of alkaloids, glycosides, tannins, saponins in the extract, but absence of flavonoids. In the incision and dead space wound models, rats were dosed orally with 300 mg/kg body weight (bw) of 10 and 5% of MCJBE solution, respectively, while in the excision wound model, rats were treated topically with 10 and 5% MCJBE ointments (MCJBEO), respectively. **Result:** The 10% MCJBE gave significantly ($P < 0.05$) highest percentage rate of wound contraction, shortest re-epithelialization and complete healing time when compared with 5% MCJBE and reference drug, framycetin sulfate. The extract of *C. jagus* showed significant ($P < 0.05$) concentration-dependent wound healing activity in incision, dead space and excision wound models. No contaminating microbial organism was isolated from wound sites of the rats dosed and treated with MCJBE throughout the study period. At day 7, post infliction of excision wound, histomorphological, and histochemical studies revealed more fibroblasts and Type 1 collagen deposits in wound site sections of rats treated with both 10 and 5% MCJBEO while those of the control showed more inflammatory cells and fewer Type 1 collagen deposits. At day 14 post infliction of excision wound, more epithelial regeneration with overlying keratin were seen in the histological sections of wounds of rats treated with both 10 and 5% MCJBEO, while histochemical study showed more Type 1 collagen deposits in wound site sections of rats in 10% MCJBEO treated group. **Conclusion:** This study established that methanolic *C. jagus* bulb extract potentiates wound healing. The study thus validated the folkloric use of *C. jagus* bulb in the management of skin sores and boils.

KEY WORDS: *Crinum jagus*, wound healing, methanolic extract, ointment

INTRODUCTION

Wound is interruption in the integrity of a tissue. Causes of cutaneous wound include: surgical, traumatic, toxic, or infectious or others [1]. To re-establish the integrity of a damaged tissue, an orderly intricate process which involve progression of events called wound healing is initiated by the damaged tissue itself from the moment an injury occurs [2,3]. Wound healing process involves complex mechanisms which involves hemostasis, inflammation, proliferation, and remodeling [1,4,5]. In each of these mechanisms, different biochemical substances are recruited to enhance the healing process. Vasoconstriction initiated by conversion of prostaglandin H2 into thromboxane A2 by thromboxane synthase results in hemostasis [6]. Intrinsic fibrinolytic factors that tend to prevent hemostasis are inhibited by plasminogen activator inhibitor Type 1 [7]. Inflammation results in accumulation of heme and heme proteins which have pro-oxidative and pro-inflammatory activities accumulate in the site of the wound to induce expression of adhesion molecules, subsequently resulting in vascular permeability, and infiltration leukocytic [4]. The infiltrating neutrophils release free radicals that kill any contaminating organism that may want to colonize the wound [8,9]. Overexpression of heme-oxygenase-1 (HO-1) which has anti-inflammatory and antioxidant (by converting heme into biliverdin/bilirubin, iron and carbon monoxide) activities accelerate wound healing process such as amelioration of the inflammation, proliferation of epithelial cells (epithelialization) and endothelial cells

(angiogenesis/neovascularization), and protection of endothelial cell apoptosis [10]. Expression of matrix metalloproteinases promotes remodeling of the extracellular matrix [11]. Overall, measurable phenomena involved in wound healing include: Wound contraction, epithelialization, and granulation tissue formation [5]. The contributions to wound healing by these phenomena depend on the type of wound. Wound contraction and epithelialization play significant roles in healing of excision wound while granulation tissue formation contributes in healing of dead space and re-sutured incision wounds [2,12]. Hence, the need for using different wound healing models in evaluation of substances for potential wound healing activity.

The necessary biochemical substances need to be available for wound healing to occur. The length of time it takes for wound healing process to be optimum and complete is directly dependent on the rate of availability of biochemical substances required for each mechanism and phenomenon to occur [13]. Wound contaminating organisms often alter or lengthen the duration of wound healing process by production of biochemical substances (enzymes) that may further destroy the wounded tissues and/or degrade the biochemical substances that enhance wound healing. Therefore, medical professionals use drugs that may be applied topically, orally or systemic to shorten the duration, minimize complications such as overwhelming microbial wound contamination of natural wound healing and achieve optimum healing [13]. In orthodox medicine, wound healing is achieved by using drugs that promote wound healing process. However, these drugs are usually costly [14] and often times they elicit side effects which are detrimental to the recipient. Hence the need for cheaper and safe alternative or complementary substances that could promote wound healing.

In traditional medicine, medicinal plants are used in the preparation of decoctions, which are applied topically to skin wounds to enable healing [14]. The efficacies of these plants in wound healing have been experienced and passed on from one generation to the other [13]. One of these medicinal plants widely used traditionally in Africa including Southeastern Nigeria in treating wounds is *Crinum jagus* (J. Thomps.) Dandy (Family Lilliaceae, formerly in family Amaryladiaceae). Its common names include Harmattan lily, St. Christopher's lily, Frest crinum or Poison bulb [15,16]. In Nigeria, it is popularly called Bush onions [16]. It is widely distributed in tropical and sub-tropical regions [17]. It is a tender perennial bulb with tulip-like (showy) white flowers, which bloom in clusters in dry season atop leafless stalks typically growing up to about 1metre tall from a clump of strap-shaped green leaves [18]. It is used in horticulture as ornamental plant [19]. Traditional practitioners in Africa including those in Southeast Nigeria, claim that the bulbs of *C. jagus* are used in form of poultices and decoctions to treat different ailments such as pain, asthma, cough, ear ache, constipation, inflammatory swellings, memory loss and skin sores, wounds, and boils [15,17,20-24]. For the treatment of wounds, the bulb is prepared in form of decoction and externally applied [20]. Scientific investigations have validated some of these claims such as antibacterial [25], antivenomous [26], antihemorrhagic, and antioxidant [27], and hepatoprotective [24] activities of *C. jagus* bulb extracts. However, the wound healing activity of *C. jagus* bulb has not been evaluated.

Phytochemical screening of extracts of *C. jagus* bulb showed that it contains alkaloids, polyphenols, and triterpenoids [16]. Specific compound contained in *C. jagus* bulb extracts as revealed by chemical investigation included crinamine, lycorine, psuedolycorine, 6-hydroxycrinamine, hamayne, tetrahydro-1, 4-oxazine (morpholine), calcium oxalate, calcium tetrata, bowdensine, and demethoxy-bowdensine [16,25,28]. Some of the bioactive compounds contained in *C. jagus* bulb extracts have been shown to exhibit properties that may promote wound healing mechanisms. For instance, crinamine from *C. jagus* bulb has been reported to exhibit strong antibacterial activity against common wound contaminant, *Staphylococcus aureus* [25]. Plant extracts that contain crinamine exhibited wound healing activity [29]. Extract of *C. jagus* bulb was shown to possess antioxidant [24,27] and hemostatic effects [27] which may promote wound healing [5]. Plants that contain phenolic compounds exhibited antioxidant activity [5,13,30]. Wound healing activity was exhibited by plants that contain alkaloids [5,13]. Alkaloids in *Crinum* species have been reported to be associated with polyphenols and resins, which exhibit anti-inflammatory and immunostimulating effects [31] which could enhance wound healing. Therefore, the presence of these compounds that exhibited mechanism involved in wound healing in *C. jagus* bulb, may contribute to its wound healing activity in humans and animals as claimed by the traditional practitioners. The objective of this study therefore was to evaluate the wound healing activity of *C. jagus* methanolic bulb extract in rats.

MATERIALS AND METHODS

The experimental protocols used in this study were approved by the Ethics Committee of the University of Nigeria, Nsukka and conforms with the guide to the care and use of animals in research and teaching of University of Nigeria, Nsukka, Enugu State Nigeria.

Animals

A total of 125 8-week-old male albino *Wistar* rats weighing between 120 and 198 g were obtained from the laboratory animal unit, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were fed on commercial growers mash (Vital feeds®) and water was provided *ad libitum*. These rats were acclimatized for 2 weeks in the animal house at the Department of Veterinary Surgery, University of Nigeria, Nsukka.

Plant Collection and Identification

Fresh *C. jagus* bulbs (J. Thomps.) Dandy were collected from Amokwe town in Udi Local Government Area Enugu State, Nigeria, in the month of May, 2014 and were identified at the International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, by a plant taxonomist, Mr. A. Ozioko. Samples were registered with a voucher specimen number FRMPC/05/14 and deposited in the center's herbarium.

Extraction

A kilogram of the *C. jagus* bulbs were sliced into smaller pieces, air dried at room temperature for 2 weeks, and then pulverized using the laboratory grinding machine at the Department of Crop Science, University of Nigeria, Nsukka. The pulverized bulbs were macerated in 80% methanol for 48 h with intermittent vigorous shaking at every 2 h. After 48 h, the mixture was filtered and the extract concentrated using a rotary evaporator set at 40°C. The dried extract was weighed and the percentage yield calculated. The extract was then stored at 4°C in a refrigerator before use.

Acute Toxicity Test

Twenty five adult rats were randomly divided into five groups of five animals per group. The animals were deprived water for 16 h before administration of the extract. The increasing doses of the extract 250, 500, 1000, and 2000 mg/kg body weight (bw) suspended in 10% Tween 20 was administered orally to the test groups, respectively, using a ball-tipped intubation needle fitted onto a syringe. The last group received 1 ml/kg of sterile distilled water and served as the control. The rats were allowed access to food and water *ad libitum* and were observed for 48 h for behavioral changes and death. The time of onset, intensity, and duration of these symptoms, if any, was recorded.

Phytochemical Analysis for Bioactive Substances

The methanolic *C. jagus* bulb extract (MCJBE) was screened for the presence of bioactive components following the methods of Trease and Evans [32].

Preparation of Ointments

The method of Okore *et al.* [33] was adopted in preparation of two herbal ointments containing 10% w/w and 5% w/w of the extract in sterile soft white paraffin. Immediately after preparation, the ointments were aseptically transferred into sterile cream tubes and sealed.

Wound Healing Studies

Incision wound model

Thirty rats were anesthetized by injecting intramuscularly with 10 and 50 mg/kg bw of xylazine hydrochloride and ketamine hydrochloride, respectively. Incision wound was created following the procedure described by Rathi *et al.* [34]. Briefly, under general anesthesia, dorsum of the animals were shaved thoroughly and prepared for aseptic surgery. Paravertebral skin incisions (5 cm in length) were made on the animals using sterile scalpel blade. The incisions were sutured using size 2/0 silk thread. Then, the rats were randomly assigned into three treatment groups consisting of 10 animals per group and treated as follows: Groups A and B were dosed orally with 300 mg/kg bw of 10% and 5% of the MCJBE once daily, respectively, while Group C was similarly given 1 ml/kg bw of sterile distilled water. The animals were treated daily for a period of 7 days. Sutures were removed at day 8 post-wounding (pw). The wound tissue breaking strength was determined at day 10 pw using the constant water flow technique described by Morton and Malone [35].

Dead Space Wound Model

Thirty rats were randomly assigned into 3 groups of 10 animals per group. They were anaesthetized by injecting intramuscularly with 10 and 50 mg/kg bw of xylazine hydrochloride and ketamine hydrochloride, respectively. Dead space wound was created following the procedure described by Rathi *et al.* [34]. Briefly, under general anesthesia, subcutaneous dead space wound were created in the region of the axilla by making a pouch through a small nip in the skin. Granulation tissue formation was induced by implanting one 30 mg sterile cotton pellets in each axilla. The wounds were sutured using size 2/0 silk and mopped with alcoholic swab. The animals were grouped and then placed individually in a clean and disinfected metal cage to avoid them licking or biting each other's wound. Groups C and D were administered orally with 300 mg/kg bw of 10% and 5% of the MCJBE once daily, respectively, while Group E was similarly given 1 ml/kg of sterile distilled water for 8 days. At day 10 pw, rats were euthanized, the cotton pellets together with the granulation tissues carefully dissected out, dried in a hot air oven at 60°C for 24 h and weighed. Weight of the granulation tissue was obtained by subtracting the post-drying weight from the pre-implantation weight.

Excision Wound Model

Totally, 40 rats were anaesthetized by injecting intramuscularly 10 and 50 mg/kg bw of xylazine hydrochloride and ketamine hydrochloride, respectively. Under general anaesthesia, dorsum of the rats were shaved and disinfected. Then, full thickness 20 mm circular wound was made on the dorsal thoracic region as per Dash *et al.* [36]. Post wounding, the rats were randomly assigned into 4 groups of 10 animals per group and treated as follows: Groups I and II were treated topically with 10% and 5% MCJBE ointment (MCJBEO), respectively, while Groups III and IV were treated with framycetin sulfate (Steritin tulle®) (reference drug) and sterile distilled water, respectively. The animals were housed individually to avoid wound biting and/or licking and treated daily with their respective ointments until complete healing occurred.

Assessment of Wound Healing Post Infliction of Excision Wound

Percentage wound contraction

Percentage wound contraction of the excision wound was determined following the procedure described by Chah *et al.* [37]. Briefly, at day 1 post infliction of excision wound, wound diameter were manually traced on a transparent white tracing paper by outlining the wound edge with a fine-tip permanent marker, and recorded as the initial wound

diameter. The wound diameters were re-measured at days 7, 14, and 21 pw. After the tracing of wound diameter of each animal, the tracing paper was appropriately labeled with the group number, rat identity and date. The area within the lines of each tracing was determined by placing the tracing paper on a 1 mm^2 graph sheet and traced out. The squares were counted and the area recorded. The degree of wound contraction was determined by subtracting the total wound area of each tracing day from the area of the initial tracing. Percentage wound contraction was then calculated as described by Chah *et al.* [37].

Wound epithelialization

Period of wound epithelialization was calculated as the number of days required for the scar to fall off leaving no raw wound [38]. The mean days for complete healing (i.e., full wound epithelialization) when epithelium covered the entire wound and hairs appear in the wound site was calculated for each group following the procedure described by Okoli *et al.* [39].

Wound Microbial Assay

Wound swabs were taken from all the animals in each group at days 3, 7, 14 and 21 using sterile swab stick moistened with sterile normal saline. Swabs were inoculated in brain heart infusion (BHI) broth (Oxoid®) and incubated at 37°C for 24 h aerobically. The broth cultures were observed for microbial growth (cloudiness/turbidity), and if any, a loopful of the broth cultures was sub-cultured on blood agar and incubated at 37°C for 24 h. Isolates of different colonial types, if any, were purified on fresh media, incubated and then used for identification following standard biochemical methods.

Histomorphological and Histochemical Examination

At days 7 and 14 post infliction of excision wound, the animals were euthanized and wound biopsies taken for histomorphological and histochemical examinations. The biopsies were fixed in 10% buffered formalin, dehydrated in graded alcohol and embedded in paraffin wax. Then, 5 μm thick sections were stained with hematoxylin and eosine (H and E) for general histomorphological analysis. Vangieson stain was used for the demonstration of Type 1 collagen (histochemical analysis) in the dermis. Semi-quantitative evaluation of the wound slides for re-epithelialization, polymorphonulcear leukocytes, tissue macrophages (TM), fibroblasts, neovascularization, collagen formation and Type 1 collagen was done using subjective scoring method on a 5-point scale from 0 to 4 by 2 independent observers blinded to the treatment protocol [40].

Statistical Analysis

Data obtained were summarized as mean ± standard error of mean. Mean values of wound breaking strength, granulation tissue weight, percentage wound contraction, wound epithelialization and time for complete healing for different groups were compared using one-way Analysis of Variance. Duncan multiple range test was used to separate variant means. $P < 0.05$ was considered significant.

RESULTS

Extraction

The MCJBE had an aromatic smell and was brownish in colour. The percentage yield was 13.6% w/w material.

Acute Toxicity Test

Administration of MCJBE extract suspended in 10% Tween 20 to rats even at the highest dose of 2000 mg/kg bw did not produce any death in the treated groups. No sign of acute toxicity was also observed except transient dullness and weakness which disappeared in few minutes.

Phytochemical Analysis

Preliminary phytochemical analysis of MCJBE qualitatively revealed the presence of alkaloids, tannins, saponins, glycosides but absence of flavonoids [Table 1].

Incision Wound Model

The result of the effect of MCJBE on breaking strength of the healed wound showed significantly ($P < 0.05$) higher wound breaking strength in animals in Group A (300 mg/kg bw of 10% MCJBE) when compared with those in Group B (300 mg/kg bw of 5% MCJBE) and the control [Table 2]. There was no significant difference ($P > 0.05$) in wound breaking strength of animals in Group B and the control.

Table 1: Phytochemical analysis of methanolic *Crinum jagus* bulb extract

Phytoconstituent	Amount
Alkaloids	+++
Tannins	+
Saponins	++
Glycosides	++
Flavonoids	-

+++: Appreciable amount, ++: Moderate amount, +: Trace amount, -: Completely absent

Table 2: Wound tissue breaking strength and granuloma weight in rats post infliction of incision and dead space wounds

Group (treatment)	Mean±SEM (g)	
	Wound tissue breaking strength	Granuloma weight
A (300 mg/kg bw of 10% MCJBE)	344.48±29.77[a]	0.22±0.01[a]
B (300 mg/kg bw of 5% MCJBE)	227.54±25.22[b]	0.10±0.00[b]
Control	224.46±24.71[b]	0.11±0.00[b]

bw: Body weight, MCJBE: Methanolic *Crinum jagus* bulb extract, Different superscripts[a,b] across a column indicate significant difference in means at $P<0.05$, SEM: Standard error of mean

Dead Space Wound Model

The result of the effect of MCJBE on granulation tissue weight showed that dry granulation tissue weight of animals in Group A was significantly ($P < 0.05$) higher when compared against that of animals in Group B and the control [Table 3]. There was no significant difference ($P > 0.05$) in granulation tissue weight of animals in Groups B and the control.

Excision Wound Model

Percentage wound contraction

The percentage rate of wound contraction in Group I (10% MCJBEO treated) at day 7 pw, was significantly ($P < 0.05$) higher compared to Group III (framycetin sulfate treated) and the control [Table 4]. Wound contraction in both MCJBEO treated groups (I and II) did not differ significantly ($P > 0.05$). Wound contraction in Group 2 did not vary significantly ($P > 0.05$) when compared with Group III. Similar trends were observed at day 14 pw. At day 21 wound contraction in both MCJBEO treated groups (I and II) and the control significantly ($P < 0.05$) increased when compared against Group III. No significant difference ($P > 0.05$) existed between the MCJBEO treated groups throughout the study period. For all groups, most wound contraction occurred between days 7 and 21 pw [Table 5].

Wound Epithelialization and Complete Wound Healing Time

Epithelialization occurred between days 11 and 17 pw [Table 6]. Epithelialization time was significantly ($P < 0.05$) shorter in animals in Groups I and II and the control compared with those in Group III. There was no significant difference ($P > 0.05$) in epithelialization time of wound between animals in Groups I-III. Mean value of epithelialization time of Group I revealed that animals in the group had the shortest wound epithelialization time. Similar trends were observed for complete healing time among the groups. Complete wound healing occurred significantly ($P < 0.05$) earlier among animals in Group I and II when compared with those in Group III.

Wound Microbial Assay

No wound contaminating bacterial organism was isolated from the wound sites of animals in Groups I-III throughout the study period, while few colonies of *Bacillus* species was isolated from the wound sites of animals in the control group at days 7 and 14 post infliction of excision wound.

Histomorphological Findings

At day 7 post infliction of excision wound, wound sections of animals in the control group revealed more inflammatory cells, that of animals in Groups I (10% MCJBEO treated) and II (5% MCJBEO treated) revealed more fibroblasts [Figure 1a-c] while those of animals in group III (framycetin sulfate treated) showed in addition to more fibroblasts, complete layer of epithelial regeneration [Figure 1d]. At day 14 pw, epithelial regeneration with overlying keratin was observed to be more in wound sections of animals in Group I-III than in wound sections of animals in the control group [Figure 2a-d].

Histochemical Findings

At day 7 pw, there were more Type 1 collagen deposits in wound sections of animals in Groups I-III compared with that of

Table 3: Wound tissue breaking strength in rats post infliction of incision wound

Group (treatment)	Mean±SEM (g)
	Wound tissue breaking strength
A (300mg/kg bw of 10% MCJBE)	344.48±29.77[a]
B (300mg/kg bw of 5% MCJBE)	227.54±25.22[b]
C (Control)	224.46±24.71[b]

bw: Body weight, MCJBE: Methanolic *Crinum jagus* bulb extract, Different superscripts[a,b] across a column indicate significant difference in means at $P<0.05$, SEM: Standard error of mean

Table 4: Granulation tissue weight in rats post infliction of incision wound

Group (treatment)	Mean±SEM (g)
	Granulation tissue weight
D (300mg/kg bw of 10% MCJBE)	0.22±0.01[a]
E (300mg/kg bw of 5% MCJBE)	0.10±0.00[b]
F (Control)	0.11±0.00[b]

bw: Body weight, MCJBE: Methanolic *Crinum jagus* bulb extract, Different superscripts[a,b] across a column indicate significant difference in means at $P<0.05$, SEM: Standard error of mean

Table 5: Percentage rate of wound contraction in rats post infliction of excision wound

Group (treatment)	Mean±SEM wound contraction (%) at days post wounding			
	1	7	14	21
I (10% MCJBEO)	0.00±0.00	89.40±3.12[a]	98.80±0.37[a]	99.60±0.40[a]
II (5% MCJBEO)	0.00±0.00	64.60±13.73[ab]	90.20±7.23[ab]	99.00±0.44[a]
III (Framycetin sulfate)	0.00±0.00	55.40±8.9[b]	85.80±1.7[b]	93.60±1.7[b]
IV (Control)	0.00±0.00	22.84±4.42[c]	81.39±4.74[b]	97.14±0.74[a]

MCJBEO: Methanolic *Crinum jagus* bulb extract ointment, Different superscripts[a,b,c] across a column indicate significant difference in means at $P<0.05$, SEM: Standard error of mean

Table 6: Wound epithelialization and complete wound healing time of rats post infliction of excision wound

Group (treatment)	Mean±SEM (days) post wounding	
	Epithelialization time	Complete healing time
I (10% MCJBEO)	11.2±1.44[a]	16.85±1.65[a]
II (5% MCJBEO)	12.14±1.47[a]	19.14±0.7[ab]
III (Framycetin sulfate)	17.80±1.2[b]	25.28±1.04[c]
IV (Control)	12.00±0.01[a]	19.43±1.1[ab]

MCJBEO: Methanolic *Crinum jagus* bulb extract ointment, Different superscripts[a,b,c] across a column indicate significant difference in means at $P<0.05$, SEM: Standard error of mean

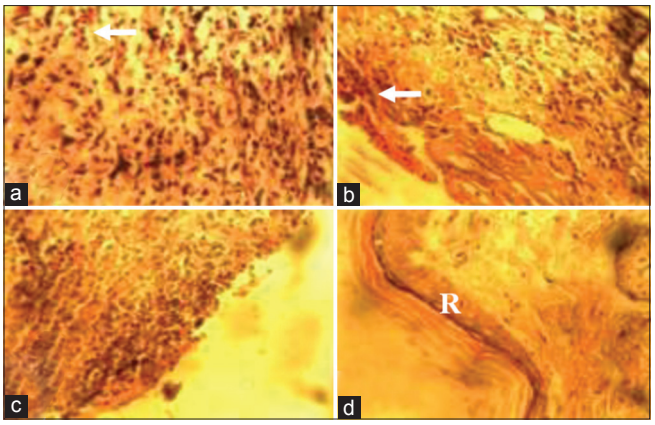

Figure 1: Photomicrographs of wound site sections at day 7 post infliction of excision wound showing moderate inflammatory cell infiltrates (arrow) and more fibroblasts in wound sections of animals in Groups I (10% methanolic *Crinum jagus* bulb extract ointment [MCJBEO] treated) (a) and II (5% MCJBEO treated) (b), more inflammatory cell infiltrates in wound section of animals in Group IV (control) (c) and complete layer of epithelial regeneration (R) with more fibroblasts in wound section of animals in Group III (framycetin sulfate treated) (d). H and E ×400

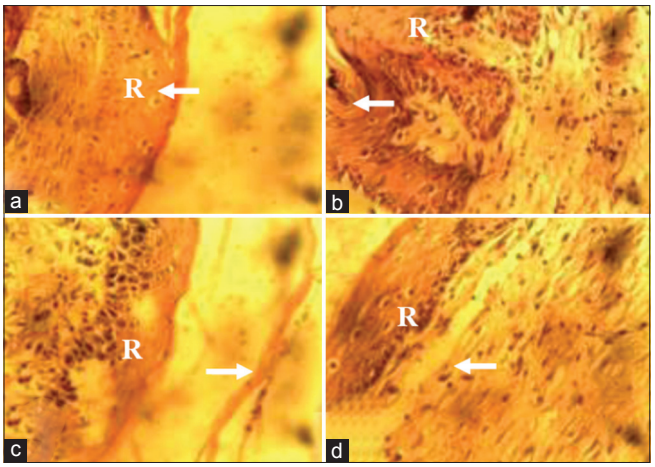

Figure 2: Photomicrograph of wound site sections at day 14 post infliction of excision wound showing a complete layer of regenerated epithelium (R) with overlying keratin (arrow) which was greater in wound sections of animals in Groups I (10% methanolic *Crinum jagus* bulb extract ointment [MCJBEO] treated) (a), II (5% MCJBEO treated) (b) and III (framycetin sulfate treated) (c) than in wound sections of animals in the control group (d). H and E ×400

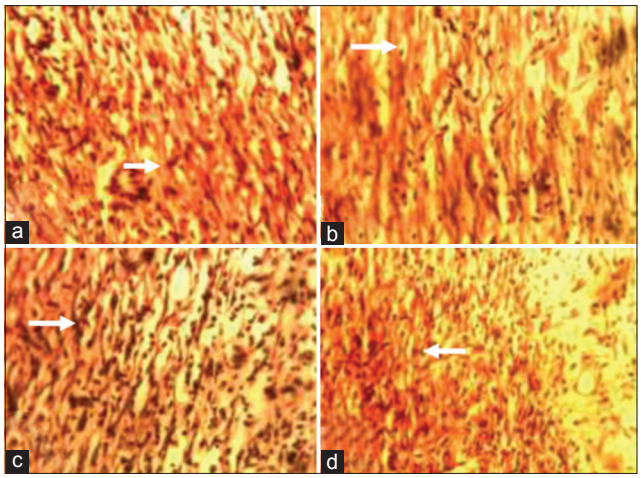

Figure 3: Photomicrograph of wound site sections at day 7 showing greater Type 1 collagen positive tissues (arrow) in wound sections of animals in Groups I (10% methanolic *Crinum jagus* bulb extract ointment [MCJBEO] treated) (a), II (5% MCJBEO treated) (b) and III (framycetin sulfate treated) (d) compared to wound section of animals in the control (c). Vangieson ×400.

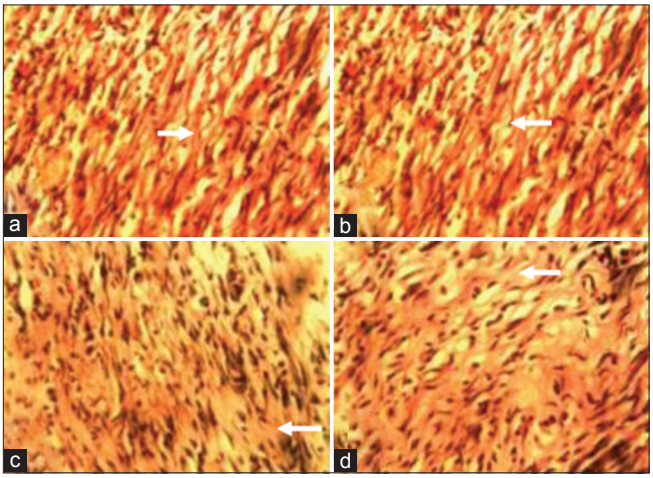

Figure 4: Photomicrograph of wound site sections at day 14 post infliction of excision wound showing more of Type 1 collagen positive tissues (arrow) in wound sections of animals in Group I (10% methanolic *Crinum jagus* bulb extract ointment [MCJBEO] treated) (a) than in wound sections of animals in Groups II (5% MCJBEO treated) (b), the control Group IV (c) and Group III (framycetin sulfate treated) (d). Vangieson ×400

animals in the control group [Figure 3a-d]. At day 14 pw, wound sections of animals in Group I revealed more Type 1 collagen deposits when compared with wound sections of animals in Groups II-IV [Figure 4a-d].

DISCUSSION

In this study, three wound models - incision, excision and dead space were used to investigate the effect of MCJBE on various events of wound healing. To ascertain the rate of healing following creation of excision wounds, rates of wound contraction and epithelialization as well as time of complete healing were assessed. The fact that animals in Group I (treated with 10% MCJBEO) had the highest percentage rate of wound contraction and shortest period of wound re-epithelialization when compared with their counterparts in the other groups, suggest that the 10% MCJBEO promoted wound healing better than at the lower concentration (5% MCJBEO) and the reference drug (framycetin sulfate) used in this study. The result also suggests that wound healing effect of *C. jagus* bulb extract occurs in a concentration-dependent manner. The shortest healing time of wounds recorded among animals in the Group I (treated with 10% MCJBEO) could be attributed to the more rapid epithelialization and wound contraction observed among animals in the group. Wound contraction occurs following increased stimulation of interleukin-8 (an inflammatory

chemokine) which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes thereby resulting in rapid maturation of granulation tissue [41]. Therefore, it is possible that the 10% MCJBEO promoted this mechanism more than the lower concentration and the reference drug used in this study. The shortest epithelialization time recorded among animals in the 10% MCJBEO treated group could be due to enhancement of collagen deposition by the extract which occurred better at a higher concentration [42]. This further suggests a concentration-dependent manner of activity exhibited by the extract.

Granulation tissue formed in a dead space wound comprises of an accumulation of modified macrophages, histological giant cells and undifferentiated connective tissue which consist largely of collagen [43,44]. Increase in granulation tissue in dead space wound is associated with enhanced collagen maturation and increased protein content as well as angiogenesis in the wound [45]. As observed in this study, the dry granulation tissue weight from dead space wounds of animals in Group D (dosed with 300 mg/kg bw of 10% MCJBE) was significantly highest when compared with Group E (dosed with 300 mg/kg bw of 5% MCJBE) and the control. This result suggests more rapid collagen maturation in the healing wounds of animals in Group D. This finding is further collaborated by the results of the histologic and histochemical studies of the excision wounds which revealed marked increase in TM, fibroblasts, collagen content, and neovascularization in wound sections of animals in Group I (10% MCJBEO treated). However, the non-significant difference between the granulation tissue weight of animals in Group E (dosed with 300 mg/kg bw of 5% MCJBE) and the control, suggests that the lower concentration of *C. jagus* bulb extract may not elicit wound healing effect better than what occurs in natural healing. Therefore, it may not be useful to use lower concentration below 10% of the *C. jagus* bulb extract in management of wound.

The tensile strength of a healing wounded tissue is dependent on the amount of collagen content and stabilization of the fibres [46]. According to Omale and Isaac [2], collagen the major component which strengthens and supports extracellular tissue is composed of amino acids and hydroxyl proline which are used as a biochemical marker for tissue collagen. Therefore, the observed significant increase in tensile strength of wounds among animals in Group A (dosed with 300 mg/kg bw of 10% MCJBE) when compared with Group B (dosed with 300 mg/kg bw of 5% MCJBE) and the control, may not only be due to increased collagen synthesis, but also due to its proper deposition and alignment [47]. The significantly higher wound breaking strength among animals in Group A when compared with their counterparts in the other groups, further suggests that the *C. jagus* bulb extract exhibited its wound healing effect in a concentration-dependent manner.

In the present study, preliminary phytochemical analysis of *C. jagus* bulb extract revealed the presence of alkaloid, tannins, saponins, and glycosides. This finding is in agreement with the report of Ode *et al.* [27] except that they reported negative saponin content. Studies with other plant extracts showed that plants containing alkaloids [5,13,48], triterpenoids [49], and tannins [50] promoted wound healing process. Therefore, higher concentration of the bioactive substances in the 10% MCJBE might have contributed to the significantly faster tissue approximation and increased tensile strength of wounds observed among animals in the groups treated with the concentration.

Non isolation of microbial organisms from the wounds in both MCJBEO treated Groups (I and II) and Group III (framycetin sulfate treated) suggests that the extract was able to prevent microbial contamination of the wounds. This may suggest that the extract induced migration of phagocytes (responsible for killing contaminating microbes in wound) to the wound site. This is supported by the histological findings of increased infiltration of phagocytic cells in wound sites of animals in the MCJBEO treated groups. Absence of microbial isolation from wounds of animals in the extract treated groups may also suggest that the *C. jagus* extract could have elicited antimicrobial effect similar to the reference drug, framycetin sulphate which is an antibiotic. Adesanya *et al.* [25] reported antistaphylococcal activity of *C. jagus* bulb extract. Plant extracts containing alkaloids and saponins have been reported to exhibit antimicrobial activity [48]. Plant extracts containing these phytochemicals including *C. jagus* bulb extract have been shown to exhibit antioxidant activity [24,27]. And wound healing properties of plants, in most cases, are associated with their significant antioxidant activities [5,30]. Therefore, the absence of microbial contamination of wound in this study may be attributed to the presence of alkaloids and saponnins in the extract. Nevertheless, isolation of *Bacillus* species from wounds of animals in the control group in this study may be because *Bacillus* is ubiquitous and the wounds were not treated with any agent that could have elicited antimicrobial effect.

CONCLUSION

The results of this study have established that the MCJBE potentiates wound healing partly by increasing collagen deposition and epithelialization. The wound healing potentials of the extract could be attributed to the presence of polyphenolic compounds including tannins, saponins, glycosides, and alkaloids in the extract. The extract elicited the best wound healing activity at a concentration of 10% and the activity was concentration-dependent. The use of *C. jagus* bulb in the management of skin sores and boils in folkloric medicine is thus validated by this study. However, further studies which would involve cell biology, immunology, and biochemistry to elucidate fully the process of wound healing by *C. jagus* bulb extract is recommended.

REFERENCES

1. Nguyen DT, Orgill DP, Murphy GF. The pathophysiologic basis for wound healing and cutaneous regeneration, biomaterials for treating skin loss. Cambridge, Boca Raton: Wood Head Publishing, CRC Press; 2009. p. 25-57.
2. Omale J, Isaac AV. Excision and incision wound healing potential of *Saba Florida* (Benth) leaf extract in *Rattus novergicus*. Int J Pharm

Biochem Res 2010;1:101-7.
3. Al-Henhena N, Mahmood AA, Al-magrami A, Nor Syuhada AR, Zahra AA, Summaya MD, *et al*. Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats. J Med Plants Res 2011;5:3660-6.
4. Pandith H, Zhang X, Liggett J, Min KW, Gritsanapan W, Baek SJ. Hemostatic and wound healing properties of *Chromolaena odorata* Leaf Extract. ISRN Dermatol 2013;2013:168269.
5. Tsala DE, Nga N, Thiery BN, Bienvenue T, Theophile D. Evaluation of the antioxidant activity and the healing action of the ethanol extract of *Calotropis procera* bark against surgical wounds. J Intercult Ethnopharmacol 2014;4:64-9.
6. Vezza R, Mezzasoma AM, Venditti G, Gresele P. Prostaglandin endoperoxides and thromboxane A2 activate the same receptor isoforms in human platelets. Thromb Haemost 2002;87:114-21.
7. Aso Y. Plasminogen activator inhibitor (PAI)-1 in vascular inflammation and thrombosis. Front Biosci 2007;12:2957-66.
8. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. Trends Cell Biol 2005;15:599-607.
9. Fialkow L, Wang Y, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 2007;42:153-64.
10. Wagener FA, van Beurden HE, von den Hoff JW, Adema GJ, Figdor CG. The heme-heme oxygenase system: a molecular switch in wound healing. Blood 2003;102:521-8.
11. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol 2003;200:448-64.
12. Kumar A. Wound healing and general management of wounds. In: Veterinary Surgical Technologies. New Delhi: Vikas Publishing House PVT Limited; 2002. p. 151-61.
13. Udegbunam SO, Udegbunam RI, Muogbo CC, Anyanwu MU, Nwaehujor CO. Wound healing and antibacterial properties of methanolic extract of *Pupalia lappacea* Juss in rats. BMC Complement Altern Med 2014;14:157.
14. Udegbunam SO, Nnaji TO, Udegbunam RI, Okafor JC, Agbo I. Evaluation of herbal ointment formulation of *Milicia excelsa* (Welw) C.C berg for wound healing. Afr J Biotechnol 2013;12:3351-9.
15. Houghton, PJ, Agbedahuns JM, Adegbulugbe A. Choline esterase inhibitory properties of alkaloids from two Nigerian *Crinum* species. Sci Dir 2004;65:2893-6.
16. Shorinwa OA, Ebong OO, Obianime AW, Siminialayi IM. The effect of acetone extracts of *Crinum jagus* bulbs on the histology of the kidney, liver and testis of albino rats. Peak J Med Plant Res 2013;2:38-44.
17. Sonibare MA, Gbile ZO. Ethnobotanical survey of anti-asthmatic plants in South Western Nigeria. Afr J Tradit Complement Altern Med 2008;5:340-5.
18. Olorode O. Taxonomy of West African Flowering Plants. London: Longman Publishing Company; 1984. p. 121.
19. Hannibal LS. A systematic review of the genus *Crinum* (Amaryllidaceae). California: Fair Oaks 95628; 1992. pp.1-15.
20. Kokwaro JO. Medicinal Plants of East Africa. Kenya: General Printers; 1976. p. 230.
21. Gill LS. Ethnomedicinal Uses of Plants in Nigeria. Benin City, Nigeria: Uniben Press; 1992. p. 27.
22. Idu M, Obaruyi GO, Erhabor JO. Ethnobotanical uses of plants among the Binis in the treatment of ophthalmic and ENT (ear, nose and throat) ailments. Ethnobot Leaflets 2008;13:480.
23. Ogunkunle AT, Olopade OR. Studies on the asthma coughs plant *Crinum jagus* L. (Amaryllidaceae) in Nigeria. Afr J Plant Sci 2011;5:108-14.
24. Nwaehujor CO, Nwinyi FC, Ode JO. Liver protective activity of the methanol extract of *Crinum jagus bulb* against acetaminophen-induced hepatic damage in rats. Asian J Biochem 2012;7:182-93.
25. Adesanya SA, Olugbade TA, Odebiyi OO, Aladesanmi JA. Antibacterial alkaloids in *Crinum jagus*. Pharm Biol 1992;30:303-7.
26. Ode OJ, Azusu IU. The anti-venom activities of the methanolic extract of the bulb of *Crinum jagus* (Amaryllidaceae). Toxicon 2006;48:331-42.
27. Ode OJ, Nwaehujor CO, Onakpa MM. Evaluation of antihaemorrhagic and antioxidant potentials of *Crinum jagus* bulb. Int J Appl Biol Pharm Tech 2010;1:1330-6.
28. Edema MD, Okieimen FE. Chemical and anticonvulsant screening of *Crinum jagus*. Niger J Chem Res 2002;7:25-8.
29. Shilpa K, Rajendra Y, Sanjeeva K, Kumar DV, Kumar RV, Gnananath K. Evaluation of wound healing potential in *Crinum defixum* Ker *gawl* bulbs. Asian J Pharm Clin Res 2013;6:61-3.
30. Suntar I, Akkol K, Nahar L, Sarker SD. Wound-healing and antioxidant properties: Do they co-exist in plants? Free Radic Antioxid 2012;2:2-7.
31. Thi Ngoc Tram N, Titorenkova TV, St Bankova V, Handjieva NV, Popov SS. Crinum L. (Amaryllidaceae). Fitoterapia 2002;73:183-208.
32. Trease EC, Evans WC. Pharmacognosy. 12th ed. London: Bailliere and Tindall; 1983. p. 115-625.
33. Okore VC, Ibezim EC, Adikwu MU, Attama AA, Esimone CO, Uzuegbu BD, *et al*. Laboratory Techniques in Pharmaceutics and Pharmaceutical Microbiology. 2nd ed. Nigeria: El'Demark Publishers; 2004. p. 1-20.
34. Rathi B, Patil PA, Baheti AM. Evaluation of aqueous extract and seeds of *Moringa oleifera* for wound healing in albino rats. J Nat Remedies 2004;4:145-9.
35. Morton JJ, Malone MM. Evaluation of vulnerary activity by open wound procedure in rats. J Trauma 1992;20:323-4.
36. Dash GK, Saresh P, Ganapaty S. Studies on hypoglycaemic and wound healing activities of *Lantana camara* Linn. J Nat Remedies 2001;1:105-10.
37. Chah KF, Eze CA, Emuelosi CE, Esimone CO. Antibacterial and wound healing properties of methanolic extracts of some Nigerian medicinal plants. J Ethnopharmacol 2006;104:164-7.
38. Nayak BS, Pinto Pereira LM. *Catharanthus roseus* flower extract has wound-healing activity in Sprague Dawley rats. BMC Complement Altern Med 2006;6:41.
39. Okoli CO, Ezike AC, Akah PA, Udegbunam SO, Okoye TC, Mbanu TP, *et al*. Studies on wound healing and antiulcer activities of extract of aerial parts of *Phyllanthus niruri* L. (Euphorbiaceae) Am J Pharmacol Toxicol 2009;4:118-26.
40. Gal P, Kilik R, Mokry M, Vidinsky B, Vasilenko T, Mozes S, *et al*. Simple method of open skin wound healing model in cortico-steroid-treated and diabetic rats: Standardization of semi-quantitative and quantitative histological assessments. Vet Med 2008;53:652-9.
41. Moyer KE, Saggers GC, Allison GM, Mackay DR, Ehrlich HP. Effects of interleukin-8 on granulation tissue maturation. J Cell Physiol 2002;193:173-9.
42. Clark RA. Wound repair: Overview and general considerations. In: Clark RA, Henson PM, editors. The Molecular and Cellular Biology of Wound Repair. New York: Plenum Press; 1996. p. 3.
43. Whaley K, Burt AD. Inflammation, healing and repair. In: Macsween RM, Whaley K, editors. Muir's Textbook of Pathology. 13th ed. London: Arnold; 1996. p. 112-65.
44. Bairy KL, Rao CM. Wound healing profiles of *Ginkigo biloba*. J Nat Remedies 2001;1:25-7.
45. Abu-Al-Basal MA. Healing potential of *Rosmarinus officinalis* L. on full-thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/c mice. J Ethnopharmacol 2010;131:443-50.
46. Udupa SL, Shaila HP, Udupa AL, Ramesh KV, Kulkami DR. Wound healing properties of some medicinal plants. Biochem Arch 1991;7:207-12.
47. Shivhare Y, Singour PK, Patil UK, Pawar RS. Wound healing potential of methanolic extract of *Trichosanthes dioica* Roxb (fruits) in rats. J Ethnopharmacol 2010;127:614-9.
48. Maatalah MB, Bouzidi NK, Bellahouel S, Merah B, Fortas Z, Soulimani R, *et al*. Antimicrobial activity of the alkaloids and saponin extracts of *Anabasis articulate*. J Biotech Pharm Res 2012;3:54-7.
49. Scortichini M, Pia RM. Preliminary *in-vitro* evaluation of the antimicrobial activity of terpenes and terpenoids towards *Erwinia amylovora* (Burril). J Appl Bacteriol 1991;71:109-12.
50. Rane MM, Mengi SA. Comparative effect of oral administration and topical application of alcoholic extract of *Terminalia arjuna* bark on incision and excision wounds in rats. Fitoterapia 2003;74:553-8.

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Original Research

Musa sapientum with exercises attenuates hyperglycemia and pancreatic islet cells degeneration in alloxan-diabetic rats

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ABSTRACT

Aim: We tested the hypothesis that administrations of methanolic extracts of *Musa sapientum* sucker (MEMS) with exercises attenuated hyperglycemia in alloxan-diabetic rats. **Materials and Methods:** A total of 40 adult male rats were divided into equal eight groups. Normoglycemic Group A was Control. Alloxan (180 mg/kg, i.p.) was administered to rats in Groups B - H to induce diabetes. Group B (diabetic control) received physiological saline. Groups C - H received MEMS (5 mg/kg), MEMS (10 mg/kg), Glibenclamide (5 mg/kg), MEMS (5 mg/kg) + exercises, MEMS (10 mg/kg) + exercises and Exercises only, respectively. Changes in body weight, blood glucose levels (BGL) and pancreatic histology were evaluated during or at the end of experiment. Body weights and BGL of rats were expressed as mean ± standard deviation and analyzed using the statistical software program SPSS 15. Statistical comparisons were done using the Student's *t*-test for unpaired samples. Differences between groups were determined as significant at $P \leq 0.05$. **Results:** Significantly ($P < 0.05$) decreased bodyweight was observed in B and H compared to A and C - G. Treatment with MEMS significantly ($P < 0.05$) decreased elevated BGL in C and D. Hypoglycemic effect of MEMS appeared enhanced with exercises in F and G. Exercises regimen alone (H) resulted in percentage reduction in BGL lower than those of C - G. Histopathological examinations revealed normal pancreas (A), atrophied islet cells (B), hyperplasia with adequate population of islet cells (C - G), and reduced hyperplasia of islet cells (H). **Conclusion:** MEMS with exercises attenuated hyperglycemia in alloxan-diabetic rats.

KEY WORDS: Alloxan, hyperglycemia, *Musa sapientum,* pancreatic islet cells, rats

INTRODUCTION

Diabetes mellitus characterized by hyperglycemia results from complete or relative deficiency of insulin. It could result from autoimmune insufficient insulin production by pancreatic islet cells or resistance of cells to insulin actions [1]. Insulin insufficiency disrupts water and electrolyte homeostasis due to adverse effects on carbohydrate, protein, and fat metabolism resulting in deranged structural and functional body systems. Diabetes is a global health burden with the estimated 171 million people affected in year 2000 expected to be doubled by 2030 [1]. Current antidiabetic therapeutic regimens (orthodox or complementary alternative medicine) are at best palliative. There is yet no curative treatment for diabetes mellitus, hence it is most relevant that further scientific efforts are made to discover plants with potential hypoglycemic properties in order to identify and produce better therapeutic anti-diabetic drugs.

M. sapientum belongs to the family Musaceae and is a food crop well-grown in villages and towns in Nigeria, and its various parts have been described to possess different medicinal properties [2]. Significant antioxidant properties have been observed in investigations of peel extracts [3], inflorescence and stalk [4] of *M. sapientum*. Its banana pulps [5] and unripe plantain bananas [6] have been reported to have anti-ulcerogenic properties; its seeds possess antioxidant, anti-diarrheal, and anti-microbial activities [7], its flowers possess hypoglycemic activities [8] while its sucker possesses hypoglycemic [9] and anti-ulcerogenic [2] potentials.

During physical activities or exercises, oxygen consumption in working muscles increases several folds (>20-fold) with the muscles making use of stored free fatty acids, triglycerides, and glycogen obtained from the catabolism of adipose tissue triglycerides and liver glucose [10]. Body muscles represent approximately 40% of the mass of the body, hence muscular actions are of great

importance to the control of blood glucose levels (BGL) and prevention of diabetes [11-13]. Intense exercises had been reported to exert positive effects on insulin release and glucoregulation [14]. Furthermore, epidemiological studies showed that the incidence of non-insulin dependent diabetes mellitus negatively correlated with measures of physical activities. This is due to the fact that exercises even at mild levels enhanced the uptake of glucose by cells in apparently normal and insulin-resistant individuals [14]. In addition, it has been reported that some mild cases of diabetes were treatable with exercises and improved diet even in the absence of insulin treatment [14]. Exercises and/or physical activities may, therefore, be of therapeutic relevance to individuals susceptible to diabetes and diabetic patients [10].

The hypoglycemic potentials of *M. sapientum* sucker in alloxan-diabetic rats have been previously noted [9]. However, in further considerations of the possible roles of exercises in the treatment of diabetes and to demonstrate whether or not that exercises could improve the hypoglycemic activities of *M. sapientum* sucker extract; we tested the hypothesis that the administrations of methanolic extracts of *M. sapientum* sucker with exercises attenuated hyperglycemia in alloxan-diabetic male Wistar rats.

MATERIALS AND METHODS

Collection and Authentication of *M. sapientum*

Fresh sucker of *M. sapientum* (10 kg) was collected from a farmland on Olabisi Onabanjo University Campus in Ikenne, Ogun State, Nigeria. The plant's identity was confirmed and authenticated at the Forest Research Institute of Nigeria, Ibadan, Oyo State, assigned the voucher number FHI 108349 and samples were deposited at the herbarium.

Animals

Totally, 40 male adult Wistar rats weighing between 200 g and 250 g and aged 22-25 weeks old were obtained from the colony bred of the Department of Physiology, University of Ibadan, Nigeria. Animals were fed throughout experimental procedures with growers feed produced by Bendel Feed and Flour Mill Limited, Nigeria. The animals were caged and kept under the standard condition in a well-ventilated animal house of the Faculty of Basic Medical Sciences of Olabisi Onabanjo University, Nigeria at the room temperature of 25°C. All rats received water ad libitum and were acclimatized for 2 weeks before the start of experimental procedures. Ethical Approval was obtained from the Ethical Committee of the Faculty of Basic Medical Sciences of Olabisi Onabanjo University, Nigeria, and the experimental procedures were carried out in accordance with the "Principles of laboratory animal care" of NIH publication number 85-23 as revised in 1985.

Preparation of Plant Extracts

Harvested *M. sapientum* suckers were rinsed and chopped into small pieces to increase the surface area for easy and fast drying. The pieces were shade dried at room temperature 25°C-30°C for 2 weeks in order to prevent direct sunlight, which can react with the active ingredients of the plant. This prevented deterioration of the phytochemical constituents of the plant material. Dried pieces of the plant material were powdered. 200 g of the dried sucker was extracted in 70% methanol for 48 h. The extract was then filtered and concentrated using rotary evaporator. The concentrated extract was further dried on a water bath. The total yield of the methanolic extract of *M. sapentium* (MEMS) was 2.61%.

Phytochemical Evaluation

M. sapientum extract was evaluated for the presence of different chemical groups using standard methods as earlier described [2,9].

Experimental Design and Treatment Groups

Totally, 40 rats were weighed, and fasting BGL determined. 5 of these normoglycemic rats were randomly selected and served as the normal control (Group A). Alloxan monohydrate (BDH Chemical Limited, Poole, England) at a dose of 180 mg/kg as determined from previous studies [9,15,16] was injected intraperitoneally to the remaining normoglycemic rats ($n = 35$) to induce diabetes. Rats with fasting BGL ≥ 190.0 mg/dl 72 h after alloxan injection were randomized into seven groups (Groups B - H) of five rats per group and treated as follows:

1. Group B: Physiological saline (diabetic control)
2. Group C: MEMS (5 mg/kg)
3. Group D: MEMS (10 mg/kg)
4. Group E: Glibenclamide (5 mg/kg)
5. Group F: MEMS (5 mg/kg) + exercises
6. Group G: MEMS (10 mg/kg) + exercises
7. Group H: Exercises only

MEMS, glibenclamide, and physiological saline were administered orally for 21 consecutive days. MEMS were administered to rats in Groups E and F, 30 min prior to their engagement in exercises.

Method for Engaging Rats in Physical Activity/Exercises

Rats in Groups E, F, and H were engaged in physical activity or exercises using the modified method of previous studies [Figure 1] [11-13]. Rats were exercised by swimming unaided for 60 min daily for 5 days/week by placing them in a 60 cm deep plastic container at 32 ± 1°C. The rats were trained for the exercise model and were exercised for 60 min/day (5 times a week) for a period of 3 weeks or 21 days (Days 5-26) to ensure adaptation of swimming into the memory of the rats so that the resulting effects on body organs/tissues were no longer due to stress.

Estimation of BGL

Fasting BGL was measured by collecting a drop of blood from the tail of each rat after incision with a sharp blade. The blood was dropped onto dextrostix (Accu-Chek system, Roche, Group, Germany) reagent pad and values read using the microprocessor digital blood glucometer (GlucotrendR2, Accu-Chek system, Roche, Group, Germany). BGL was measured and recorded on days 1, 2, 5, 12, 19, and 26 of experiments.

Histology

Rats were sacrificed under diethyl ether (BDH Chemical Limited, Poole, England) anesthesia at the end of all treatments. The pancreas was removed and immediately fixed in 10% formalin solution. Fine microscopic sections were obtained and mounted on glass slides. The mounted sections were then counterstained with hematoxylin and eosin for light microscopic analyses [9,15,16].

Statistical Analysis

Body weights and BGL of rats were expressed as mean ± standard deviation and analyzed using the statistical software program SPSS 15. Statistical comparisons were done using the Student's *t*-test for unpaired samples. Differences between groups were determined as significant at $P \leq 0.05$.

RESULTS

Phytochemical Analysis

Phytochemical screening of *M. sapientum* sucker revealed the presence of saponins, saponin glycosides, tannins, alkaloids, and indole alkaloids.

Changes in Body Weight

Body weights of rats significantly decreased ($P < 0.05$) in rats belonging to Group B (untreated alloxan-diabetic Group) or alloxan-diabetic rats in Group H treated with exercises only. Body weight increased significantly ($P < 0.05$) in control rats (Group A) and alloxan-diabetic rats in Groups C - G treated with MEMS and exercises or glibenclamide [Figure 2].

Blood Glucose Level

BGL of alloxan-diabetic rats significantly increased ($P < 0.05$) in Group B rats when compared with normoglycemic rats (Group A). Treatment with MEMS, MEMS with exercises, glibenclamide or exercises only significantly ($P < 0.05$) reduced alloxan-induced hyperglycemia as observed in Groups C - H [Figure 3]. Treatment with exercises only (Group H) produced a significantly lower reduction in BGL when compared with glibenclamide-treated rats (Group E). However, a significantly higher reduction in BGL were observed in rats belonging to Groups C, D, F, and G treated with MEMS (with or without exercises) when compared with glibenclamide-treated rats (Group E) [Figure 3]. The best hypoglycemic effect was observed in Group D rats treated with MEMS (10 mg/kg) [Figure 3]. There was no significant difference ($P > 0.05$) in the extent of BGL reduction of diabetic rats treated with MEMS only (Groups C and D) and those treated with MEMS plus exercises (Groups F and G) [Figure 3].

Histological Examination

Histological examination revealed a significant reduction in population of pancreatic islet cells of diabetic control rats of Group B [Figure 4b] when compared with normoglycemic control rats of Group A that showed normal cyto-architectural components of the pancreas with adequate population of pancreatic islet cells [Figure 4a]. Alloxan diabetic rats in

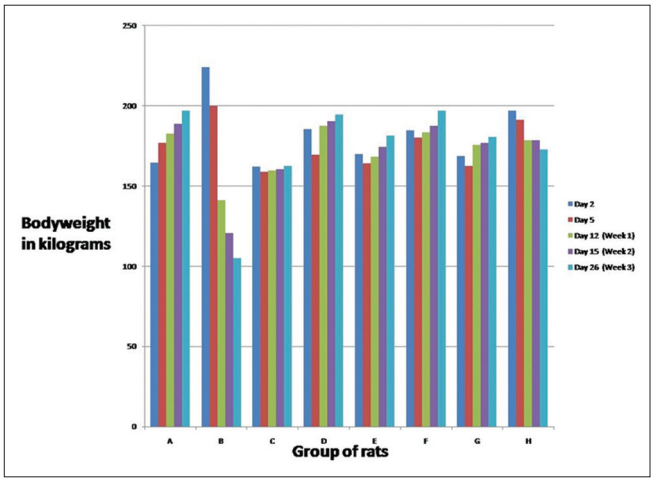

Figure 2: Changes in body weights in kg of rats

Figure 1: Rats of Group F undergoing swimming exercises

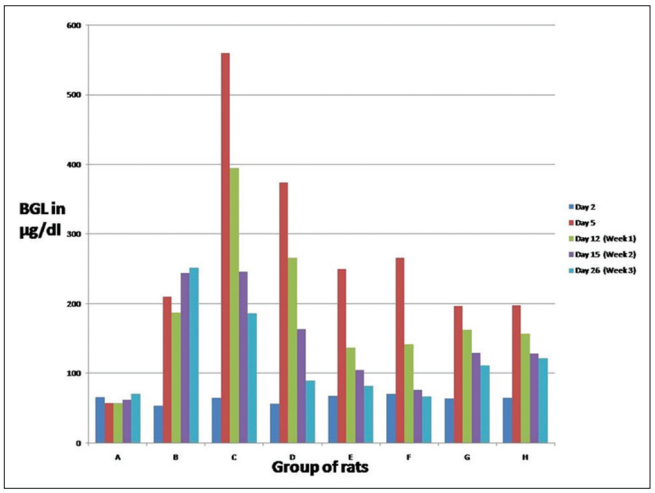

Figure 3: Mean blood glucose levels in µg/dl of Rats

Groups C – G treated with MEMS, MEMS plus exercises or glibenclamide exhibited pancreatic histology similar to those of the normal control [Figures 4a, 5a-c, and 6a-b]. Mild regeneration or hyperplasia of pancreatic islet cells was observed in the rats belonging to Group H [Figure 6c].

DISCUSSION

Pancreatic islet beta cells have the functional responsibility to produce insulin that controls the levels of blood glucose in the body. A dramatic rise in BGL occurs with insufficient release or lack of insulin [1]. The cytotoxic role of alloxan is effected via generation of reactive oxygen species, which lead to the release of highly reactive hydroxyl radicals and increased concentration of cytosolic calcium. The end result is the fast destruction of pancreatic islet beta cells and hyperglycemia [17]. If untreated, the resultant effects could lead to adverse effects on body systems or clinical conditions such as weight loss, neuropathy, impaired renal, and retinal functions [1].

Comparisons of percentage changes in body weights of rats (day 1 vs. day 26) showed statistically significant decreases ($P < 0.05$) in Groups B and H; while statistically significant increases ($P < 0.05$) were observed in control Group A and treated alloxan-diabetic rats of Groups C - G [Figure 2]. This implied that alloxan administrations induced hyperglycemia and weight loss in untreated Alloxan-diabetic rats of Group B. Treatment of hyperglycemia with exercises only could, however, not significantly attenuated hyperglycemia with the resultant weight loss in rats

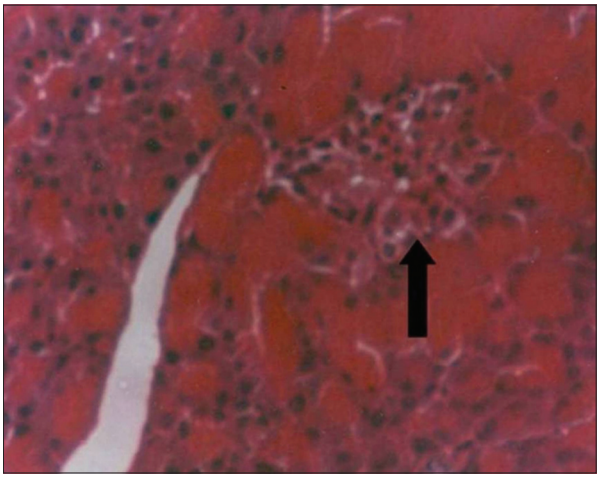

Figure 4a: Photomicrograph sample of pancreas of rats of the normoglycemic Group A that received only physiological saline (hematoxylin and eosin × 200), Solid black arrow indicates normal population of pancreatic islets cells

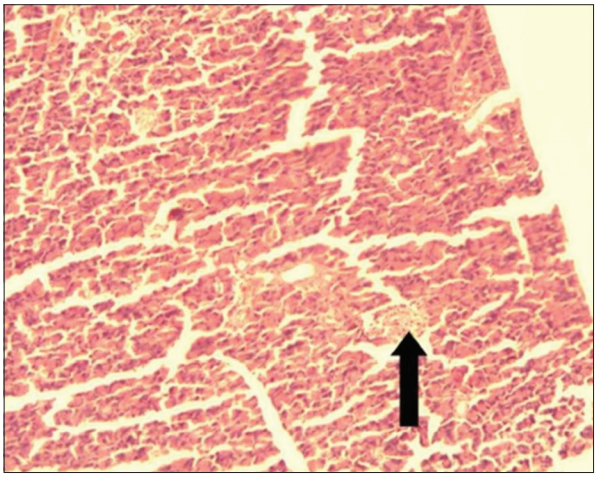

Figure 4b: Photomicrograph sample of pancreas of rats of Group B that received 80 mg/kg/bodyweight of alloxan and treated with only physiological saline (hematoxylin and eosin × 200), Solid black arrow indicates greatly reduced population or atrophy of pancreatic islets cells. This implied loss of beta cells population due to actions of alloxan via production of reactive oxygen species

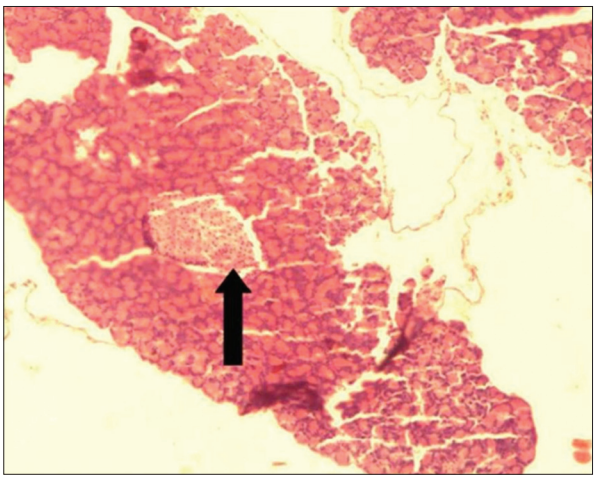

Figure 5a: Photomicrograph sample of pancreas of rats of Group C that received 180 mg/kg/bodyweight of alloxan and treated with 5 mg/kg/bodyweight of methanolic extract of *Musa sapientum* sucker (hematoxylin and eosin × 200), Solid black arrow indicates normal population of pancreatic islets cells. This implied possible regeneration of beta cells population lost due to actions of Alloxan via production of reactive oxygen species

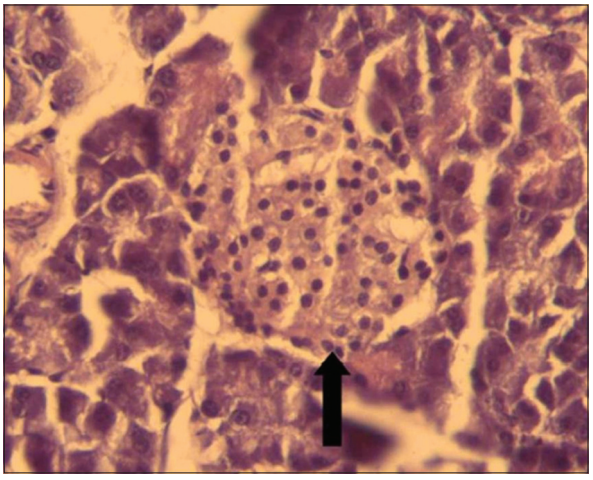

Figure 5b: Photomicrograph sample of pancreas of rats of Group D that received 180 mg/kg/bodyweight of alloxan and treated with 10 mg/kg/bodyweight of methanolic extract of *Musa sapientum* sucker (hematoxylin and eosin × 200), Solid black arrow indicates normal population of pancreatic islets cells. This implied possible regeneration of beta cells population lost due to actions of alloxan via production of reactive oxygen species

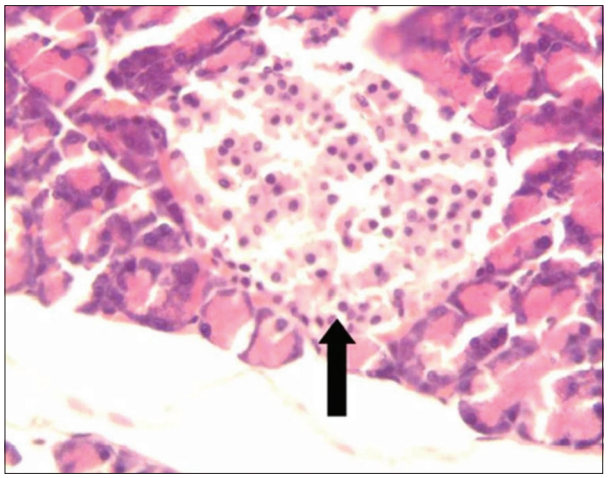

Figure 5c: Photomicrograph sample of pancreas of rats of Group E that received 180 mg/kg/bodyweight of alloxan and treated with 5 mg/kg/bodyweight glibenclamide (hematoxylin and eosin × 200) Solid black arrow indicates normal population of pancreatic islets cells. This implied possible regeneration of beta cells population lost due to actions of alloxan via production of reactive oxygen species

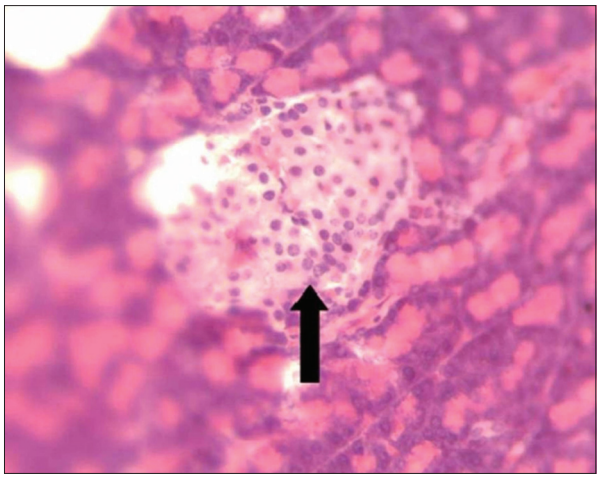

Figure 6b: Photomicrograph sample of pancreas of rats of Group G that received 180 mg/kg/bodyweight of alloxan and treated with 10 mg/kg/bodyweight of methanolic extract of *Musa sapientum* sucker plus 60 min daily exercises for 5 days per week (hematoxylin and eosin × 200), Solid black arrow indicates normal population of pancreatic islets cells. This implied possible regeneration of beta cells population lost due to actions of alloxan via production of reactive oxygen species

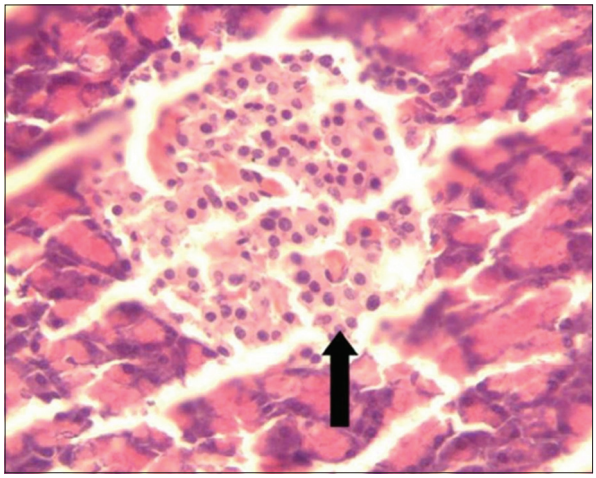

Figure 6a: Photomicrograph sample of pancreas of rats of Group F that received 180 mg/kg/bodyweight of alloxan and treated with 5 mg/kg/bodyweight of methanolic extract of *Musa sapientum* sucker plus 60 min daily exercises for 5 days per week (hematoxylin and eosin × 200), Solid black arrow indicates normal population of pancreatic islets cells. This implied possible regeneration of beta cells population lost due to actions of Alloxan via production of reactive oxygen species

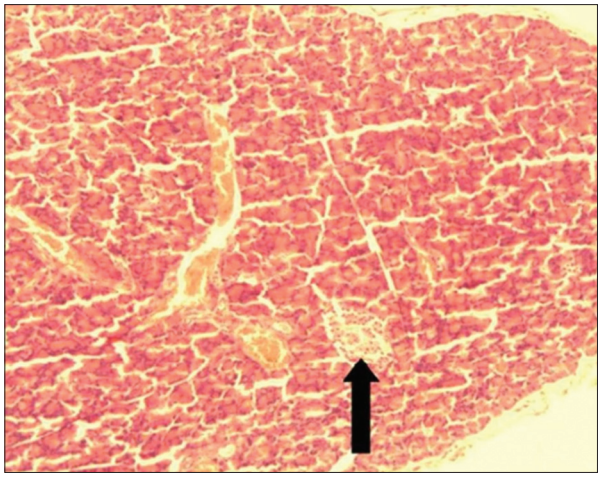

Figure 6c: Photomicrograph sample of pancreas of rats of Group H that received 180 mg/kg/bodyweight of alloxan and treated with swimming exercises only (hematoxylin and eosin × 200), Solid black arrow indicates mild reduction in the population of pancreatic islets cells. This implied mild regeneration of beta cells population lost due to actions of alloxan via production of reactive oxygen species

of Group H. However, the possible resultant weight loss due to hyperglycemia was significantly attenuated in alloxan-diabetic rats treated with 5 and 10 mg/kg/body weight of extract (C and D), 5 and 10 mg/kg/body weight of extract with exercises (F and G), and 5 mg/kg/body weight of glibenclamide (E). Furthermore, the observed effects of the extract on weight loss compared favorably with glibenclamide. Our observations are in agreement with the only previously reported protective effects of *M. sapientum* sucker extracts against weight loss in alloxan-diabetic rats [9].

Histopathological examinations at the end of experimental procedures showed normal pancreas (Group A), atrophied pancreatic islet cells (Group B), hyperplasia with adequate populations of islet cells (Groups C - G), and reduced hyperplasia of islet cells (Group H) [Figures 4a, 5a-c, and 6a-c]. These findings implied that treatments of alloxan-diabetic rats with *M. sapientum* sucker extracts (with or without exercises) were possibly able to aid the regeneration of destroyed pancreatic islet beta cells induced by alloxan in rats. This is in agreement with previously reported pancreatic histoprotective effects of the different parts of *M. sapientum* such as its peel extracts [3], inflorescence, and stalk [4] and sucker extracts [9].

Evaluations of measured BGL on day 1, at the induction of diabetes (day 2) and weekly (weeks 1-3) after the induction of diabetes [Figure 3] showed that alloxan administration resulted

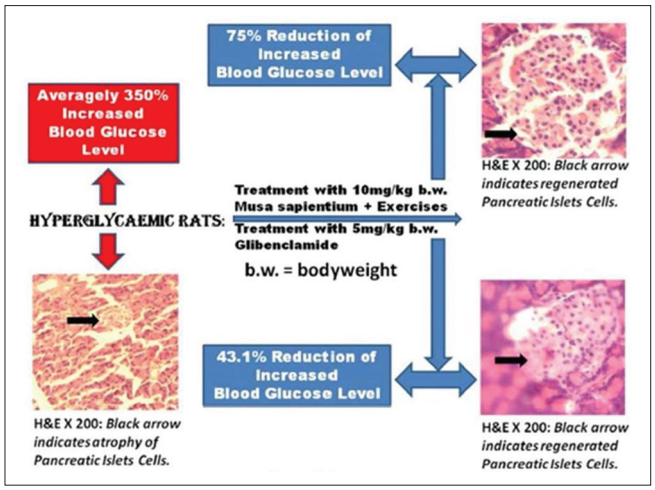

Figure 6d: Graphical abstract shows the hypoglycemic effects of 5 mg/kg bodyweight of glibenclamide (Group E) and 10 mg/kg body weight methanolic extracts of *Musa sapientum* sucker + exercises (Group G) in alloxan diabetic rats

in non-reversible hyperglycemia in untreated alloxan-diabetic rats of Group B. Treatment of hyperglycemia with exercises only significantly attenuated hyperglycemia but at a lower rate when compared with glibenclamide-treated rats. However, hyperglycemia was significantly attenuated in alloxan-diabetic rats treated with extract doses (Groups C and D) and extracts doses with exercises (Groups F and G) comparatively with glibenclamide-treated rats of Group E [Figures 3 and 6d]. The possible hypoglycemic potentials of *M. sapientum* sucker extracts appeared enhanced and improved with exercises. This could suggest that increased muscular activities might have significant reversal roles on hyperglycemia in rats confirming possible supportive anti-diabetic roles of exercises as previously noted [10-13].

Phytochemical screenings of *M. sapientum* sucker showed the ubiquitous existence of tannins, saponins, alkaloids, and glycosides. [2]. The direct mechanism of action of *M. sapientum* sucker is not clear, however, its anti-diabetic properties might be due to its phytochemical components such as tannins, saponins, and alkaloids. Tannins as phenolic compounds promote the status of oxidative stress biomarkers with the ability to scavenge free radicals, which could also have enhanced its anti-diabetic activity [2,18,19]. Saponins possess immunomodulatory, anti-inflammatory, and vasoprotective effects, which could have aided the observed anti-diabetic activity of *M. sapientum* sucker [2,18]. Alkaloids have similarly been noted to possess hypoglycemic properties [19].

CONCLUSIONS

The findings of this study confirmed the hypoglycemic potentials of *M. sapientum* sucker extracts (with or without exercises) in Alloxan-diabetic rats. Furthermore, our findings suggest that treatments with *M. sapientum* sucker extracts promote the restorations of destroyed pancreatic islet cells in alloxan-diabetic rats. This observation could be of relevance in the development of new therapeutic anti-diabetic agents from *M. sapientum* sucker extracts.

REFERENCES

1. Boon AN, Colledge NR, Walker BR, Hunter JA. Davidson's Principles and Practice ofMedicine. 20th ed. India: Churchill Livingstone, Elsevier Limited.; 2006.
2. Akinlolu AA, Ghazali OK, Salau, BA, Jubril O, Omowunmi M. *Musa sapientum* attenuates total antioxidant and lipid profile values in rats with indomethacin-induced gastric ulceration. Cell Membr Free Radic Res 2013;5:236-43.
3. Ramakrishnan B, Selvaraj S, Babu S, Radhakrishnan S, Radhakrishnan N, Palanisamy P. Antioxidant potential of peel extracts of banana varieties (*Musa sapientum*). Food Nutr Sci 2011;2:1128.
4. Jayamurthy P, Aparna B, Gayathri G, Nisha P. Evaluation of antioxidant potential of inflorescence and stalk of plantain (*Musa sapientum*). J Food Biochem 2013;37:2-7.
5. Lewis DA, Fields WN, Shaw GP. A natural flavonoid present in unripe plantain banana pulp (*Musa sapientum* L. var. *paradisiaca*) protects the gastric mucosa from aspirin-induced erosions. J Ethnopharmacol 1999;65:283-8.
6. Prabha P, Karpagam T, Varalakshmi B, Packiavathy AS. Indigenous anti-ulcer activity of *Musa sapientum* on peptic ulcer. Pharmacognosy Res 2011;3:232-8.
7. Hossain MS, Alam MB, Asadujjaman M, Zahan R, Islam MM, Mazumder ME, *et al.* Antidiarrheal, antioxidant and antimicrobial activities of the *Musa sapientum* Seed. Avicenna J Med Biotechnol 2011;3:95-105.
8. Dhanabal SP, Sureshkumar M, Ramanathan M, Suresh B. Hypoglycemic effect of ethanolic extract of *Musa sapientum* on alloxan induced diabetes mellitus in rats and its relation with antioxidant potential. J Herb Pharmacother 2005;5:7-19.
9. Salau BA, Ajani EO, Akinlolu AA, Ekor MN, Soladoye MO. Methanolic extract of *Musa sapientum* sucker moderates fasting blood glucose and body weight of alloxan- induced diabetic rats. Asian J Exp Biol Sci 2010;1:30-5.
10. American Diabetes Association. Physical Activity/Exercises and Diabetes. Diabetes Care 2004. Available from: http://www.care.diabetesjournals.org/content/27/suppl_1/s58.full. doi:10.2337/diacare.27.2007.S58. [Last Accessed on 2015 Apr 30].
11. de Oliveira CA, Luciano E, de Mello MA. The role of exercise on long-term effects of alloxan administered in neonatal rats. Exp Physiol 2005;90:79-86.
12. Rossi DM, Valenti VE, Navega MT. Exercise training attenuates acute hyperalgesia in streptozotocin-induced diabetic female rats. Clinics (Sao Paulo). 2011;66:1615-9.
13. Edite T, Sara N, Frederico T, Flávio R. Regular physical exercise training assists in preventing type 2 diabetes development: Focus on its antioxidant and anti-inflammatory properties. Cardiovasc Diabetol 2011;10:12.
14. Marliss EB, Vranic M. Intense exercise has unique effects on both insulin release and its roles in glucoregulation: Implications for diabetes. Diabetes 2002;51:S271-83.
15. Gbolade A, Ekor MN, Akinlolu A, Ayoola MD. Antidiabetic activity of ethanolic extracts of *Spondias mombin* stem bark on alloxan-induced diabetic rats. J Pharmt Res 2008;7:192-5.
16. Gbolade AA, Akinlolu AA, Odewabi AO. Anti-hyperglycaemic and hypolipidemic activities of stem bark ethanol extract of *spondias mombin* l. (anacardiaceae). Niger Journ Pharm Sci 2011;10:42-51.
17. Szkudelski T. The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiol Res 2001;50:537-46.
18. Oyedemi S, Bradley G, Afolayan A. Antidiabetic Activities of Aqueous Stem Bark Extract of Strychnos henningsii Gilg in Streptozotocin-nicotinamide Type 2 Diabetic Rats. Iran J Pharm Res 2012;11:221-8.
19. Adelaja AA, Ayoola MD, Otulana JO, Akinola OB, Olayiwola A, Ejiwunmi AB. Evaluation of the histo - gastroprotective and antimicrobial activities of *heliotropium indicum* linn (boraginaceae). Malays J Med Sci 2008;15:22-30.

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Original Research

ScopeMed

NaCl stress-induced changes in the essential oil quality and abietane diterpene yield and composition in common sage

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ABSTRACT

Aim: The purpose of this study was to evaluate how increasing NaCl salinity in the medium can affects the essential oils (EOs) composition and phenolic diterpene content and yield in leaves of *Salvia officinalis* L. The protective role of such compounds against NaCl stress was also argued with regard to some physiological characteristics of the plant (water and ionic relations as well as the leaf gas exchanges). **Materials and Methods:** Potted plants were exposed to increasing NaCl concentrations (0, 50, 75, and 100 mM) for 4 weeks during July 2012. Replicates from each treatment were harvested after 0, 2, 3, and 4 weeks of adding salt to perform physiological measurements and biochemical analysis. **Results:** Sage EOs were rich in manool, viridiflorol, camphor, and borneol. Irrigation with a solution containing 100 mM NaCl for 4 weeks increased considerably 1.8-cineole, camphor and β-thujone concentrations, whereas lower concentrations (50 and 75 mM) had no effects. On the contrary, borneol and viridiflorol concentrations decreased significantly under the former treatment while manool and total fatty acid concentrations were not affected. Leaf extracts also contained several diterpenes such as carnosic acid (CA), carnosol, and 12-*O*-methoxy carnosic acid (MCA). The concentrations and total contents of CA and MCA increased after 3 weeks of irrigation with 75 or 100 mM NaCl. The 50 mM NaCl had no effect on these diterpenes. Our results suggest a protective role for CA against salinity stress. **Conclusion:** This study may provide ways to manipulate the concentration and yield of some phenolic diterpenes and EOs in sage. In fact, soil salinity may favor a directional production of particular components of interest.

KEY WORDS: Antioxidants, carnosic acid, essential oil quality, plant nutrition, salinity

INTRODUCTION

High demand for natural products such as essential oils (EOs) and phenolic diterpenes has fuelled the increased interest in the cultivation of medicinal and aromatic plants (MAPs) as alternatives to traditional crops. The cultivation of EOs-containing species from different families has been initiated in a number of countries to respond to food and cosmetic industry needs. Common sage (*Salvia officinalis*) is among several Lamiaceae species, which received much interest [1-3]. The main sage oil-producing countries in the Mediterranean basin are Spain, France, and Tunisia [4]. Still, growing such species under arid and semi-arid conditions is a big duty. For instance, abiotic stresses such as heat, water shortage, and salinity, apart from restricting the growth and yield, provoke various metabolic changes in most plants [2,5,6]. All the above abiotic factors result in a build-up of salt in the soil imposing a major limitation to the growth and yield of crops. Although there is a substantial literature on the behavior of common species under stressful conditions [5], data on MAPs are less available.

Common sage is a Mediterranean shrub of the Lamiaceae family, which has been used for a long time as food spice and folk medicine. The biological properties of EO of sage are attributed mainly to α- and β-thujone, camphor, and 1.8-cineole [7]. Still the percentage of these EOs depends on several factors including the geographic origin of the plant, environmental factors, plant organ, and genetic differences [8]. Apart from volatiles and flavonoids, sage contains the relatively large amount of polyphenolic compounds with antioxidant activity [9,10]. Carnosic acid (CA), 12-*O*-methyl carnosic acid (MCA) and carnosol (CAR) were shown to be the major diterpene constituents in leaves of both sage and rosemary [9]. CA metabolism plays a double role in several Labiatae plants. First, CA can protect plants from biotic and abiotic stresses by scavenging free radicals within the chloroplasts. Second, CA metabolism may also play a role in the stability of cell membranes [11]. In addition, CA and its derivatives have a wide range of pharmacological and biological activities. These compounds are responsible for up to 90% of the antioxidant activity of the plant extract [12,13], so there is an increasing

interest in using sage as a source of natural antioxidants in food preservatives. Besides, CA and CAR were found to possess antibacterial, anticancer, anti-inflammatory, antitumor, anti-obesity, and photoprotective activities [10,14,15]. Recent reports have showed that these compounds may be useful for the treatment of neurodegenerative diseases [16]. Such biological and commercial potential of the sage diterpenes makes them an attractive target for developing strategies intended for their extensive production [17]. Besides, the extraction of these compounds from plants needs sufficient plant biomass together with high amounts of these metabolites. In this regard, several studies [18-20] have targeted enhancing the production of these high-value plant compounds by choosing the accurate plant species or accession, the richest plant part or the proper plant development stage. Besides, the use of some agricultural techniques has been also reported [20]. It was shown, for instance, that the essential cations (K^+, Ca^{2+}, Mg^{2+} and Fe^{2+}) can favor the foliar biosynthesis of several phenolic diterpenes in both sage and rosemary [2,20]. As well, it was shown that the upper young leaves contained higher levels of diterpenes when compared to bottom senescent ones [20]. Furthermore, it has been shown that drought-induced oxidative stress reduces the CA and CAR contents of the sage leaves and enhances the formation of the highly oxidized diterpenes isorosmanol (ISO) and dimethyl isorosmanol [12]. Still, the effects of moderate and severe NaCl salinity stress on the biosynthesis of such high-value plant compounds are not well studied.

In several countries, water shortages have forced growers to use more and more treated wastewater and saline water for plant irrigation. The soil and water salinity possess serious limitation to plant productivity. Crops are more and more exposed to this problem accentuated by increasing climate aridity. Plants exposed to relatively high concentrations of salt undergo several changes in their metabolism in order to cope with their stressful environment. For instance, excessive soil salinity alters EOs biosynthesis and composition in several species of industrial interest [6,21,22]. The impact of salinity on the EO yield and composition was studied on sage's fruits in hydroponic culture [1] or with relatively low level of NaCl in the soil (lower than 4.7 dS/m) [3]. Still the effect of moderate and higher NaCl concentrations in the soil on the EOs composition and phenolic diterpenes are not well studied on the common sage. It is, therefore, of interest to evaluate if irrigation with increasing saline water can be used as an agricultural technique to increase their foliar concentrations in sage. The protective role of CA against NaCl stress was also argued with regard to some physiological features of the plant (water and ionic relations as well as the photosynthetic exchanges).

MATERIALS AND METHODS

Plant Material and Culture

Common sage used in the present study was propagated by cuttings at the floral budding stage. Sixty-four plants (1-year-old) were grown in 5 L plastic pots containing desert dune sand (≤1 mm in diameter, 0.1 g soluble salt 100/g DW of sand) in a glasshouse covered with a shade net. The experiment was carried out during July 2012. Plants were exposed, in average, to a PPFD of 1000 mmol/m/s. During 3 weeks after transplanting into the pots, all plants were irrigated with a complete nutrient solution having an initial total ion concentration of 4.5 mM and electrical conductivity (EC) of 3.0 dS/m. These plants were divided into two batches: For the first batch (kinetic batch), forty-eight plants (48) have received increasing NaCl concentrations (0, 50, 75, and 100 mM) in the irrigation solution. The resulted soil ECs were, respectively, 3.0, 9.0, 11.1, and 14.9 dS/m. To avoid osmotic shock, NaCl concentrations were increased gradually, by 25 mM/day, until the desired concentration was reached. Each solution was used to irrigate twelve pots every 4 days. Four plants were randomly harvested from each treatment after 2, 3, and 4 weeks of adding NaCl. The experimental design was a completely randomized block with 4 replicates (each pot being a replicate). This batch of plants was used to determine the effect of salinity on total leaf dry weight, the water content (WC), and the kinetic of accumulation of the phenolic abietanes diterpenes and their yields. The second batch of plants was used to determine the effect of increasing salinity on EOs composition, ion contents, and plant gas exchange. Sixteen plants were irrigated with the same NaCl as mentioned above and were harvested only at the end of the treatment.

Determination of Plant Growth and WC

Plants (4 replicates for each treatment) were randomly harvested after 0, 2, 3, and 4 weeks of adding salt to determine plant growth and leaf WC. After recording their total leaf fresh biomass (FW), they were oven-dried at 80°C for 48 h and total leaf dry biomass (DW) was measured. Plant growth was estimated by determining the total dry weight of the leaves. The leaf WC was determined as follow:

$$WC = FW - DW/DW$$

Ion Contents and Gas Exchange Measurements

Ion contents and gas exchange were performed on fully expanded leaves collected between 9 and 11 am after 4 weeks of NaCl treatment. These evaluations were completed with an infrared, portable CO_2 gas analyzer (ADC, BioScientific Ltd., Hoddesdon, UK). The photosynthetic rate (*A*), transpiration (*E*), and stomatal conductance (*gs*) were all evaluated. The instantaneous water use efficiency (*iWUE*) was calculated as *A*/*E*.

EOs Extraction and Gas Chromatography/Mass Spectrometry (GC/MS) Analyses

EOs analyses were performed on leaves collected between 9 and 11 am after 4 weeks of treatment. The EOs were extracted from 10 g sub-samples of fresh leaves by steam distillation at atmospheric pressure, using a modified Clevenger type apparatus. Distillation lasted 3 h. The EOs were separated from the aqueous phase by adding chloroform. The organic layer was dried with anhydrous sodium sulfate and concentrated under atmospheric pressure to eliminate the chloroform. The residue

was solubilized in 30 volumes of 100% (v/v) hexane and analyzed by GC/MS as described by Tounekti *et al*. [6].

Abietanes Diterpenes Analyses

For the abietanes diterpenes analyses, a sub-sample of fresh leaves was taken from each harvested plant. The leaves were collected, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. Diterpenes were determined as described by Munné-Bosch *et al*. [12].

Statistical Analyses

Variance of data was analyzed with a two-way ANOVA (salt concentration and duration of treatment being the independent variables) using a GLM procedure of SAS software 1996 for a Randomized Complete Block design with 4 replicates. To correct for departure from normality, EOs data (percentages) were Log-transformed before analyses; untransformed means were reported. Where applicable, means were separated by Duncan's Multiple Range Test ($P \leq 0.05$).

RESULTS

Growth, Water Relations, Ionic Contents, and Gas Exchanges in Salt-stressed Sage Plants

The present results revealed that soil salinity had no significant effect on total leaf dry weight per plant [Figure 1]. Still such growth parameter tended to decrease after 4 weeks in all salt treatments. Furthermore, the lower NaCl levels (50 and 75 mM) have not affected noticeably the leaf WC either after 4 weeks of treatment. However, a significant decrease was seen for the 100 mM NaCl treatment starting from the 3rd week [Figure 1].

After 4 weeks soil salinity has led to a significant decrease in the assimilation rate (*A*), stomatal conductance (g_s), transpiration rate (*E*) and therefore the instantaneous water use efficiency (*iWUE*) mainly for 75 and 100 mM treatments [Table 1]. The largest decreases were shown at 100 mM NaCl, leading to *A* values of 10 μmol/m/s after 4 weeks of stress which was 50% lower than control plants. Besides, our results demonstrate that *gs* decreased significantly more than *A* in all salt treatments and mainly when the lowest NaCl concentrations were provided, which suggests a stomatal limitation of the photosynthetic capacity mainly after 4 weeks of treatments. After the same period, salinity has strongly increased Na^+ and reduced the K^+ content in the sage leaves, with the 100 mM dose causing the largest effect. For instance, the leaf Na^+ contents increased by 140% and the K^+ contents decreased by 32% in the 100 mM-treated plants relative to controls [Table 1].

Salt Stress-induced Changes in EOs

The GC-MS analyses of sage EOs obtained by steam distillation of leaves allowed the identification of 31 compounds representing about 96% of the total EOs [Table 2]. The oil contained different chemical classes such as monoterpenes (24%), sesquiterpenes (29%), diterpenes (19%), saturated fatty acids (23.6%), and alkanes (1.3%). The majority of monoterpenes and sesquiterpenes recovered were oxygen-containing compounds. The most abundant were viridiflorol (28.1%), manool (17.5%), camphor (13.4%), borneol (8.9%), and β-thujone (3.4%). The analyses also revealed a relatively large fraction made of saturated fatty acids [Table 2]. The increased concentration of NaCl in the soil medium changed the composition of sage EOs but did not induce the synthesis of new oils. These changes depended on salt concentration. 1.8-cineole, β-thujone and camphor increased considerably in the plants fed with 100 mM NaCl; lower salt concentrations had no effect. In contrast, borneol and viridiflorol levels were reduced by the 100 mM NaCl treatment. Manool and total fatty acid fraction did not change [Table 2].

Salt Stress-induced Changes in Phenolic Diterpenes

In common sage, CA and CAR are the major phenolic diterpenes in the leaves [Figure 2]. Our results demonstrated that the CA content varied between 1.2 and 1.7 mg/g DW and CAR between 1.2 and 1.6 m/g DW in the leaves of control plants. We found

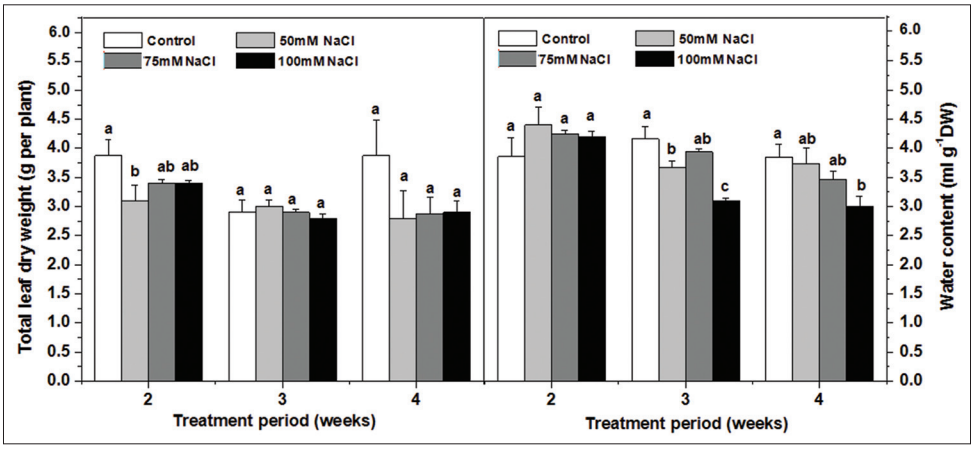

Figure 1: Effect of increasing soil NaCl concentrations (mM) on foliar water content (ml (H_2O) g^{-1} Dry weight) and total leaves dry weight (g per plant) in *Salvia officinalis* L plants. Each value is the mean of 3 replicates ± standard deviation. Values marked by different small letters are significantly different at $P < 5\%$

Table 1: Ions contents, net photosynthesis (*A*), stomatal conductance (*gs*), transpiration rate (*E*), and instantaneous water use efficiencies (*iWUE=A/E*) in leaves of sage plants grown in a medium without NaCl (control), or containing increasing NaCl concentrations (0, 50, 75 and 100 mM)

NaCl treatments	K^+ (mg/g DW)	Na^+ (mg/g DW)	*A* (μmol/m/s)	*gs* (mol/m/s)	*E* (mmol/m/s)	*WUE* (mmol CO_2/mol H_2O)
Control	51.03±1.86[a]	10.92±1.01[c]	20.72±0.68[a]	1.30±0.05[a]	4.90±0.22[a]	4.23±0.12[a]
50 mM	43.67±1.22[b]	19.84±1.26[b]	20.46±1.54[a]	1.12±0.03[b]	4.93±0.17[a]	4.15±0.14[a]
75 mM	37.42±1.61[c]	23.98±0.80[a]	16.46±1.54[b]	0.96±0.04[c]	4.33±0.13[b]	3.80±0.15[b]
100 mM	34.64±1.63[c]	26.36±1.45[a]	9.87±2.35[c]	0.89±0.04[d]	2.85±0.32[c]	3.47±0.22[c]

The plants were harvested after 4 weeks of salt treatments. Each value represents the mean (±SE) of 4 replicates; values marked by different small letters are significantly different at $P<5\%$. SE: Standard error

Table 2: Leaf EOs composition (% of total) in *Salvia officinalis* L. after 4 weeks of culture on increasing NaCl concentrations

Compounds	Treatments			
	0 mM	50 mM	75 mM	100 mM
1.8-cineole	1.61±0.41[b]	0.84±0.48[b]	1.23±0.32[b]	10.69±1.06[a]
Hydrate trans-sabinene	0.23±0.11	0.28±0.07	0.55±0.11	0.18±0.08
Linalool	0.23±0.10	0.52±0.20	0.79±0.23	0.37±0.27
β-thujone	3.39±0.61[b]	1.25±0.16[b]	1.74±0.36[b]	15.53±5.32[a]
α-thujone	0.29±0.04	0.22±0.01	0.21±0.09	2.11±1.90
Camphor	13.45±1.96[b]	12.86±0.92[b]	14.5±2.56[b]	27.80±2.67[a]
Borneol	8.90±1.33[b]	6.35±0.03[ba]	0.95±0.11[c]	1.73±0.37[c]
Terpinene-4-ol	2.47±0.37	1.45±0.10	Tr	0.97±0.25
α-terpinolene	1.35±0.22	1.10±0.08	Tr	0.42±0.11
Heptacosane	0.41±0.41	0.31±0.05	0.68±0.12	0.25±0.15
Bornyle acetate	0.76±0.17	0.55±0.05	Tr	0.40±0.30
Unidentified	0.20±0.20	0.26±0.16	1.04±0.26	0.20±0.10
Bornane-2-6-dione	1.07±0.54	2.73±0.16	0.78±0.10	0.36±0.26
α-Octene	0.29±0.14	0.57±0.47	1.63±0.44	0.30±0.20
Unidentified	0.32±0.21	0.20±0.10	1.07±0.35	0.19±0.09
Heneicosane	0.27±0.15	Tr	0.94±0.28	0.23±0.13
Cresol	Tr	0.25±015	Tr	0.32±0.22
2-methyl-4-nitrosoresorcinol	2.36±0.92	2.82±1.10	2.83±0.96	1.96±0.71
5-(trimethylsilyl) furfural	1.43±0.63	10.91±6.22	3.70±0.89	4.13±0.93
α-humulene	0.50±0.23	2.27±1.05	1.07±0.11	1.08±0.46
Nonadecane	0.31±0.16	0.30±0.09	0.81±0.12	0.20±0.10
Caryophyllene oxide	0.94±0.22	1.73±0.49	2.08±0.19	0.76±0.66
Viridiflorol	28.13±2.45[a]	30.29±8.53[a]	33.03±4.22[a]	7.75±3.56[b]
Humulene oxide	0.65±0.25	1.27±0.32	1.75±0.12	1.07±0.17
Heptane 3-ethyl-3-methyl	0.38±0.24	0.19±0.09	0.63±0.23	0.16±0.06
Hexadecanoic acid	5.70±0.88	2.78±0.17	5.34±0.45	5.79±0.01
Unidentified	0.78±0.68	0.10±0.00	Tr	0.20±0.01
Manool	17.55±1.06	14.80±0.09	17.39±2.66	9.40±5.48
Longiborn-9-ene	Tr	0.16±0.06	Tr	0.34±0.02
Octadecanoic acid	1.84±0.50	0.78±0.59	1.70±0.33	1.33±0.02
Stearic acid	1.00±0.54	0.15±0.05	Tr	1.18±0.02
Fatty acids	8.53±1.63	3.71±0.82	7.05±1.66	8.30±0.03

Each percentage is the mean of 3 replicates ± SD. To correct deviation from normality, EOs data (percentages) were log-transformed before analyses; untransformed means were reported. Within rows, means followed by different letters are significantly different at $P < 5\%$ (Duncan's test). tr: Compound found at a level ≤0.1%. SD: Standard deviation, EOs: Essential oils

that NaCl in the soil medium did not affect significantly the oxidized diterpenes rosmanol (ROS) and dimethyl isorosmanol (DIM) as well as their total contents regardless of the duration of the treatment [Table 3]. Whereas, the effect of salinity on CA, CAR, MCA, ISO, and the total reduced diterpene contents changed with time (treatment x duration significant). After 2 weeks of treatment, NaCl had only a limited effect on sage abietane diterpene content. However, after 3 weeks, plants fed with 75 mM or 100 mM NaCl had considerably more CA, MCA and reduced forms in their leaves than those fed with 0 mM or 50 mM NaCl [Figure 2]. After 4 weeks, plants which received 75 mM or 100 mM NaCl contained more CA, MCA, and CAR than those irrigated with 50 mM. The latter had similar CA but lower MCA and CAR content than control plants. The reduced diterpenes content tended to decrease when plants were fed with 50 mM NaCl and to increase for higher concentrations (after 3 or 4 weeks). Still, the levels of the oxidized compounds did not change significantly in the 50 and 75 mM-treated plants of the present study. Higher salt concentrations enhanced CA, MCA, and CAR biosynthesis [Figure 2].

DISCUSSION

In all plant species, salinity leads to a reduction in plant biomass when it exceeds a certain threshold [5]. According to Maas and Hoffman [23], a crop is considered moderately salt sensitive

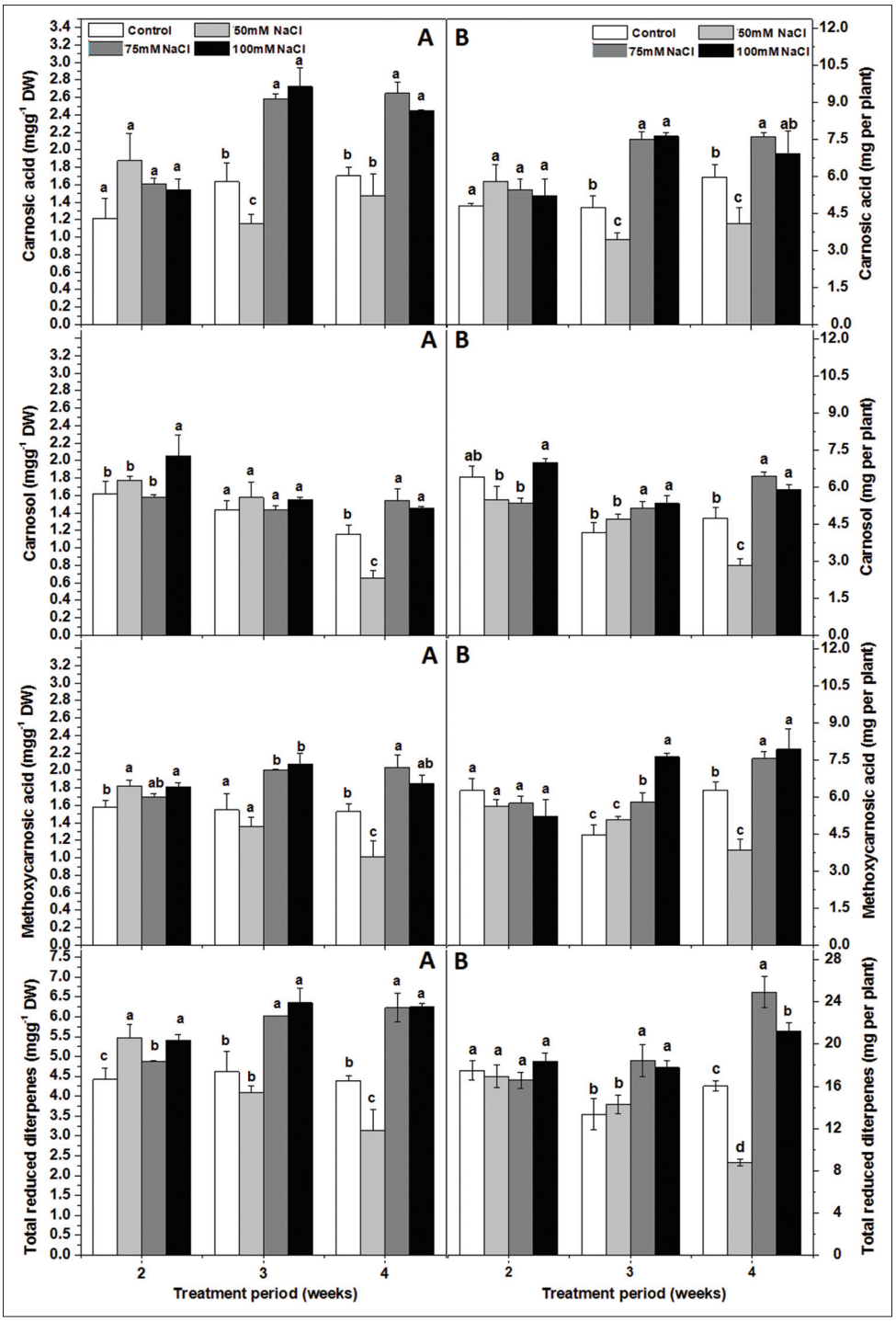

Figure 2: Effect of increasing soil NaCl concentrations (mM) on foliar reduced diterpene concentrations (mg/g Dry weight) (A) and contents (mg per plant) (B) in *Salvia officinalis* L plants. CA: Carnosic acid, CAR: Carnosol, MCA: Methoxycarnosic acid; RED: Total reduced diterpenes (RED = CA + CAR + MCA). Each concentration is the mean of 4 replicates ± standard deviation. In values marked by different small letters are significantly different at $P < 5\%$

when 50% of the reduction in biomass occurred at 90 mM NaCl, which is not the case for sage plant as revealed by our results. In fact, despite its trend for decreasing, for all NaCl applied levels, at the treatment end, total leaf dry weight per plant was not significantly affected by soil salinity [Figure 1]. Hence, the present study confirms that sage is a moderately salt-resistant glycophyte [2,23]. The detrimental effects of NaCl on plants are typically due to osmotic effects and/or ionic imbalances resulting from nutritional deficiency or excess ions [20,24,25]. The abscission of the oldest leaves, and therefore the slight decrease of the total leaf dry weight (either not statistically significant), has been considered an adaptive mechanism that prevents the accumulation of toxic ions (Na^+ and Cl^-) and reduces water loss in salt-stressed plants [5,24,25]. According to our results,

Table 3: Summary of two-way ANOVA of effects of growth medium NaCl concentration and treatment duration as fixed independent variables

Parameter	d.f.	F_{NaCl}	$F_{duration}$	$F_{NaCl*duration}$
CA	11	7.04**	3.59^{ns}	2.79*
CAR	11	2.81^{ns}	13.76**	3.31*
MCA	11	8.16**	1.49^{ns}	2.99*
ROS	11	1.30^{ns}	1.05^{ns}	1.75^{ns}
ISO	11	5.27*	4.08*	3.09*
DIM	11	0.77^{ns}	0.10^{ns}	0.96^{ns}
RED	11	10.40**	1.60^{ns}	3.94*
OX	11	1.87^{ns}	0.18^{ns}	1.43^{ns}

d.f., total error degrees of freedom; **, $P<0.001$; *, $P<0.05$; ns: Not significant. CA: Carnosic acid, CAR: Carnosol, MCA: 12-*O*-methoxy carnosic acid, ROS: Rosmanol, DIM: Dimethyl isorosmanol, ISO: Isorosmanol, OX: Total oxidized diterpenes, RED: Total reduced diterpenes

the application of the 50 mM NaCl in the soil did not affect noticeably the leaf WC either after 4 weeks of treatment, while at higher levels plants were unable to suitably hydrate their tissues which cause water stress [6,24,25]. As a consequence, salinity has led to an early decrease in the assimilation rate (*A*), stomatal conductance (g_s), and the transpiration rate (*E*) [Table 1], as it was seen in several other species [20,24]. Still the largest decreases were shown for the higher NaCl levels. Such simultaneous decrease in g_s and *E* has kept away the salt-stressed plants from an acute loss of the leaf cell turgor. To maintain leaf cell turgor, plants generally accumulate solutes from the soil (Na^+ and Cl^-) [24]. According to our results, after 4 weeks of treatment, the Na^+ increased by 140% and the K^+ decreased by 32% in the 100 mM NaCl-treated plants relative to controls, while the effects were more moderate in the 75 and 50 mM-treated plants. Still, this increased uptake of Na^+ (inclusion mechanism), combined with a limited production of new leaves, led to a build-up of Na^+ to toxic levels, which likely caused the leaf senescence and abscission.

The response of sage to changes in its environment involves among others the regulation of the levels of its secondary metabolites including EOs and phenolic diterpenes [2,3,12,26]. The effect of salinity on isoprenoids biosynthesis is expected as it affects the photosynthetic assimilation (A) and, therefore, the glycolysis pathway from where their precursor, D-glyceraldehyde 3-phosphate and pyruvate, were provided. It was already found that adding 100 mM NaCl to the plant growing medium almost increased concentrations and contents of several abietane diterpenes in rosemary leaves [20]. Herein we test for the first time to our knowledge the effect of increasing NaCl concentrations on the leaf abietanes diterpenes yield and composition in the sage plant.

The GC-MS analyses of sage EOs obtained by steam distillation of leaves allowed the identification of 31 compounds representing about 96% of the total EOs [Table 2]. The most abundant oils were viridiflorol (28.1%), manool (17.5%), camphor (13.4%), borneol (8.9%), and β-thujone (3.4%); this composition is somewhat similar to that of Eastern Lithuanian sage (i.e. 9.3-35.6% α-thujone; 6.9-29.1% camphor; 6-24% viridiflorol; 3.1-13.6% α-humulene; 3-13.3% manool; 8.6-12.7% 1.8-cineole, and 2-5.5 % borneol) [27,28]. The relatively high levels of oxygenated compounds mainly thujones, 1.8-cineole and camphor confer to the sage EO its antiseptic, astringent, carminative, and antispasmodic values. Still lesser concentrations of 1.8-cineole, camphor, and borneol were recorded herein as compared to our earlier studies, which may be due to seasonal variations, cultivar diversity or plant age [29]. For example, the low camphor levels found in this study seems to be due at least to the fact that the bulk of the leaves used were mature. Many studies showed that camphor is used in the recovery and recycling mechanisms for the carbon and energy accumulated during leaf development [30]. The analyses also revealed a relatively large fraction made of saturated fatty acids. This fraction was found in the extracts of many Lamiaceae species mainly in the genus *Salvia* [31-33].

As many other abiotic stresses, soil salinity can increase the synthesis of some secondary metabolites and encourage the formation of new compounds [34,35]. In agreement with this, our results showed that increasing NaCl in the soil medium changed the composition of sage Eos, but did not induce the synthesis of new oils. These changes depended on NaCl levels applied into the soil. For instance, 1.8-cineole, β-thujone and camphor increased considerably in the plants fed with 100 mM NaCl; lower salt concentrations had no effect. In contrast, borneol and viridiflorol levels were reduced by the 100 mM NaCl treatment. Manool and total fatty acid fraction did not change. Similarly, Hendawy *et al.* [36] reported that sage plants fed with NaCl (2500 ppm) in combination with different zinc concentrations had higher β-thujone, camphor and 1.8-cineole concentrations, less viridiflorol, and no manool. In the same way, Aziz *et al.* [3] have reported that increasing NaCl up to 4.7 dS/mimproved the α-thujone, cis-thujone and camphor contents. They also found that 1.8-cineole decreased and viridiflorol increased which is different to our results. The lower NaCl level they used could explain these differences since the percentage of some EO compounds are dependent on the medium salinity among others [6,35]. Besides, the effects of NaCl on the EO composition vary between species. For example the 50 mM NaCl applied during 3 weeks, increased 1.8-cineole, borneol and camphor in coriander leaves; while higher concentrations decreased these monoterpenes [35], which is not the case for sage as shown by the present results. The 1.8-cineole and camphor were also stimulated in two *Ocimum* varieties subject to an excess of water (125% of field capacity) or to a water deficit (75 and 50% of field capacity) [37]. NaCl appears to affect the activity of key enzymes in the biosynthesis pathways of EOs which concentrations are altered by salinity [38,39]. According to Putievsky *et al.* [40], good quality sage oil should contain a high percentage (>50%) of the epimeric α- and β-thujones and a low percentage (<20%) of camphor. Still more recent studies have stated that the biological properties of the sage's EO are attributed mainly to α- and β-thujone, camphor, and 1.8-cineole [7]. It appeared, therefore, that the soil salinity has beneficial effects on the sage's EO quality.

In common sage, CA and CAR are the major phenolic diterpenes in the leaves [Figure 2]. Dried sage leaves can contain up to 3% CA, depending on the plant variety, the

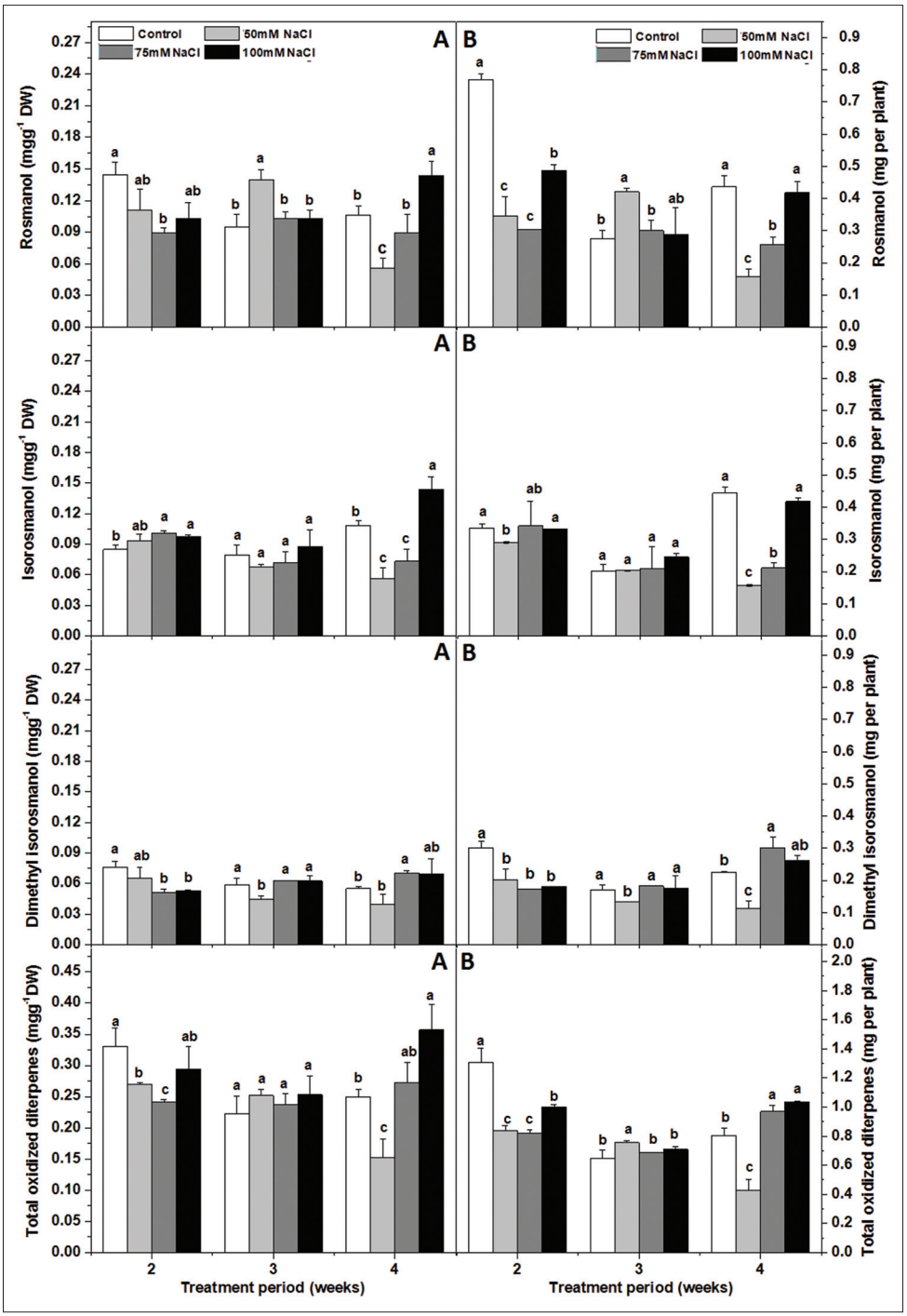

Figure 3: Effect of increasing soil NaCl concentrations (mM) on foliar oxidized diterpene concentrations (mg/g Dry weight) (A) and contents (mg per plant) (B) in Salvia officinalis L plants. ROS: Rosmanol, ISO: Isorosmanol, DIM: Dimethyl isorosmanol, OX: Total oxidized diterpenes (OX = ROS + ISO + DIM). Each concentration is the mean of 4 replicates ± standard deviation. In values marked by different small letters are significantly different at $P < 5\%$

growth conditions, the sample treatment and the method of preparing the extract. According to our results, the CA content varied between 1.2 and 1.7 mg/g DW and CAR between 1.2 and 1.6 mg/g DW in the leaves of control plants. Still our result agreed that environmental constraints produce substantial differences in flavonoid and phenolic acids and esters within plants particularly members of the Lamiaceae family [12,41,42]. We found that NaCl in the soil medium did not affect significantly the oxidized diterpenes ROS and DIM as well as their total contents regardless of the duration of the treatment [Table 3]. Whereas, the effect of salinity on CA, CAR, MCA, ISO, and the total reduced diterpene contents changed with time (treatment × duration significant) [Table 3]. After 2 weeks of treatment, NaCl had only a limited

effect on sage diterpene content. However, after 3 weeks, plants fed with 75 or 100 mM NaCl had considerably more CA, MCA, and reduced forms in their leaves than those fed with 0 or 50 mM NaCl [Figure 2]. After 4 weeks, plants which received 75 or 100 mM NaCl contained more CA, MCA, and CAR than those irrigated with 50 mM. The latter had similar CA, but lower MCA and CAR content than control plants. Therefore, the NaCl-induced changes in leaf CA, MCA, and the total reduced diterpene composition depended more on stress intensity (concentration of salt in the irrigation solution); but, variability in CAR content is better explained by treatment duration. Still most isoprenoids and phenolic diterpenes are induced to protect plant cells from drought, salt, heat stress or mechanical wounding [2,39,43]. The tendency of the reduced diterpenes content to decrease when plants were fed with 50 mM NaCl and to increase for higher concentrations (after 3-4 weeks), suggests that the plants reacted differently to these two ranges of salinity. It appears that the 50 mM NaCl treatment induced only a mild osmotic stress, which caused the degradation (oxidation) of the normally occurring pools of reduced diterpenes. Still, the levels of the oxidized compounds did not change significantly in the 50 and 75 mM-treated plants of the present study, thus further supporting the view that plants can withstand photooxidative stress at these salinity levels. Whereas, higher salt concentrations, led to a more acute stress (oxidative or Na^+ or Cl^- toxicity) to which plants responded with enhanced CA, MCA and CAR biosynthesis [Figure 2]. In the latter situation, *de novo* biosynthesis of reduced diterpenes exceeded their degradation to oxidized forms. This appears especially true for the 100 mM NaCl-treated plants whose leaves had high concentrations of oxidized diterpenes basically at the 14th week [Figure 3]. It is already known that CA may give rise to CAR after enzymatic dehydrogenation or to highly oxidized diterpenes such as ROS or ISO after enzymatic dehydrogenation and free radical attack [9,12,42]. Thus, CA may function as a "cascading" antioxidant, in which oxidation products are further oxidized, thus improving antioxidative protection by CA. In addition, CA can be *O*-methylated to form MCA. The *O*-methylated diterpenes can reinforce and stabilize the lipid chain [42]. Another indication that the 100 mM-treated plants faced different type of stress compared to the lower-treated plants is the decrease of the leaf WC especially on the 14th week [Figure 1] meaning that their ability for osmotic adjustment decreased. Furthermore, our results demonstrate that the *gs* decreased significantly more than A in all salt treatments and mainly at 50 mM NaCl, which suggests a stomatal limitation of the photosynthetic capacity (restriction of CO_2 availability for carboxylation). However, at higher concentrations mainly at 100 mM NaCl and apart from a stomatal limitation of photosynthesis, the plants suffered from a reduction in the *iWUE* and more acute stress, which could harm the photosynthetic apparatus [24,25]. These findings are in agreement with previous results that found that the increase in salinity levels decreased *iWUE* in tomato plants [44]. Under these conditions, the formation of reactive oxygen species can occur, which possibly leads to photoinhibition and/or photo-oxidative impairment. Besides, the effect of 50 mM NaCl on CA, MCA, and CAR resembles that of drought- or high light-induced oxidative stress conditions, which reduced their concentrations in rosemary leaves [12].

CONCLUSION

In conclusion, NaCl in the soil medium affected EOs composition of common sage leaves by stimulating biosynthesis pathways of several monoterpene families such as *p*-menthane (1.8-cineole) and bornane (camphor). The increased concentration of 1.8-cineole under NaCl stress should enhance the commercial value of sage EOs [45] since 1.8-cineole and thujone are the main parameters of sage oil quality [46]. In addition, NaCl decreased borneol and the sesquiterpene viridiflorol. Still, our study provides ways to manipulate the concentration of phenolic diterpenes in sage leaves. We have shown that sage plant subjected to increasing NaCl concentrations (>50 mM) in the medium had higher CA and MCA content, but slightly lower total leaf dry mass compared to non-treated plants. Therefore, it may be possible to obtain higher yields of antioxidants, such as CA, from plants grown with saline water. It may be feasible to increase the commercial value of sage leaf extracts by manipulating the concentration of phenolic diterpenes and EOs. Nevertheless, larger scale field studies are needed to ascertain the potentially beneficial effects of NaCl on phenolic diterpene and EOs concentration against the potential loss in plant biomass production and soil fertility in the long run.

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REFERENCES

1. Ben Taarit M, Msaada K, Hosni K, Hammami M, Kchouk ME, Marzouk M, *et al*. Plant growth, essential oil yield and composition of sage (*Salvia offcinalis L.*) fruits cultivated under salt stress conditions. Ind Crop Prod 2009;30:333-7.
2. Tounekti T, Munné-Bosch S, Vadel AM, Chtara C, Khemira H. Influence of ionic interactions on essential oil and phenolic diterpene composition of Dalmatian sage (*Salvia officinalis* L.). Plant Physiol Biochem 2010;48:813-21.
3. Aziz EE, Sabry RM, Ahmed SS. Plant growth and essential oil production of sage (*Salvia officinalis* L.) and curly-leafed parsley (*Petroselinum crispum* ssp. *crispum* L.) cultivated under salt stress conditions. World Appl Sci J 2013;28:785-96.
4. Leung AY, Foster S. Encyclopedia of Common Natural Ingredients Used in Food, Drugs, and Cosmetics. 2nd ed. New York: John Wiley & Sons, Inc.; 1996.
5. Chnnusamy V, Jagendorf A, Zhu J. Understanding and improving salt tolerance in plants. Crop Sci 2005;45:437-48.
6. Tounekti T, Vadel AM, Bedoui A, Khemira H. NaCl stress affects growth and essential oil composition in rosemary (*Rosmarinus officinalis* L.). J Hortic Sci Biotechnol 2008;83:267-73.
7. Raal A, Orav A, Arak E. Composition of the essential oil of *Salvia officinalis* L. from various European countries. Nat Prod Res 2007;21:406-11.
8. Perry NB, Anderson RE, Brennan NJ, Douglas MH, Heaney AJ, McGimpsey JA, *et al.* Essential oils from dalmatian sage (*Salvia officinalis* l.): Variations among individuals, plant parts, seasons, and sites. J Agric Food Chem 1999;47:2048-54.

9. Schwarz K, Ternes W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*: I. isolation of carnosic acid and formation of other phenolic diterpenes. Z Leben Unters Forsch 1992;195:99-103.
10. Brückner K, Božic D, Manzano D, Papaefthimiou D, Pateraki I, Scheler U, *et al.* Characterization of two genes for the biosynthesis of abietane-type diterpenes in rosemary (*Rosmarinus officinalis*) glandular trichomes. Phytochemistry 2014;101:52-64.
11. Munné-Bosch S, Alegre L. Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of rosemary. Plant Physiol 2001;125:1094-102.
12. Munné-Bosch S, Schwarz K, Alegre L. Enhanced formation of alpha-tocopherol and highly oxidized abietane diterpenes in water-stressed rosemary plants. Plant Physiol 1999;121:1047-052.
13. Thorsen MA, Hildebrandt KS. Quantitative determination of phenolic diterpenes in rosemary extracts. Aspects of accurate quantification. J Chromatogr A 2003;995:119-25.
14. Paris A, Strukelj B, Renko M, Turk V, Pukl M, Umek A, *et al.* Inhibitory effect of carnosic acid on HIV-1 protease in cell-free assays [corrected]. J Nat Prod 1993;56:1426-30.
15. Rau O, Wurglics M, Paulke A, Zitzkowski J, Meindl N, Bock A, *et al.* Carnosic acid and carnosol, phenolic diterpene compounds of the labiate herbs rosemary and sage, are activators of the human peroxisome proliferator-activated receptor gamma. Planta Med 2006;72:881-7.
16. Fischedick JT, Standiford M, Johnson DA, Johnson JA. Structure activity relationship of phenolic diterpenes from *Salvia officinalis* as activators of the nuclear factor E2-related factor 2 pathway. Bioorg Med Chem 2013;21:2618-22.
17. Tounekti T, Munné-Bosch S. Enhanced phenolic diterpenes antioxidant levels through non-transgenic approaches. Crit Rev Plant Sci 2012;31:505-19.
18. del Baño MJ, Lorente J, Castillo J, Benavente-García O, del Río JA, Ortuño A, *et al.* Phenolic diterpenes, flavones, and rosmarinic acid distribution during the development of leaves, flowers, stems, and roots of *Rosmarinus officinalis*. Antioxidant activity. J Agric Food Chem 2003;51:4247-53.
19. Abreu ME, Muller M, Alegre L, Munne-Bosch S. Phenolic diterpenes and α-tocopherol contents in leaf extracts of 60 *Salvia* species. J Sci Food Agric 2008;88:2648-53.
20. Tounekti T, Vadel AM, Ennajeh M, Khemira H, Munné-Bosch S. Ionic interactions and salinity affect monoterpene and phenolic diterpene composition in rosemary (*Rosmarinus officinalis*). J Plant Nutr Soil Sci 2011;174:504-14.
21. El-Keltawi NE, Croteau R. Salinity depression of growth and essential oil formation in spearmint and marjoram and its reversal by foliar applied cytokinin. Phytochemistry 1987;26:1333-4.
22. Heuer B, Yaniv Ravina I. Effect of late salinization of chia (*Salvia hispanica*), stock (*Matthiolatri cuspidata*) and evening primrose (*Oenothera abiennis*) on their oil content and quality. Ind Crop Prod 2002;15:162-7.
23. Maas EV, Hoffman GJ. Crop salt tolerance - current assessment. J Irrigation Drainage Div ASCE 1977;103:115-34.
24. Delfine S, Alvino A, Villani MC, Loreto F. Restrictions to carbon dioxide conductance and photosynthesis in spinach leaves recovering from salt stress Plant Physiol 1999;119:1101-6.
25. Loreto F, Centritto M, Chartzoulakis K. Photosynthetic limitations in olive cultivars with different sensitivity to salt stress. Plant Cell Environ 2003;26:595-601.
26. Ben Taarit M, Msaada K, Hosni K, Marzouk B. Changes in fatty acid and essential oil composition of sage (*Salvia officinalis* L.) leaves under NaCl stress. Food Chem 2010;9:951-6.
27. Maric S, Maksimovic M, Milos M. The impact of the locality altitudes and stages of development on the volatile constituents of *Salvia officinalis* L. from Bosnia and herzegovina. J Essent Oil Res 2006;18:178-80.
28. Bernotiene G, Nivinskiene O, Butkiene R, Mockute D. Essential oil composition variability in sage (*Salvia officinalis* L.). Chemija 2007;18:38-43.
29. Giannouli AL, Kintzios SE. Essential oils of *Salvia* spp: Examples of intra-specific and seasonal variation. In: Kintzios SE, editor. Sage: The Genus *Salvia*. Amsterdam: Harwood Academic Publishers; 2000. p. 69-80.
30. Croteau R, El-Bialy H, Dehal SS. Metabolism of monoterpenes: Metabolic fate of (+)-camphor in sage (*Salvia officinalis*). Plant Physiol 1987;84:643-8.
31. Senatore F, Arnold NA, Piozzi F, Formisano C. Chemical composition of the essential oil of *Salvia microstegia* Boiss. et Balansa growing wild in Lebanon. J Chromatogr A 2006;1108:276-8.
32. Özek G, Özek T, Işcan G, Başer KH, Hamzaoglu E, Duran A. Comparison of hydrodistillation and microdistillation methods for the analysis of fruit volatiles of *Prangos pabularia* Lindl., and evaluation of its antimicrobial activity. S Afr J *Bot* 2007;73:563-9.
33. Liang Q, Liang Z, Wang J, Xu W. Essential oil composition of *Salvia miltiorrhiza* flower. Food Chem 2009;113:592-4.
34. Simon JE, Bubenheim DR, Joly RJ, Charles DJ. Water stress induced alterations in essential oil content and composition of sweet basil. J Essent Oil Res 1992;4:71-5.
35. Neffati M, Marzouk B. Changes in essential oil and fatty acid composition in coriander (*Coriandrum sativum* l.) leaves under saline conditions. Ind Crop Prod 2008;28:137-42.
36. Hendawy SF, Khalid KA. Response of sage (*Salvia officinalis* L.) plants to zinc application under different salinity levels. J Appl Sci Res 2005;1:147-55.
37. Khalid KH. Influence of water stress on growth, essential oil, and chemical composition of herbs (*Ocimum* Sp.). Int Agrophysics 2006;20:289-96.
38. Burbott AJ, Loomis WD. Evidence for metabolic turnover of monoterpenes in peppermint. Plant Physiol 1969;44:173-9.
39. Munné-Bosch S, Alegre L. Drought-induced changes in the redox state of α-tocopherol, ascorbate and the diterpene carnosic acid in chloroplasts of Labiatae species differing in carnosic acid contents. Plant Physiol 2003;131:1816-25.
40. Putievsky E, Ravid U, Sanderovich D. Morphological observations and essential oils of sage (*Salvia officinalis* L.) under cultivation. J Essent Oil Res 1992;4:291-3.
41. Hidalgo P, Ubera J, Tena M, Valcárcel M. Determination of carnosic acid in wild and cultivated *Rosmarinus officinalis*. J Agri Food Chem 1998;46:2624-7.
42. Munné-Bosch S, Alegre L, Schwarz K. The formation of phenolic diterpenes in *Rosmarinus officinalis* L. under Mediterranean climate. Eur Food Res Technol 2000;210:263-7.
43. Byun-McKay A, Godard KA, Toudefallah M, Martin DM, Alfaro R, King J, *et al.* Wound-induced terpene synthase gene expression in Sitka spruce that exhibit resistance or susceptibility to attack by the white pine weevil. Plant Physiol 2006;140:1009-21.
44. Kutuk C, Cayci G, Heng LK. Effects of increasing salinity and N-15 labelled urea levels on growth, N uptake, and water use efficiency of young tomato plants. Aust J Soil Res 2004;42:345-51.
45. Boelens MH. Essential oils and aroma chemicals from *Eucalyptus globulus* Labill. Perfum Flavor 1985;9:1-14.
46. Areias F, Valentão P, Andrade PB, Ferreres F, Seabra RM. Flavonoids and phenolic acids of sage: Influence of some agricultural factors. J Agric Food Chem 2000;48:6081-4.

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Original Research

Herbal medicine use among patients with chronic diseases

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ABSTRACT

Aim: Complementary and alternative medicine (CAM) is commonly used all over the world, and herbal medicines are the most preferred ways of CAM. The aim of this study was to determine the frequency of herbal medicine use among patients with chronic diseases. **Methodology:** A cross-sectional descriptive study was conducted from April 2014 to December 2014 among patients who had been diagnosed with diabetes mellitus (DM), hypertension (HT), and hyperlipidemia (HL) in Family Medicine Department of Dışkapı Yıldırım Beyazıt Training and Research Hospital, in Ankara. A questionnaire about herbal drug use was applied by face to face interview to the participants. **Results:** A total of 217 patients were included in this study. The mean age of the participants was 56.6 ± 9.7 years (55 male and 162 female). The rate of herbal medicine use was 29%. Herbal medicine use among female gender was significantly higher ($P = 0.040$). Conventional medication use was found to be lower among herbal medicine consumers. There was no relationship between herbal medicine use and type of chronic disease, living area, and occupation or education level. Most frequently used herbs were lemon (39.6%) and garlic (11.1%) for HT, cinnamon (12.7%) for DM, and walnut (6.3%) for HL. **Conclusions:** In this study, herbal medicine use was found to be higher among patients who had been diagnosed with chronic diseases. Therefore, physicians should be aware of herbal medicine usage of their patients and inform them about the effectivity and side effects of herbal medicines.

KEY WORDS: Diabetes, herbal medicines, hyperlipidemia, hypertension, phytotherapy

INTRODUCTION

Complementary and alternative medicine (CAM) is commonly used all over the world [1]. Herbal medicines are the most preferred ways of CAM [2]. In USA, 38% of the population use herbal medicines [3]. Soner *et al*. found that 48.8% of people use herbal medicine in Turkey [4]. People use herbal medicines to be healthier, prevent or treat diseases. Although herbal products are believed to be harmless among consumers, they might have side effects, and they have potential to cause drug interactions [5,6]. This is a particular concern in patients with chronic diseases who utilize polypharmacy.

Although Turkey has a long tradition of using herbal medicine, the data about the frequency of utilization of herbal medicine in chronic diseases are scarce [4,7-12]. Therefore, in this study, we aimed to determine the frequency of the use of herbal medicine among patients who had been diagnosed with diabetes mellitus (DM), hypertension (HT), and hyperlipidemia (HL) in family medicine department.

METHODOLOGY

A cross-sectional descriptive study was conducted among adult patients ($n = 232$) who had been followed with DM, HT, HL from April 2014 to December 2014 in Hasköy Outpatient Clinics of Family Medicine Department of Dışkapı Yıldırım Beyazıt Training and Research Hospital in Ankara. Eligible patients who gave informed consent were included in the study.

A face-to-face questionnaire was administered to the participants by the first author. The questionnaire included information about socio-demographic features, chronic diseases, conventional medications, and herbal medicine use. Compliance to conventional medicine use was defined as compliance and non-compliance whether the patients regularly had received their prescribed drugs according to the clinicians' instructions. In addition, data about the name of the herbal medicine, the reasons for herbal use, belief about effectivity, knowledge about potential adverse effects, and by whom (e.g., families or friends, media or health care providers) its use was recommended were collected.

The study was approved by Local Ethical Committee of Dışkapı Yıldırım Beyazıt Training and Research Hospital.

Data Analysis

The data obtained from the study were analyzed using Statistical Package for Social Sciences version 15 for Windows. Descriptive

statistics is presented as mean ± standard deviation, range, and frequency (% values). Chi-square test or Fisher's exact test was used for categorical variables and Student's *t*-test for normally distributed data with equal variances. A $P < 0.05$ considered as statistically significant.

RESULTS

A total of 217 patients were included in this study. The mean age of the participants was 56.6 ± 9.7 years. There were 55 male (25.4%) and 162 female (74.6%) patients. It was found that 96.2% of women were housewives and 63.6% of men were retired. There were 35 patients (16.1%) who had DM, 59 patients (27.2%) who had HT, 7 patients (3.2%) who had HL, and there were 116 patients (53.5%) who had more than one disease. Among those patients, 212 of them (97.7%) used conventional medicine and 122 of them (56.2%) went check-up regularly. The socio-demographic and clinical features of the study population are presented in Table 1.

The number of herbal medicine users was 63. Herbal medicine use was found to be significantly higher among female gender ($P = 0.04$). Conventional medication use was found to be lower among herbal medicine consumers (97.3% vs. 99.4%).

Herbal medicines were commonly recommended to the users by their families or friends (61.9%), by media (27%), and by health care providers (11.1%), respectively. Herbal medicines were merely recommended by professional health care providers (7.9% by physicians, 3.2% by pharmacists).

Among those herbal medicine users, 68.3% thought that herbal medicines had a good effect, 11.1% had a minor effect, and 20.6% had no effect on their medical conditions. A total of 54 herbal medicine users (85.7%) thought that herbal medicines had no adverse effects, 7 (11.1%) thought they might have adverse effects, 2 (3.2%) had no idea.

Table 1: The socio-demographic and clinical features of the study population

Patients characteristics	*N* (%)			*P**
	Herbal medicine users	Herbal medicine non-users	Total	
Participants	63 (29)	154 (71)	217 (100)	
Male	10 (15.9)	45 (29.2)	55 (25.4)	0.04
Female	53 (84.1)	109 (70.8)	162 (75.6)	
Age (years, mean±SD)	56.9±8.7	56.4±9.9	56.6±9.7	0.81
Education level				0.41
Uneducated	15 (23.8)	30 (19.5)	45 (20.7)	
Primary/secondary school	36 (57.2)	98 (63.1)	134 (61.7)	
High school	6 (9.5)	19 (12.3)	25 (11.5)	
College	6 (9.5)	7 (4.5)	13 (6.0)	
Location				0.86
Urban	53 (84.1)	128 (83.1)	181 (83.4)	
Rural	10 (15.9)	26 (16.9)	36 (16.6)	
Number of chronic diseases				0.52
1	33 (52.4)	68 (44.2)	101 (46.5)	
2	17 (27)	51 (33.1)	68 (31.3)	
3	13 (20.6)	35 (22.7)	48 (22.1)	
Check-up regularly				0.30
Yes	32 (50.8)	90 (58.4)	122 (56.2)	
No	31 (49.2)	64 (41.6)	95 (43.8)	

*$P<0.05$ considered as statistically significant, SD: Standard deviation

The most common reasons of herbal medicine use were to increase the effectiveness of the conventional medications (39.7%), to believe that they were harmless (33.3%), to find them more effective (25.6%), and cheaper (1.6%) than the conventional drugs.

Most frequently used herbs were lemon (39.6%) and garlic (11.1%) for HT, cinnamon (12.7%) for DM, and walnut (6.3%) for HL. Four patients (6.3%) were using herbal mixtures without knowing the ingredients. Patients mostly use roots, leaves, fruits or seeds of plants which can be found easily.

DISCUSSION

DM, HT, and HL are the most prevalent chronic diseases in the world. According to TURDEP-2 and TEKHARF studies, the prevalence of DM is 16.6%, the prevalence of HT is 31.6%, and the prevalence of HL is 37.3% in Turkey [13,14].

Recent studies support the popularity and increasing use of CAM by individuals in western countries as well as in Turkey [2,4,9,10]. We found approximately one-third of our patients had used herbal medicine for the treatment of chronic diseases. The differences in the reported rates of herbal medicine use might depend on socio-demographic features of study population even in the same country. Biçen *et al.* reported 53% herbal medicine use for HT in a study population similar to our study population (most of the individuals were female, mean age was 57.6 years, and education level was low) [11]. Soner *et al*. found 48.8% herbal use in a population mostly consisted of female inpatients with the median age 37.0 years and with higher education levels [4]. However, Gücük *et al*. reported lower rates (16%) herbs use in cardiovascular disease among male predominant patients with the mean age of 49 ± 13 years [7].

The proposed reasons of the prevalent use of herbal medicines were insufficient controlling in the market, ease of reaching to the products, and over advertising of those products by media [15].

It was shown that women are more prone to use herbal medicines [3,4,7]. Our findings were also compatible with those previous reports. Hence, most of the women were housewives (96.2%) in our study population. The television programs, especially for women may be the possible explanation for this cause. Herbal medicines are mentioned a lot on these kinds of programs.

In contrast to the studies [3,4,7], we did not find a relationship between herbal medicine use and education level. Previous studies found that people who had high education level were more likely to use herbal medicines.

It is known that compliance to conventional drugs in chronic diseases is low [16]. Although the effectiveness of the herbal

medicines in the treatment of chronic diseases has not been clearly documented, we found that non-compliance with conventional medication was found to be higher in herbal medicine users. This preference has potential to jeopardize the patients' health. Furthermore, we found that most of the patients believed that herbal medicine was effective (68.3% good effect, 11.1% minor effect) and had no adverse effects (85.7%). Although there were 7 patients (11.1%) who thought herbal medicines might have adverse effects, we did not ask their experiences about adverse effects. Unfortunately, we did not collect data about the impact of those medications on our patients' medical conditions (e.g., blood pressure, hemoglobin A1c levels or lipid profiles). Soner *et al*. found that 53.2% of the study population believed that herbal medicines were not harmful, had less side effects or completely safe; they also found that 11.3% of the herbal medicine users had adverse effects such as palpitation, abdominal pain or hot flushing [4].

In addition, we found that herbal medicines were merely recommended by professional health care providers (7.9% by physicians, 3.2% by pharmacists). Family and friends or media were the main sources of information as seen in previous studies [4,7,11,17]. This is another important point, which could lead to negative outcomes in the treatment. The potential side-effect and outcome influence may be brought about by combinated use of herbal medicine and conventional drugs, and these circumstances are always difficult to predict because elucidating the exact mechanism of each extract of an herb that affects drug's pharmacokinetic is still incomplete. Hence, adverse drug reactions, toxicities, and treatment failure are more likely to occur when drugs are consumed with herbs [18]. To date, an increasing number of studies in evaluating herbal medicine-drug interaction have been reported, and many herbal drugs and products interactions and side effects are well-known [5,19,20]. For example, concomitant use of danshen (*Salvia miltiorrhiza*) and warfarin may exaggerate the anticoagulant effect of warfarin and possibly cause bleeding [21]. Korean/Asian ginseng (*Panax ginseng*) and American ginseng (*Panax quinquefolius*) have antidiabetic effects [22-24] using ginseng concurrently with oral hypoglycemic agents or insulin injections may increase the risk of hypoglycemia [25]. Therefore, herbs should be used with the control of a professional health-care provider. We do not know the attitudes of the health care professionals for herbal medicines in Turkey which should be further evaluated.

Although 20.6% of the herbal medicine users thought that herbal medicine was ineffective for their treatment, and 11.1% of them believed that herbal medicines might have had adverse effects, interestingly they continued to use herbal medicines. Efe *et al*. found that 45.3% of the patients believed that herbal medicines were effective for HT [17].

Patients mostly use roots, leaves, fruits or seeds of plants which can be found easily, according to our study. Studies on this subject are limited in order to compare our results. Efe *et al*. found that 74.7% of patients used CAM (mostly herbal medicines) for HT and lemon was the most preferred one [17]. The results are consistent with our study. Although lemon was the most preferred one for HT, its effectiveness for decreasing blood pressure was not shown before certainly [26].

According to our knowledge, this is the first study in Turkey about herbal medicine use among three, most prevalent chronic diseases. However, our study also has limitations. First of all, this is a local study and the number of participants is limited. The sample is not likely to be representative of the entire population. Second, the source of information was subjective and according to the declaration of patients.

CONCLUSION

In this study, herbal medicine use was found to be higher among patients with chronic diseases and most of the patients used herbal medicine without professional recommendations. There were also patients who prefer to use herbal medicines instead of their prescribed drugs. Therefore, physicians should be aware of herbal medicine usage of their patients and inform them about the effectivity and side effects of herbal medicines.

REFERENCES

1. Wu CH, Wang CC, Kennedy J. Changes in herb and dietary supplement use in the U.S. adult population: A comparison of the 2002 and 2007 National health interview surveys. Clin Ther 2011;33:1749-58.
2. Fox P, Coughlan B, Butler M, Kelleher C. Complementary alternative medicine (CAM) use in Ireland: A secondary analysis of SLAN data. Complement Ther Med 2010;18:95-103.
3. Barnes PM, Bloom B, Nahi RL. Complementary and alternative medicine use among adults and children: United States. 2007. Natl Health Stat Rep 2008;12:1-23.
4. Soner BC, Şahin AS, Şahin TK. A survey of Turkish hospital patients' use of herbal medicine. Eur J Integrat Med 2013;547-52.
5. Cvijovic K, Boon H, Barnes J, Brulotte J, Jaegar W, Murty M, *et al.* A tool for rapid identification of potential herbal medicine-drug interactions. Can Pharm J 2009;142:1-2.
6. Lin SS, Tsai CL, Tu CY, Hsieh CL. Reducing drug-herb interaction risk with a computerized reminder system. Ther Clin Risk Manag 2015;11:247-53.
7. Gücük Ipek E, Güray Y, Demirkan B, Güray U, Kafes H, Basyigit F. The prevalence of alternative herbal medicine and nutritional complementary product intake in patients admitted to out-patient cardiology departments. Turk Kardiyol Dern Ars 2013;41:218-24.
8. Keskin A, BIlge U. Mental disorders frequency alternative and complementary medicine usage among patients with hypertension and type 2 diabetes mellitus. Niger J Clin Pract 2014;17:717-22.
9. Koc Z, Avci IA, Saglam Z. The use of complementary and alternative medicine by older patients that have chronic diseases. Health Med 2012;6:413-22.
10. Aydın S, Bozkaya AO, Mazıcıoğlu M, Gemalmaz A, Özçakır A, Öztürk A. What influences herbal medicine use? Prevalence and related factors. Turk J Med Sci 2008;38:455-63.
11. Biçen C, Elver Ö, Erdem E, Kaya C, Karataş A, Dilek M, *et al*. Herbal product use in hypertension patients. J Exp Clin Med 2012;29:109-12.
12. Toprak D, Demir S. Treatment choices of hypertensive patients in Turkey. Behav Med 2007;33:5-10.
13. Satman I, Omer B, Tutuncu Y, Kalaca S, Gedik S, Dinccag N, *et al.* Twelve-year trends in the prevalence and risk factors of diabetes and prediabetes in Turkish adults. Eur J Epidemiol 2013;28:169-80.
14. Yumuk VD. Prevalence of obesity in Turkey. Obes Rev 2005;6:9-10.
15. Krasuski RA, Michaelis K, Eckart RE. The cardiovascular patient's perceptions of complementary and alternative medicine. Clin Cardiol 2006;29:161-4.
16. Hacıhasanoğlu R. Treatment compliance affecting factors in hypertension. TAF Prev Med Bull 2009;8:167-72.
17. Efe D, Akca NK, Kiper S, Aydin G, Gumus K. Supportive methods

used by individuals with hypertension to decrease blood pressure. Spatula DD 2012;2:201-12.
18. Chen XW, Sneed KB, Pan SY, Cao C, Kanwar JR, Chew H, *et al.* Herb-drug interactions and mechanistic and clinical considerations. Curr Drug Metab 2012;13:640-51.
19. Brantley SJ, Argikar AA, Lin YS, Nagar S, Paine MF. Herb-drug interactions: Challenges and opportunities for improved predictions. Drug Metab Dispos 2014;42:301-17.
20. Liu MZ, Zhang YL, Zeng MZ, He FZ, Luo ZY, Luo JQ, *et al.* Pharmacogenomics and herb-drug interactions: Merge of future and tradition. Evid Based Complement Alternat Med 2015;2015:321091.
21. Tsai HH, Lin HW, Lu YH, Chen YL, Mahady GB. A review of potential harmful interactions between anticoagulant/antiplatelet agents and Chinese herbal medicines. PLoS One 2013;8:e64255.
22. Jeon WJ, Oh JS, Park MS, Ji GE. Anti-hyperglycemic effect of fermented ginseng in type 2 diabetes mellitus mouse model. Phytother Res 2013;27:166-72.
23. Xie JT, Mchendale S, Yuan CS. Ginseng and diabetes. Am J Chin Med 2005;33:397-404.
24. Sen S, Querques MA, Chakrabarti S. North American Ginseng (*Panax quinquefolius*) prevents hyperglycemia and associated pancreatic abnormalities in diabetes. J Med Food 2013;16:587-92.
25. Kim DH. Chemical diversity of *Panax ginseng*, panax quinquifolium, and *Panax notoginseng*. J Ginseng Res 2012;36:1-15.
26. Kato Y, Domoto T, Hiramitsu M, Katagiri T, Sato K, Miyake Y, *et al.* Effect on blood pressure of daily lemon ingestion and walking. J Nutr Metab 2014;2014:912684.

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Original Research

ScopeMed

The subtle central effect of nutraceuticals: Is it placebo or nocebo?

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ABSTRACT

Background: Herbal medicines are often perceived by the general public as a "soft" alternative to Western Medicine, but the use of these substances can be risky since they can induce nocebo effect. **Aim:** The aim was to evaluate the nocebo effects of *Nigella sativa* oil, garlic and coenzyme Q10 (CoQ10) on the integrative function of the central nervous system and psychomotor performance. **Materials and Methods:** This is a randomized, double-blind, controlled, and prospective study conducted in the Department of Pharmacology, College of Medicine, Al-Mustansiriya University, Baghdad, Iraq during February 2013. A total of 160 medical students participated in this study were randomly assigned equally to one of the following groups: Group A: Received single dose of *N. sativa* oil (500 ml capsule); Group B: Received single dose of garlic (500 mg capsule); Group C: Received single dose of CoQ10 (120 mg capsule) and; Group D: received single dose of matching oral placebo (300 mg starch capsule). For all participants, reaction time and flicker fusion threshold were measured by the Leeds psychomotor performance test battery before and after 3 h of taking the drugs **Results:** Neither placebo nor nutraceuticals exerted significant effect on total reaction time. Although the recognition reaction time is insignificantly reduced by 2.77% (placebo), 5.83% (*Nigella sativa*), 7.21% (garlic) and 12.64% (CoQ10) from the pretreatment values, they are adversely affect the motor reaction time to reach the significant level in subjects pretreated with Garlic ($P = 0.02$). **Conclusion:** Nutraceuticals are not free from nocebo effect on psychomotor performance.

KEY WORDS: Garlic and coenzyme Q10, *Nigella sativa* oil, psychomotor performance

INTRODUCTION

Herbal medicines are often perceived by the general public as a "soft" alternative to Western Medicine, but the use of these substances can be risky since they can induce nocebo effect [1]. In 1961 Walter Kennedy chosen the term nocebo (Latin for I will harm) as the counterpart of placebo [2]. This term was introduced a few years after Henry Beecher published his paper on the placebo effect [3]. Most clinical studies explored the beneficial effects of nutraceuticals and ignored their nocebo effects, The seeds/oil of *Nigella sativa* has anti-inflammatory, analgesic, antipyretic, antimicrobial, hypotensive, hypoglycemic, antiepileptic and antineoplastic activity [4-7].

Garlic, considered either food or herbal medicine, possesses antimutagenic and antiproliferative properties that can be used in anticancer interventions, hypoglycemic [8-10].

Coenzyme Q10 (CoQ10) is an essential electron carrier in the mitochondrial respiratory chain and an important antioxidant. It exerts a beneficial effect on cognitive, digestive, cardiovascular and immune systems, and modulates inflammatory and degenerative processes in the body [11-13].

Nutraceuticals derived from such spices as turmeric, red pepper, black pepper, licorice, clove, ginger, garlic, coriander, and cinnamon target inflammatory pathways, thereby may prevent neurodegenerative diseases e.g., Parkinson's disease [14,15]. On the other hand, most clinical studies ignored the subtle central effect of the nutraceuticals, therefore the aim of this study is to show the nocebo effects of the nutraceuticals notably black cumin, garlic and CoQ10 on the integrative function of central nervous system and psychomotor performance in human using Leeds battery testing.

MATERIALS AND METHODS

This is a randomized, controlled, double-blind, prospective study conducted in the Department of Pharmacology, College of Medicine, Al-Mustansiriya University, Baghdad, Iraq in February 2013. A total of 160 healthy male volunteers were entered into the present study. They were randomly chosen from college medical students, aged ranges between 19 and 24 years (mean age 21 years). All participants were in good health, without any significant clinical history of physical or mental illness and not taking any concomitant medication that was likely to interfere with the study. Furthermore, caffeine and other beverages were forbidden on the study day. The study was approved by Local Scientific Committee of the Institution and written informed consent was obtained from all participants.

Prior to the study participants were trained to perform the psychomotor performance tests and practiced on three

separate occasions to exclude any learning bias. Then, they were randomly assigned equally to one of the following groups:

Group A: Received single dose of *N. sativa* oil (500 ml capsule, Sehapharma, Egypt).

Group B: Received single dose of garlic (500 mg capsule, private label nutraceuticals, USA).

Group C: Received single dose of CoQ10 (120 mg capsule, MaritzMayer Laboratories, USA).

Group D: Received a single dose of matching oral placebo (300 mg starch capsule).

All participants in the above 4 groups were subjected to Leeds battery psychomotor instrument before and after 3 h of taking the corresponding interventional agent (nutraceuticals or placebo) in order to assess their pre- and post-treatment psychomotor performance in term of choice reaction time (CRT), critical flicker fusion (CFF) and total reaction time (TRT).

It is well-known that CRT can be used to assess the sensory-motor alertness through measuring the speed of recognition and motor responding to particular sensory stimulus. In the present study, by using Leeds psychomotor tester, the participants instructed to respond to the bright red light that illuminated in one of six position in random way by pressing the button where the light presented. It's important to mention that the mean of 9 consecutive presentations is recorded as a response measure of three components of reaction time: recognition, motor, and TRT.

The recognition reaction time (RRT) represent the time spent between red light illumination in the Leeds psychomotor device and the beginning of participant responding toward that light (motor action), manifested by lifting of their finger from specific site (start button). The motor reaction time (MRT) represented by the time spent from the start of motor action to the end of it i.e., the time through which the finger of the participant moves from start button to the response button. Summation of RRT and MRT represent TRT.

The CFF task assessed the integrative capacity of the central nervous system (CNS) and, more specifically, the ability to discriminate discrete "bits" of sensory information. In this, the participants are asked to concentrate on 4 illuminated points and to response to the changes in illumination in these points from flickering to fusion and vice versa. Individual thresholds are determined by the psychophysical method of limits on 3 ascending (flicker to fusion) and 3 descending (fusion to flicker) scales. A decrease in the CFF threshold is indicative of a reduction in the overall integrative activity of the CNS.

All data were analyzed using the statistical package of social sciences (SPSS) version 15 for Windows program on the computer. Data were given as mean ± standard deviation (SD). Student's *t*-test was used to compare mean values between groups. Statistical significance was accepted as $P < 0.05$.

RESULTS

Table 1 shows that the neither placebo nor nutraceuticals exerted a significant effect on TRT. The RRT is insignificantly reduced by 2.77% (placebo), 5.83% (*N. Sativa*), 7.21% (Garlic) and 12.64% (CoQ10) from the pretreatment values [Table 1]. Nutraceuticals adversely affect the MRT to reach the significant level in subjects pretreated with Garlic [Table 1]. Placebo treatment reduced the MRT by 1.4% while the nutraceutical preparations prolonged the MRT by 12.22% (*N Sativa*), 30% (Garlic) and 8.82% (CoQ10).

Integrative activity of central nervous assessed by measuring the CFF threshold; ascending and descending components remained stable with placebo pretreatment and nutraceuticals [Table 2].

DISCUSSION

The results of this study show that garlic produces nocebo effect on the motor component of reaction time while *N. sativa* and CoQ10 produce placebo effect. All the nutraceuticals produce placebo like effect on the integrative activity of the CNS. The effects of *N. sativa* on the brain were extensively done on small animals and *in vitro* utilizing cell lines. These studies showed that *N. sativa* exert antiepileptic, anxiolytic, and cerebral protection against ischemia and reperfusion injury [16-19]. This study shows that *N. sativa* does not produce any effect on the healthy human brain in term of psychomotor performance or integration activity of CNS, i.e.; it is a safe nutraceutical. The neuroprotective effect of garlic was extensively studied in different experimental models of cerebral ischemia and

Table 1: Placebo and nocebo effects of nutraceuticals preparations

	N. Sativa oil	Garlic	CoQ10	Placebo
Total reaction time (min)				
Before	527.1±51,33	530.7±57.64	541.8±53.45	581.7±98.25
After	525.6±56.56	547.9±53.70	515.9±49.97	568.4±51.24
Recognition reaction time (min)				
Before	365.2±79.89	381.6±49.99	343.4±41.26	375.5±77.97
After	343.9±25.14	354.1±47.85	300.0±50.26	365.1±65.12
Motor reaction time (min)				
Before	161.9±63.16	149.1±47.64	198.4±66.24	206.2±49.20
After	181.7±49.17	193.8±42.12*	215.9±29.99	203.3±33.23

*P=0.02 compared with pretreatment value, *N. sativa: Nigella sativa*, CoQ10: Coenzyme Q10

Table 2: Effect of nutraceuticals on the integrative activity of CNS in humans

Nutraceutical	Critical flicker fusion threshold (ascending component)		Critical flicker fusion threshold (descending component)	
	Before	After	Before	After
N. Sativa oil	31.33±2.44	32.39±3.12	31.32±3.60	31.69±2.18
Garlic	31.61±4.03	31.04±3.18	30.62±2.25	31.83±3.59
CoQ10	32.39±3.12	32.39±1.91	32.11±4.22	32.21±2.24
Placebo	30.82±1.76	30.79±1.85	32.83±2.96	32.02±2.14

N. Sativa: Nigella sativa, CNS: Central nervous system

neurodegenerative diseases [20-22]. Other experimental animal studies showed that garlic improved the memory and cognitive function [23,24]. This study demonstrates the nocebo effect rather than the beneficial effect on the psychomotor function in the healthy human. Therefore, the garlic nutraceutical should prescribe with caution in conditions associated with impaired psychomotor performance, e.g., elderly age group or in concomitant use with CNS depressant agents, e.g., alcohol or antihistamines. CoQ10 protects the neurons from degenerative changes, e.g., Parkinson's disease, Huntington's chorea, optic nerve atrophy and it exerts a beneficial effect in the management of headache including migraine [25-27]. In this study, the effect CoQ10 on the psychomotor performance does not differ from the placebo effect. Short term therapy and one dose testing of each nutraceutical are the limitations of the study. It concludes that nutraceuticals are not free from nocebo effect on psychomotor performance.

REFERENCES

1. Nosbaum A, Ben Said B, Halpern SJ, Nicolas JF, Bérard F. Systemic allergic contact dermatitis to black cumin essential oil expressing as generalized erythema multiforme. Eur J Dermatol 2011;21:447-8.
2. Kennedy WP. The nocebo reaction. Med World 1961;95:203-5.
3. Rajagopal S. Nocebo effect. Available from http://priory.com/medicine/nocebo.htm (2007). [Last retrieved on 2011 Jul 29].
4. Butt MS, Sultan MT. Nigella sativa: reduces the risk of various maladies. Crit Rev Food Sci Nutr 2010;50:654-65.
5. Bamosa AO, Kaatabi H, Lebdaa FM, Elq AM, Al-Sultanb A. Effect of Nigella sativa seeds on the glycemic control of patients with type 2 diabetes mellitus. Indian J Physiol Pharmacol 2010;54:344-54.
6. Akhondian J, Kianifar H, Raoofziaee M, Moayedpour A, Toosi MB, Khajedaluee M. The effect of thymoquinone on intractable pediatric seizures (pilot study). Epilepsy Res 2011;93:39-43.
7. Nagoor Meeran MF, Jagadeesh GS, Selvaraj P. Thymol attenuates inflammation in isoproterenol induced myocardial infarcted rats by inhibiting the release of lysosomal enzymes and downregulating the expressions of proinflammatory cytokines. Eur J Pharmacol 2015;754:153-61.
8. Miroddi M, Calapai F, Calapai G. Potential beneficial effects of garlic in oncohematology. Mini Rev Med Chem 2011;11:461-72.
9. Ashraf R, Khan RA, Ashraf I. Garlic (Allium sativum) supplementation with standard antidiabetic agent provides better diabetic control in type 2 diabetes patients. Pak J Pharm Sci 2011;24:565-70.
10. Yun HM, Ban JO, Park KR, Lee CK, Jeong HS, Han SB, *et al.* Potential therapeutic effects of functionally active compounds isolated from garlic. Pharmacol Ther 2014;142:183-95.
11. Molyneux SL, Florkowski CM, Richards AM, Lever M, Young JM, George PM. Coenzyme Q10; an adjunctive therapy for congestive heart failure. N Z Med J 2009;122:74-9.
12. Lee BJ, Huang YC, Chen SJ, Lin PT. Coenzyme Q10 supplementation reduces oxidative stress and increases antioxidant enzyme activity in patients with coronary artery disease. Nutrition 2012;28:250-5.
13. Sandhir R, Sethi N, Aggarwal A, Khera A. Coenzyme Q10 treatment ameliorates cognitive deficits by modulating mitochondrial functions in surgically induced menopause. Neurochem Int 2014;74:16-23.
14. Kannappan R, Gupta SC, Kim JH, Reuter S, Aggarwal BB. Neuroprotection by spice-derived nutraceuticals: you are what you eat! Mol Neurobiol 2011;44:142-59.
15. Nasri H, Baradaran A, Shirzad H, Rafieian-Kopaei M. New concepts in nutraceuticals as alternative for pharmaceuticals. Int J Prev Med 2014;5:1487-99.
16. Ezz HS, Khadrawy YA, Noor NA. The neuroprotective effect of curcumin and Nigella sativa oil against oxidative stress in the pilocarpine model of epilepsy: a comparison with valproate. Neurochem Res 2011;36:2195-204.
17. Gilhotra N, Dhingra D. Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels. Pharmacol Rep 2011;63:660-9.
18. Hosseinzadeh H, Parvardeh S, Asl MN, Sadeghnia HR, Ziaee T. Effect of thymoquinone and Nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomedicine 2007;14:621-7.
19. Bin Sayeed MS, Shams T, Fahim Hossain S, Rahman MR, Mostofa A, Fahim Kadir M, *et al.* Nigella sativa L. seeds modulate mood, anxiety and cognition in healthy adolescent males. J Ethnopharmacol 2014;152:156-62.
20. Guo JP, Yu S, McGeer PL. Simple *in vitro* assays to identify amyloid-beta aggregation blockers for Alzheimer's disease therapy. J Alzheimers Dis 2010;19:1359-70.
21. Ashafaq M, Khan MM, Shadab Raza S, Ahmad A, Khuwaja G, Javed H, *et al.* S-allyl cysteine mitigates oxidative damage and improves neurologic deficit in a rat model of focal cerebral ischemia. Nutr Res 2012;32:133-43.
22. Lau KK, Chan YH, Wong YK, Teo KC, Yiu KH, Liu S, *et al.* Garlic intake is an independent predictor of endothelial function in patients with ischemic stroke. J Nutr Health Aging 2013;17:600-4.
23. Haider S, Naz N, Khaliq S, Perveen T, Haleem DJ. Repeated administration of fresh garlic increases memory retention in rats. J Med Food 2008;11:675-9.
24. Pintana H, Sripetchwandee J, Supakul L, Apaijai N, Chattipakorn N, Chattipakorn S. Garlic extract attenuates brain mitochondrial dysfunction and cognitive deficit in obese-insulin resistant rats. Appl Physiol Nutr Metab 2014;39:1373-9.
25. Beal MF. Therapeutic approaches to mitochondrial dysfunction in Parkinson's disease. Parkinsonism Relat Disord 2009;15 Suppl 3:S189-94.
26. Tarazi FI, Sahli ZT, Wolny M, Mousa SA. Emerging therapies for Parkinson's disease: from bench to bedside. Pharmacol Ther 2014;144:123-33.
27. Pringsheim T, Davenport W, Mackie G, Worthington I, Aubé M, Christie SN, *et al.* Canadian Headache Society guideline for migraine prophylaxis. Can J Neurol Sci 2012;39 2 Suppl 2:S1-59.

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Original Research

ScopeMed

Analgesic and free radical scavenging activities of hydromethanolic extract of *Crateva adansonii* stem bark

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ABSTRACT

Objective: *Crateva adansonii* is a moderately sized deciduous tree found throughout the tropics especially along the river banks. This study was aimed at the evaluation of the analgesic and antioxidant activities of the methanolic extract of *C. adansonii* stem-bark. **Methods:** The analgesic activity of *Crateva* extract was investigated using both chemical and thermal models of nociception in rodents while the antioxidant activity was evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) photometric model. **Results:** The extract produced a minute concentration-dependent increase in free radical scavenging activities. The extract (100, 200, and 400 mg/kg) caused a significant ($P < 0.05$) dose-dependent reduction in the number of writhing in treated rats when compared to the negative control. The extract at 100, 200, 400 mg/kg, and pentazocine (3 mg/kg) increased the pain reaction time in the treated rats by 58.05%, 66.67%, 94.76%, and 79.40%, respectively, when compared to the negative control. **Conclusion:** The *C. adansonii* stem bark possesses analgesic activity against peripheral and central mediated pain sensation and also antioxidant properties. This study justifies the ethnomedical use of *C. adansonii* in pain treatment.

KEY WORDS: Acetic acid, analgesic, aspirin, *Crateva adansonii*, pentazocine, tail flick

INTRODUCTION

Medicinal plants have been used as a form of medication for the relief of pain throughout history [1]. The treatments of pain disorders are an area in which the practitioners of traditional medicine enjoy patronage and success [2]. Herbs are believed to be an important source of new chemical substances with potential therapeutic efficacy. Salicylic acid and morphine were originally derived from the plant sources, thus study of plant species traditionally used as pain killers should be encouraged as a useful research strategy in the search of new analgesic and anti-inflammatory drugs [3].

Crateva adansonii DC belonging to the family *Capparidaceae* is commonly called "Varun" or "garlic pear" in English [4,5]. *C. adansonii* is a moderately sized deciduous tree found throughout the tropics especially along the river banks. The leaves are trifoliate and ovate to oblong in shape. The flowers are white or creamy and occur at the terminal corymbs. The barks are gray, smooth, and sometimes horizontally wrinkled [6]. Different parts of the plant are extensively used in folkloric medicine for the cure of many disease conditions. The powdered bark is used in the treatment of urinary, renal tubules, gastro-intestinal, and uterine affection [5]. In ethnomedicine, the plant is used in inflammatory conditions, asthma, snakebites, and as astringent [7]. In Senegal, the roots are used in the treatment of syphilis, jaundice, and yellow fever [8]. The scientific evaluation of the antimicrobial, anti-gout, and antitrypanosomal activities has been reported [4,9,10]. Abdullahi *et al.* [9] reported the presence of phenolics, alkaloids, flavonoids, and saponins in the leaves of *C. adansonii*.

Despite the widespread use of *C. adansonii* in ethnomedicine by many cultural groups in the relief of rheumatic and other pain conditions, there is a paucity of scientific information

on the analgesic and antioxidant properties of the plant. This study was designed to evaluate the analgesic and antioxidant activities of the methanolic extract of *C. adansonii* stem-bark.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The stem barks of *C. adansonii* were collected in May, 2014 from Orba in Udenu local government, Enugu State, Nigeria. They were identified by Mr. A. O. Ozioko, a Taxonomist at Bioresource Development and Conservation Programme, Enugu state, Nigeria. A voucher specimen catalogued MOUAU/VPP/2014/012 was deposited in the departmental herbarium for reference purposes.

Preparation of the Plant Material

The stem bark of the plant were dried at room temperature on a laboratory bench and pulverized into coarse powder. The powdered plant material was extracted using cold maceration method in 80% methanol for 48 h with intermittent shaking at 3 h interval. The extract was filtered using Whatmann No. 1 filter papers. The filtrate was concentrated in a hot air oven at 40°C and the extract was stored in a refrigerator at 4°C as *Crateva* extract until required for the experiment. The percentage yield was calculated using the formula below:

(Weight of extract/weight of starting plant material) × 100

Experimental Animals

Totally, 30 albino rats of both sexes weighing 100-130 g and 30 mice of both sexes weighing 28-34 g, sourced from the laboratory animal unit of the Department of Veterinary Physiology, Pharmacology and Biochemistry, Michael Okpara University of Agriculture Umudike, Abia State were used for the study. The animals were housed in aluminum cages at room temperature and under natural light/darkness cycles. The rats were supplied with clean drinking water and fed *ad libitum* with standard commercial pelleted grower feed (Vital feed® Nigeria). The rats were acclimatized for 2 weeks prior to the study. They were maintained in accordance with the recommendations of the Guide for the care and use of laboratory animals [11] and the experimental protocol was approved by the Institution's Ethical Committee.

Determination of Free Radical Scavenging Activities of *Crateva* Extract using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) Photometric Assay

The free radical scavenging activity of *Crateva* extract was analyzed by the DPPH photometric assay [12] using a spectrophotometer. The test extract (2 ml) at different concentrations (25, 50, 100, 200, and 400 μg/ml) were mixed with 0.5 mM DPPH (in 1 ml of methanol) in a cuvette. The absorbance at 517 nm was taken after 30 min of incubation in the dark at room temperature. The concentrations were prepared in triplicates and the percentage antioxidant activity calculated as follows.

% antioxidant activity (AA) = 100-([[absorbance of sample — absorbance of blank] ×100]/absorbance of control)

A volume of 1 mL of methanol plus 2.0 ml of the extract was used as the blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol were used as the negative control. Ascorbic acid (vitamin C) was used as reference standard [13].

Effect of *Crateva* Extract on Acetic Acid-induced Abdominal Writhing in Mice

The method of Vale *et al.* [14] was used. Five groups of mice consisting of 6 mice each were fasted for 12 h but free access to tap water was allowed. Group A received distilled water (10 ml/kg) and served as negative control. Group B received aspirin (100 mg/kg) orally and served as positive control, while Groups C-E received 100, 200, and 400 mg/kg of *Crateva* extract by oral administration, respectively. 45 min later, the mice received 10 ml/kg of 0.7% acetic acid intraperitoneally. The number of writhing or abdominal stretches produced in each mouse was counted for 30 min.

Effects of *Crateva* Extract on Tail Flick Response in Rats

The experiment was carried out by measuring tail withdrawal time from hot water as described by Adzu *et al.* [15]. Thirty rats were randomly divided into 5 Groups (A-E) of 6 rats each and fasted for 12 h. The rats were treated as follows: Group A served as negative control and received distilled water (10 ml/kg) orally, Group B served as positive control and received pentazocine (3 mg/kg) intraperitoneally, while Group C-E received *Crateva* extract (50, 300 and 600 mg/kg, respectively) orally. 1 h post drug treatment about 3 cm of the tail of each rat was dipped into a water bath containing warm water maintained at a temperature of 50 ± 1°C. The time taken for the mouse to flick the tail known as the pain reaction time (PRT) was recorded for all the mice.

Statistical Analysis

Data obtained were presented as mean ± standard error of mean and analyzed using one-way Analysis of Variance of SPSS software. The variant mean was separated by least significant difference of the different groups. Significance was accepted at the level of $P < 0.05$.

RESULT

DPPH Radical Scavenging Effect

The extract produced a minute concentration-dependent increase in free radical scavenging activities. The effects of the extract were significantly ($P < 0.05$) lower when compared to the effects of the ascorbic acids. The IC_{50} of the extract is >400 μg/ml [Figure 1].

Effect of *Crateva* Extract on Acetic Acid-induced Abdominal Writhing in Mice

The effects of *Crateva* extract on acetic acid induced writhing are presented in Table 1. The extract (100, 200, and 400 mg/kg) caused a significant ($P < 0.05$) dose-dependent reduction in the number of writhing in treated rats when compared to the negative control. The effects of the *Crateva* extract were comparable to that of aspirin (100 mg/kg). The extract (100, 200, and 400 mg/kg) and aspirin (100 mg/kg) produced 56.30%, 57.97%, 61.30%, and 50.93% reduction in the number of writhing respectively, when compared to the negative control.

Effects of *Crateva* Extract on Tail Flick Response in Rats

The pretreatment of the rats with *Crateva* extract (100, 200, and 400 mg/kg) caused a significant ($P < 0.05$) increase in the PRT in a dose-dependent manner when compared to the negative control group [Table 2]. The extract (100, 200, and 400 mg/kg) and pentazocine (3 mg/kg) increase the PRT in the treated rats by 58.05%, 66.67%, 94.76%, and 79.40% respectively, when compared to the negative control.

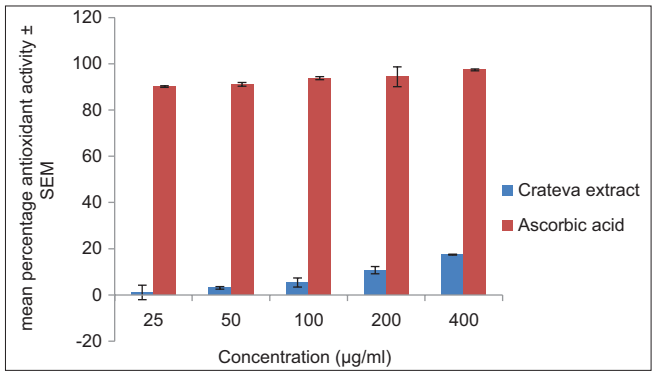

Figure 1: Effect of *Crateva* extract on 2, 2-diphenyl-1-picrylhydrazyl radical scavenging assay

Table 1: Effects of *Crateva* extract on acetic acid induced writhing reflex in mice

Treatment	Mean number of writhing±SEM	% inhibition
Distilled water 10 ml/kg	135±9.00	-
Aspirin 100 mg/kg	66.25±0.98***	50.93
Crateva extract 100 mg/kg	59.00±1.11***	56.30
Crateva extract 200 mg/kg	56.75±1.56***	57.97
Crateva extract 400 mg/kg	52.25±1.08***	61.30

*$P<0.05$; **$P<0.01$; ***$P<0.001$ when compared to distilled water treated group. SEM: Standard error of mean

Table 2: Effects of *Crateva* extract on tail flick response in rats

Treatment	Mean PRT±SEM (sec)	% increase on PRT
Distilled water 10 ml/kg	2.67±0.30	-
pentazocine 3 mg/kg	4.79±0.53*	79.40
Crateva extract 100 mg/kg	4.22±0.43*	58.05
Crateva extract 200 mg/kg	4.45±0.49*	66.67
Crateva extract 400 mg/kg	5.20±0.75*	94.76

*$P<0.05$ when compared to distilled water treated group, PRT: Pain reaction time; Sec: Second; SEM: Standard error of mean

DISCUSSION

The analgesic activity of *Crateva* extract was investigated using both chemical and thermal models of nociception in rodents while the antioxidant activity was evaluated using DPPH photometric model. The choice of the doses used in this study was based on the report of previous studies [7]. Acetic acid-induced writhing test was used for detecting both the peripheral and central analgesia, whereas the tail flick test are most sensitive to central acting analgesic drugs. The extract demonstrated a weak antioxidant activity in the DPPH photometric assay, with 50% inhibitory concentration (IC_{50}) >400 μg/ml.

Intraperitoneal injection of acetic acid induces the release of prostaglandins and sympathomimetic system mediators in the peritoneal fluid, which sensitize the peritoneal nociceptors [3]. The pretreatment of the mice with *Crateva* extract produced a dose-dependent analgesia comparable to the analgesia produced by the standard analgesic drug (Aspirin). This indicates that the *Crateva* extract and aspirin may have a similar mechanism of action. Aspirin is a non-steroidal anti-inflammatory drug that reduces the synthesis of prostaglandins and thromboxanes by irreversible inactivation of cyclooxygenase enzyme [16-18].

The *Crateva* extract caused a significant ($P < 0.05$) increase in PRT in the treated rats in a dose-dependent manner. The quality of the analgesia produced by the *Crateva* extract in the thermal model is comparable to the analgesia produced by pentazocine [Table 2]. This indicates that the *Crateva* extract has a central acting analgesic activity and may have a similar mechanism of action as pentazocine. Pentazocine is a synthetic opioid agonist-antagonist, which produces analgesia through interaction with μ and κ-receptors [19].

The analgesic and antioxidant activities of *Crateva* extract may be mediated by the phytochemical constituents [20]. Abdullahi *et al.* [9] reported the presence of saponins, flavonoids, alkaloids, and phenolics in *C. adansonii* leaves. The above listed phytochemical components of *C. adansonii* have been demonstrated to possess analgesic and antioxidant properties [20,21]. The antioxidant effect may help to counteract the adverse effect of oxidative stress that may arise due to pain sensation and drug biotransformation [22].

CONCLUSION

The *C. adansonii* stem bark possesses analgesic activity against peripheral and central mediated pain sensation and also antioxidant properties. This study justifies the ethnomedical use of *C. adansonii* in pain treatment. Further work is desired for the characterization of active analgesic principle.

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REFERENCES

1. Almeida RN, Navarro DS, Barbosa-Filho JM. Plants with central analgesic activity. Phytomedicine 2001;8:310-22.
2. Akah PA, Nwambie AI. Evaluation of Nigerian traditional medicines: Plants used for rheumatic disorder. J Ethnopharmacol 1994;42:179-82.
3. Shanmugasundaram P, Venkataraman S. Anti-nociceptive activity of *Hygrophilous auriculata* (Schum) Heine. Afr J Tradit Complement Altern Med 2005;2:62-9.
4. Igoli NP, Gray AI, Clements CJ, Igoli JO, Nzekwe U, Singla RK. Scientific investigation of antitrypanosomal activity of *Crateva Adansonii* DC leaves extracts. Indo Global J Pharm Sci 2012;2:226-9.
5. Gitte TA, Kare MA, Deshmukh AM. Ethno- medicinal studies on barks of some medicinal plants in Marathwada (M. S.) India –I. Recent Res Sci Technol 2012;4:08-10.
6. Flowers of India. Available from: http://www.flowersofindia.net/catalog/slides/Garlic%20Pear%20Tree.html. [Last accessed on 2015 Mar 23].
7. Akanji MA, Salau AK, Yakubu MT. Safety evaluation of aqueous extract of *Crateva adansonii* leaves on selected tissues of rats. Fount J Nat Appl Sci 2013;2:17-28.
8. Burkill HM. The useful plants of west tropical Africa. Vol. 5; 1985. Available from: http://www.plants.jstor.org/stable/10.5555/al.ap.upwta.5537. [Last accessed on 2015 Mar 19].
9. Abdullahi A, Hamzah RU, Jigam AA, Yahya A, Kabiru AY, Muhammad H, *et al.* Inhibitory activity of xanthine oxidase by fractions *Crateva adansonii*. J Acute Dis 2012;1:126-9.
10. Agboke AA, Attama AA, Momoh MA. Evaluation of the antimicrobial activities of crude extract of *Cryptolepis sanguinolenta* and *Crateva adansoni*i leaves and their interactions. J Appl Pharm Sci 2011;1:85-9.
11. Ward JW, Elsea JR. Animal case and use in drug fate and metabolism. In: Edward RG, Jean LH, editors. Methods and Techniques. 1st ed. New York: Markel Dekker; 1997.
12. Onoja SO, Anaga AO. Evaluation of the antidiabetic and antioxidant potentials of methanolic leaf extract of *Helianthus annuus* L. on alloxan-induced hyperglycemic rats. Comp Clin Pathol 2014;23:1565-73.
13. Iwalewa EO, Adewale IO, Aiwo BJ, Arogundabe T, Osinowo A, Daniyan OM, *et al.* Effects of *Harungana madagascariensis* stem bark extract on the antioxidant markers in alloxan induced diabetic and carrageenan induced inflammatory disorders in rats. J Complement Integr Med 2008;5:1-18.
14. Vale ML, Marques JB, Moreira CA, Rocha FA, Ferreira SH, Poole S, *et al.* Antinociceptive effects of interleukin-4, -10, and -13 on the writhing response in mice and zymosan-induced knee joint incapacitation in rats. J Pharmacol Exp Ther 2003;304:102-8.
15. Adzu B, Amos S, Wambebe C, Gamaniel K. Antinociceptive activity of Zizyphus spina-christi root bark extract. Fitoterapia 2001;72:344-50.
16. Abebe W. Herbal medication: Potential for adverse interactions with analgesic drugs. J Clin Pharm Ther 2002;27:391-401.
17. Uionne RA, Gordo SM. Non-steriodal anti-inflammatory drugs for acute pain control. Dent Clin N Am 1994;38:645-64.
18. Welch SP, Martin BR. Opiod and non-opioid analgesics. In: Graig CR, Stitzel RE, editors. Modern Pharmacology with Clinical Applications. 5th ed. Boston: Little, Brown and Company; 1997. p. 323.
19. Brunton LL, Parker KL, Blumenthal DK, Buxton IL. Goodman & Gilman's The Pharmacological Basis of Therapeutics. 11th ed. New York: McGraw Hill Medical; 2007.
20. Calixto JB, Beirith A, Ferreira J, Santos AR, Filho VC, Yunes RA. Naturally occurring antinociceptive substances from plants. Phytother Res 2000;14:401-18.
21. Bittar M, Souza MM, Cechinel FV, Yunes RA, Lento R, Delle-Monache F. Antinociceptive activity of 13,118-binaringenin, a bifavonoid present in plants of the family *Guttiferae*. Planta Med 2000;66:84-6.
22. Rang HP, Dale MM, Ritter JM, Moore RK. Pharmacology. 6th ed. Edinburgh: Churchill Livingstone; 2007.

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Original Research

ScopeMed

Comparative hypoglycemic activities of aqueous and ethanolic extracts of four medicinal plants (*Acanthus montanus, Asystasia gangetica, Emilia coccinea and Hibiscus rosasinensis*) in Type I diabetic rats

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ABSTRACT

Background: The present study ascertained the capacities of crude aqueous and ethanolic leaf extracts of *Acanthus montanus* (ACMO), *Asystasia gangetica* (ASGA), *Emilia coccinea* (EMCO), and *Hibiscus rosasinensis* (HIRO), as well as their combinatorial formulations to ameliorate hyperglycemia in Type I diabetic rats. **Materials and Methods:** Hyperglycemia was induced by single intraperitoneal injection of alloxan monohydrate in phosphate buffer saline (PBS) solution (pH = 7.4) dosage = 120 mg/kg; bw. Individual hyperglycemic rats (HyGR) received separate doses of either 20 mg/kg bw/24 h of ACMO, ASGA, EMCO or HIRO, as well as their combinatorial formulations (AAEH) for 14 days. Preparation of aqueous extracts (AQx) and ethanolic extracts (ETHx) of the four herbal samples was according to standard methods. Blood samples were drawn from 12 h post-fasted rats at regular intervals of 24 h for 14 days and measured for fasting blood glucose concentration (FBGC) using the glucose oxidase spectrophotometric method. **Results:** Cumulatively, ETHx of the herbal samples exhibited the greater capacity to lower FBGC in HyGR than that of the AQx. ETHx of AAEH exhibited the highest capacity to lower FBGC in HyGR by 53.55 ± 1.04%, whereas AQx of EMCO exhibited the lowest capacity to lower FBGC, which corresponded to 36.19 ± 0.88%. **Conclusion:** The study showed that ETHx of the herbal samples were comparatively more potent than the corresponding AQx as agents of glycemic control and for the management of hyperglycemia. Furthermore, the combination of the herbal extracts synergistically improved the therapeutic potentials of the individual herbal extracts.

KEY WORDS: Aqueous extracts, ethanolic extracts, hyperglycemia, medicinal plants

INTRODUCTION

Diabetes mellitus (DM) is the most common serious metabolic disorder considered to be one of the five leading causes of death in the world [1,2]. Among several pathophysiologic indicators [3-5], DM is primarily characterized by hyperglycemia. The multiple etiologies, classifications and complications of DM have been described elsewhere [6-9].

The use of plant materials as sources of food, cosmetics, and medicine for the benefit of human and domestic animals is as old as the existence of mankind. More so, a recent survey showed that 80% of Africa population relies on traditional herbal remedies, often referred to as alternative or complementary medicine in the industrialized countries, for the alleviation of pathologic conditions [10]. Medicinal plants contain active principles known to ancient and modern civilizations, for their healing properties, before the advent of synthetic therapeutic organic compounds at the dawn of the 19th century.

Due to plant diversity, the available active principles in plant materials exhibit diverse variability in terms of their physicochemical properties and corresponding medicinal usefulness [6,11]. The phytochemical contents and medicinal usefulness of *Acanthus montanus* (ACMO), *Asystasia gangetica* (ASGA), *Emilia coccinea* (EMCO), and *Hibiscus rosasinensis* (HIRO) have been mentioned by several authors [12-19].

The present study was restricted to chemically induced DM using animal models as previously described [1,7,20,21], which depicted the pathophysiology of Type I DM, and the capacities of crude aqueous and ethanolic leaf extracts of ACMO, ASGA, EMCO, and HIRO, as well as their combinatorial formulation to ameliorate hyperglycemia in experimental rats. In addition, the administration of combinatorial herbal formulations to hyperglycemic rats (HyGR) was on the premise that herbal concoctions from different plant species or genera might serve to potentiate the efficacy of the herbal extracts toward mitigating hyperglycemia.

MATERIALS AND METHODS

Collection and Preparation of Herbal Samples

Fresh leaves of ACMO (Nees) T. Anderson, EMCO G. Don and HIRO L. were collected from uncultivated lands in Umuamacha Ayaba Umaeze, Osisioma Ngwa Local Government Area (LGA), Abia State, Nigeria, whereas fresh leaves of ASGA L. T. Anderson were collected from Ubowuala, Emekuku, Owerri North LGA, Imo State, Nigeria. The four herbs were identified and authenticated by Dr. M. Ibe, School of Agriculture and Agricultural Technology, Federal University of Technology, Owerri. All the leaves were collected between the months of July and August, 2014.

The leaves of individual plants were washed with a continuous flow of distilled water for 15 min and allowed to dry at laboratory ambient temperature (24 ± 5°C). A 500 g part of each herbal samples were weighted using a triple beam balance (OHAU 750-50: Burlington, NC, USA) and dried in an oven (WTC BINDER, 7200 Tuttlingen, Germany) at 60°C until a constant weight was achieved. The dried leaves were packaged in dark polyethylene bags and kept in a cold room (7 ± 3°C) for 24 h before pulverization. Next, the separate dried leaves were pulverized using Thomas-Willey milling machine (ASTM D-3182, INDIA), after which the ground samples were stored in air-tight plastic bottles with screw caps pending extraction.

Extraction of Herbal Samples

Preparation of ethanolic extracts (ETHx) of the four herbal samples was according to the methods previously described [19], whereas the corresponding aqueous extracts (AQx) was obtained according to the methods of Chikezie [22].

The separate extracts were reconstituted in phosphate buffered saline (PBS) solution (extract vehicle), osmotically equivalent to 100 g/L PBS (90.0 g NaCI, 17.0 g $Na_2HPO_4.2H_2O$, and 2.43 g $NaH_2PO_4.2H_2O$), before appropriated doses were administered to the experimental animals.

Experimental Animals

Male albino (Wistar) rats weighing between 150 g and 160 g were maintained at room temperature of 24 ± 5°C, 30-55% of relative humidity on a 12-h light/12-h dark cycle, with access to water and standard commercial feed (SCF) (Ewu Feed Mill, Edo State, Nigeria) *ad libitum* for 2 weeks acclimatization period. The handling of the animals was in accordance with the standard principles of laboratory animal care of the United States National Institutes of Health (NIH, 1978).

Induction of Diabetes/Experimental Design

Hyperglycemia was induced in the experimental rats by intraperitoneal injection of 0.1 mol/L alloxan monohydrate (Sigma, St. Louis, MO., USA) as previously described by Ojiako and Chikezie [9] with minor modification to the dose administered to the rats (dose = 120 mg/kg; bw). A total of 72 male Wistar rats were allotted into 12 groups of 6 rats each. The animals were deprived of food and water for additional 16 h before the commencement of treatment as described elsewhere [8]. The animal groups were designated on the basis of treatments received at regular intervals of 24 h for 14 days.

- NORM: Normoglycemic rats received SCF + water *ad libitum* + 1.0 mL/kg of PBS.
- DIAB: HyGR (Diabetic Control) received SCF + water *ad libitum* + 1.0 mL/kg of PBS.
- Hr-AQx-ACMO: HyGR received SCF + water *ad libitum* + AQx of ACMO (20 mg/kg in PBS; i.p.).
- Hr-ETHx-ACMO: HyGR received SCF + water *ad libitum* + ETHx of ACMO (20 mg/kg in PBS; i.p.).
- Hr-AQx-ASGA: HyGR received SCF + water *ad libitum* + AQx of ASGA (20 mg/kg in PBS; i.p.).
- Hr-ETHx-ASGA: HyGR received SCF + water *ad libitum* + ETHx of ASGA (20 mg/kg in PBS; i.p.).
- Hr-AQx-EMCO: HyGR received SCF + water *ad libitum* + AQx of EMCO (20 mg/kg in PBS; i.p.).
- Hr-ETHx-EMCO: HyGR received SCF + water *ad libitum* + ETHx of EMCO (20 mg/kg in PBS; i.p.).
- Hr-AQx-HIRO: HyGR received SCF + water *ad libitum* + AQx of HIRO (20 mg/kg in PBS; i.p.).
- Hr-ETHx-HIRO: HyGR received SCF + water *ad libitum* + ETHx of HIRO (20 mg/kg in PBS; i.p.).
- Hr-AQx-AAEH: HyGR received SCF + water *ad libitum* + combined dose (ratio: 1:1:1:1 *w/w*) of AQx of ACMO + ASGA + EMCO + HIRO (20 mg/kg in PBS; i.p.).
- Hr-ETHx-AAEH: HyGR received SCF + water *ad libitum* + combined dose (ratio: 1:1:1:1 *w/w*) of ETHx of ACMO + ASGA + EMCO + HIRO (20 mg/kg in PBS; i.p.).

Blood volumes of 0.5 mL were drawn from 12 h post-fasted rats at regular intervals of 24 h for 14 days and measured for fasting blood glucose concentration (FBGC).

Fasting Plasma Glucose Concentration

FBGC was measured by the glucose oxidase spectrophotometric method according to Randox® kit manufacturer's procedure (Randox® Laboratories Ltd. Ardmore, United Kingdom).

Percentage Reduction in Fasting Blood Glucose Concentrations of Hyperglycemic Rats

Relative reduction in FBGC of the HyGR within the 14-day treatment period was calculated as the quotient of the

difference between FBGCs at the commencement of the experiment (i.e., t = day 0) and at the end of the experiment (i.e., t = day 14) and FBGC at t = day 0; thus.

$$\%RFBGC = \frac{(FBGC_{(day0)} - FBGC_{(day14)})}{FBGC_{(day0)}} \times 100 \quad (1)$$

%RFBGC: Percentage reduction in fasting blood glucose concentrations.

Statistical Analysis

The results were expressed as mean ± standard error of the mean, and statistically analyzed by one-way ANOVA followed by Dunnett test, with the level of significance set at $P < 0.05$.

RESULTS

An overview of Table 1 showed that ETHx of the four herbal samples gave relatively higher yield (g%; *w/w* ratio) than the corresponding AQx. The average cumulative yield of the herbal extracts was AQx = 14.39 g%, whereas ETHx = 17.07 g%. Figure 1 showed that FBGC of normoglycemic rats (NORM group), within the experimental time of 14 days, was relatively constant and ranged between 3.80 ± 0.24 mM/L and 4.83 ± 0.45 mM/L; $P > 0.05$. In addition, the FBGC of the untreated HyGR (DIAB group; Diabetic Control) was significantly ($P < 0.05$) higher than that of the NORM group. Specifically, FBGC of the DIAB group was within the range of 15.10 ± 1.11 mM/L – 19.91 ± 1.53 mM/L [Figure 1].

Table 1: Percentage yields of aqueous and ETHx of herbal samples

Herbal samples	Yield g%; *w/w* ratio	
	AQx	ETHx
ACMO	14.86	16.35
ASGA	12.02	16.69
EMCO	16.14	17.99
HIRO	14.55	17.23

AQx: Aqueous extract; ETHx: Ethanolic extract; ACMO: *Acanthus montanus* (Nees) T. Anderson; ASGA: Asystasia gangetica L.T. Anderson; EMCO: *Emilia coccinea* (SIMS) G. Don; HIRO: *Hibiscus rosasinensis* L.

Hr-AQx-ACMO was hyperglycemic ([FBGC] > 11.00 mM/L) for 12 consecutive days but became normoglycemic when $t \geq 13$ days. However, FBGC of Hr-AQx-ACMO was significantly ($P < 0.05$) higher than that of the NORM group. Likewise, Hr-ETHx-ACMO was normoglycemic at $t \geq 8$ days and FBGC of Hr-ETHx-ACMO was significantly ($P < 0.05$) higher than that of the NORM group within the experimental time of 14 days.

Figure 2 showed that Hr-AQx-ASGA was normoglycemic at $t \geq 6$ days. Estimations showed that FBGCs of both Hr-AQx-ASGA and Hr-ETHx-ASGA declined by ≈1.94 folds on the 14th day. Furthermore, at the end of the experimental time, FBGCs of Hr-AQx-ASGA and Hr-ETHx-ASGA were significantly ($P < 0.05$) higher than that of the NORM group. Furthermore, Hr-ETHx-ASGA was normoglycemic at $t \geq 7$ days.

Within the experimental time of 14 days, FBGC of Hr-AQx-EMCO ranged between 16.91 ± 0.91 mM/L and 10.54 ± 0.79 mM/L, whereas FBGC of Hr-AQx-EMCO gave: 15.91 ± 0.83 mM/L - 9.00 ± 0.71 mM/L. Hr-AQx-EMCO was normoglycemic at $t \geq 10$ days, whereas Hr-ETHx-EMCO was normoglycemic at $t \geq 11$ days. At the end of the experimental time, FBGC of Hr-AQx-EMCO represented 36.20% reduction in circulating blood glucose concentration. By the same estimation, FBGC of Hr-ETHx-EMCO corresponded to 43.43% reduction in circulating glucose concentration. FBGCs of Hr-AQx-EMCO and Hr-ETHx-EMCO were significantly ($P < 0.05$) higher than that of the NORM group [Figure 3].

Figure 4 showed that Hr-AQx-HIRO and Hr-ETHx-HIRO were normoglycemic $t \geq 9$ days and $t \geq 6$ days, respectively.

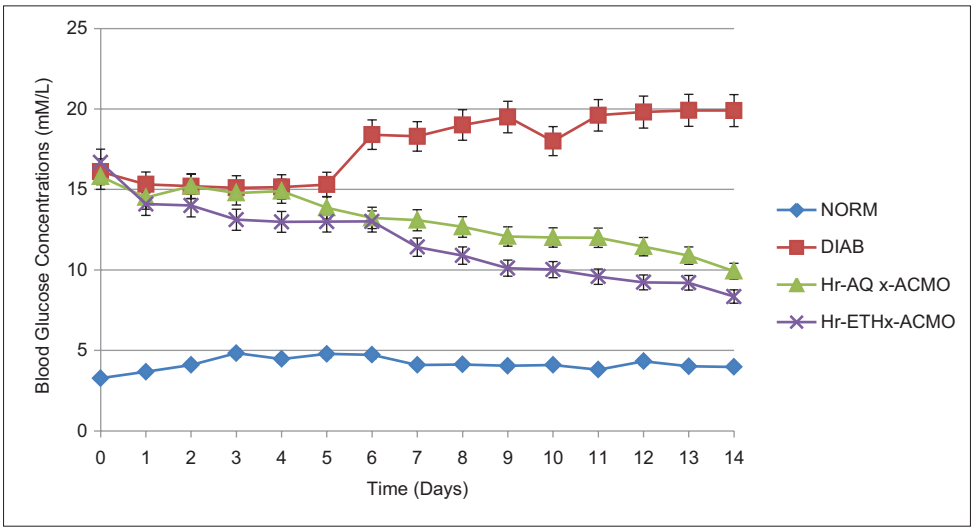

Figure 1: Comparative fasting blood glucose concentrations of normoglycemic, untreated hyperglycemic and hyperglycemic rats treated with aqueous and ethanolic extracts of *Acanthus montanus* (ACMO)

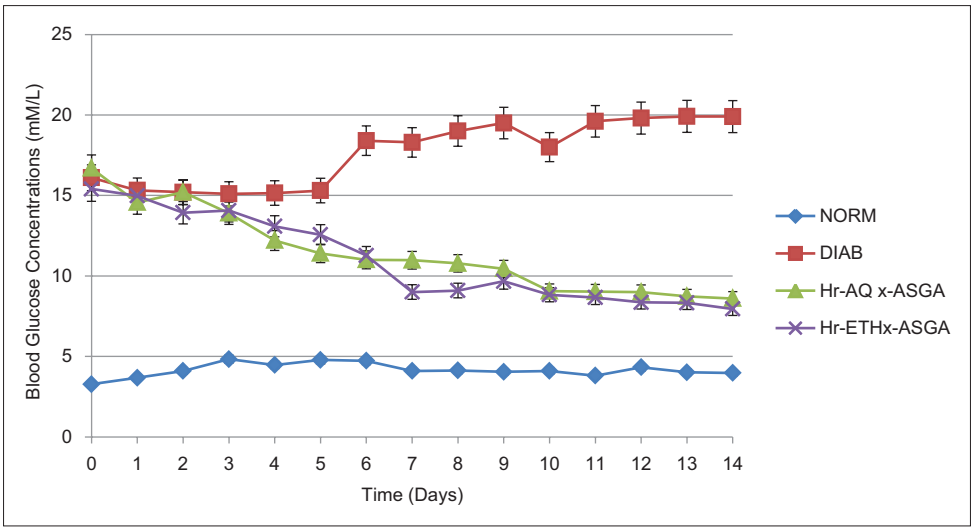

Figure 2: Comparative fasting blood glucose concentrations of normoglycemic, untreated hyperglycemic and hyperglycemic rats treated with aqueous and ethanolic extracts of *Asystasia gangetica* (ASGA)

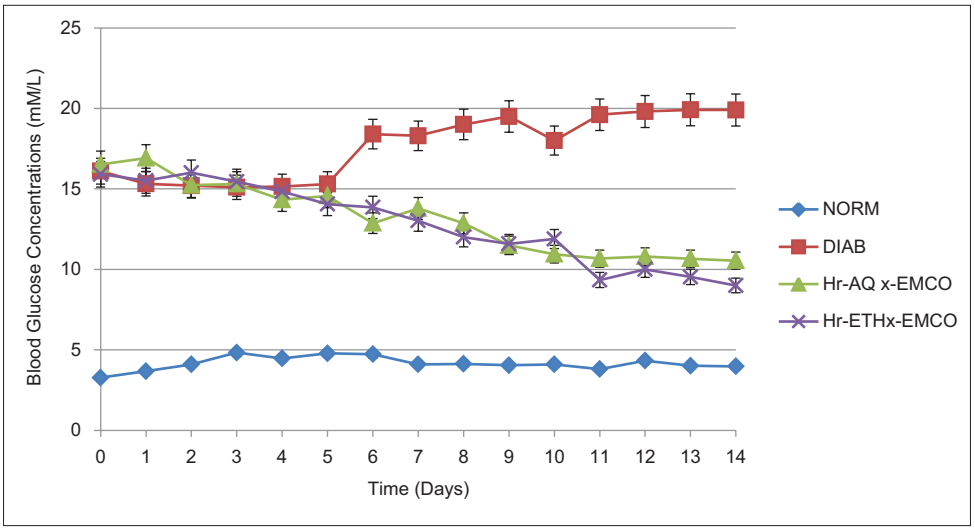

Figure 3: Comparative fasting blood glucose concentrations of normoglycemic, untreated hyperglycemic and hyperglycemic rats treated with aqueous and ethanolic extracts of *Emilia coccinea* (EMCO)

FBGC of Hr-AQx-HIRO declined by 1.53 folds on the 14th day, whereas that of Hr-ETHx-HIRO declined by 1.93 folds within the same experimental period. FBGCs of Hr-AQx-HIRO and Hr-ETHx-HIRO were significantly ($P < 0.05$) higher than that of the NORM group at $t = 14$ days.

Figure 5 showed that Hr-AQx-AAEH and Hr-ETHx-AAEH were normoglycemic at t ≥ 4 days. Reduction in FBGCs at t = 14 days were as follows: Hr-AQx-AAEH = 1.87 folds and Hr-ETHx-AAEH = 2.15 folds. In addition, a cursory view of Figure 5 showed that the lowest FBGC occurred at $t \geq 11$ days; thus, Hr-AQx-AAEH$_{[FBGC]}$ = 8.10 ± 0.08 mM/L and Hr-ETHx-AAEH$_{[FBGC]}$ = 6.88 ± 0.81 mM/L; $P > 0.05$. FBGCs of Hr-AQx-AAEH and Hr-ETHx-AAEH were significantly ($P < 0.05$) higher than that of the NORM group.

Finally, an overview of Figures 1-5 showed that AQx and ETHx of the herbal samples caused a reduction in FBGCs in HyGR in the order: ETHx > AQx.

The relative reduction in FBGC in HyGR following the administration of the herbal extracts within the 14-day treatment period is presented in Table 2. Specifically, Table 2 showed that the capacities of the herbal extracts to reduce FBGC in HyGR were in the following order: Hr-ETHx-AAEH > Hr-ETHx-ACMO > Hr-AQx-ASGA > Hr-ETHx-ASGA > Hr-ETHx-HIRO > Hr-AQx-AAEH > Hr-ETHx-EMCO > Hr-AQx-ACMO > Hr-AQx-HIRO > Hr-AQx-EMCO.

DISCUSSION

The present study showed that herbal extracts and its combinatorial formulations exhibited blood glucose lowering effect in HyGR, which conformed to previous reports [6,7,9,21,23, 24]. According to those reports, the capacity of plant extracts to ameliorate hyperglycemia in experimental

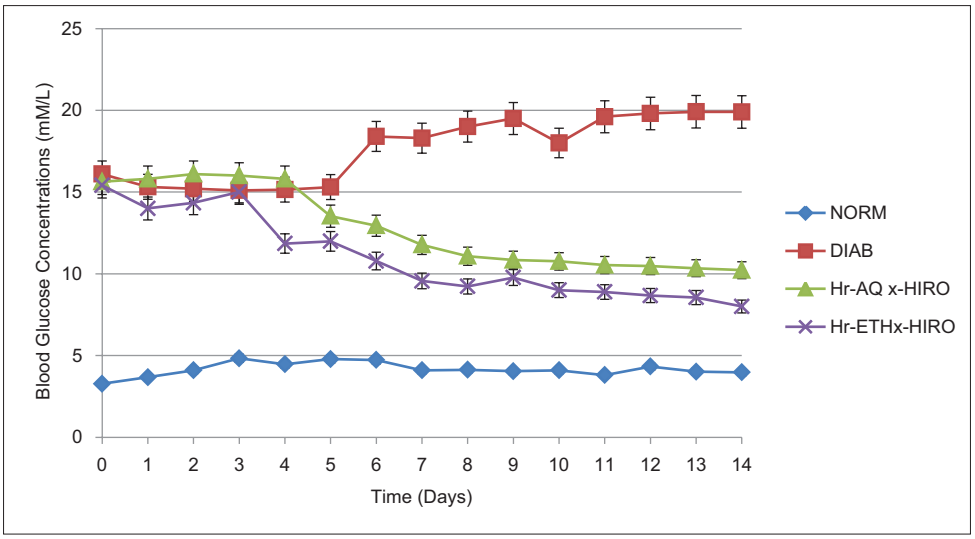

Figure 4: Comparative fasting blood glucose concentrations of normoglycemic, untreated hyperglycemic and hyperglycemic rats treated with aqueous and ethanolic extracts of Hibiscus rosasinensis (HIRO)

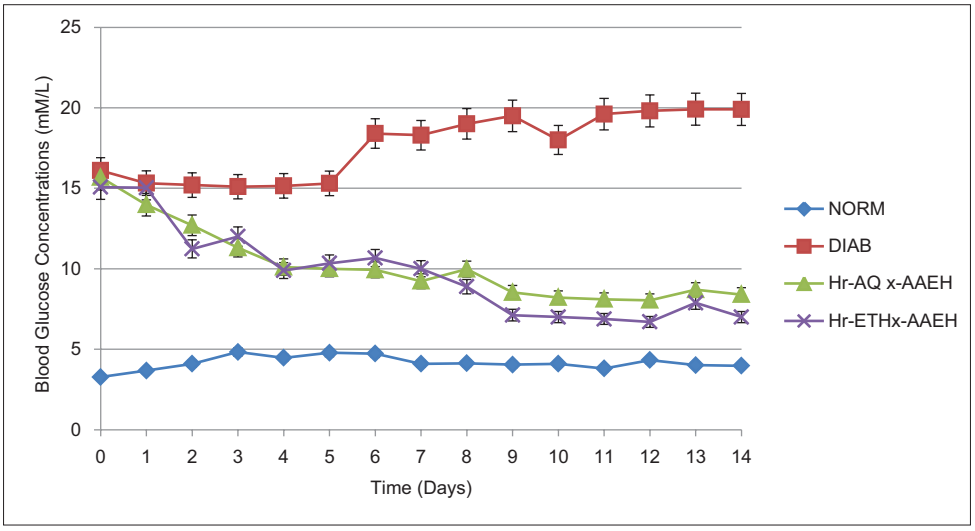

Figure 5: Comparative fasting blood glucose concentrations of normoglycemic, untreated hyperglycemic and hyperglycemic rats treated with aqueous and ethanolic extracts of combinatorial formulations of four herbal samples (AAEH)

animals was attributed to their phytochemical contents; notably the flavonoids, alkaloids, polyphenols, terpenoids, coumarins, and several other bioactive constituents, which in turn dictated the cumulative pharmacognostic potencies of the herbal formulations. Anti-diabetic bioactive principles exhibit a variety of biologic activities and therapeutic mode of actions that have been previously described [6,9,25]. Accordingly, the relatively high flavonoid content, in particular, in addition to the presence and mutual effects of variety of anti-diabetic phytochemicals in the herbal extracts [19] obviously contributed to their divergent capacities to exert hypoglycemic effect in the experimental rats as represented in the present study [Table 2].

Likewise, studies have shown that the absolute concentrations and available bioactive principles from herbal samples depended on the nature of solvent used in the extraction process, which among other experimental factors dictated the hypoglycemic potency of the medicinal preparations [1,25,26]. The results of the present study showed that EHx of the herbal samples was comparatively more efficacious than their corresponding AQx as an agent of glycemic control and for the management of hyperglycemia.

The comparative capacity of the combinatorial herbal extracts to exert high glycemic control in HyGR, as exemplified by Hr-ETHx-AAEH, appears to suggest synergy among the anti-diabetic bioactive principles of the component herbal extracts. Previous reports have confirmed that combinations of different herbal extracts in solution altered the biologic and pharmacologic properties of the constituent bioactive principles as a result of inter-phytochemical interactions [27,28].

Table 2: Percentage reduction in fasting blood glucose concentrations of HyGR administered with herbal extracts within experimental time (0 day ≥ *t* ≥14 days)

Rat groups	Reduction in FBGC (%)
Hr-AQx-ACM0	$37.21\pm0.75^{f,g,h}$
Hr-ETHx-ACM0	$49.97\pm1.05^{a,b}$
Hr-AQx-ASGA	$48.47\pm1.01^{a,b,c}$
Hr-ETHx-ASGA	$48.37\pm0.93^{a,b,c,d}$
Hr-AQx-EMC0	$36.19\pm0.88^{f,g,h,i,j}$
Hr-ETHx-EMC0	$43.43\pm0.95^{b,c,d,e,f,g}$
Hr-AQx-HIR0	$36.61\pm0.88^{f,g,h,i}$
Hr-ETHx-HIR0	$48.09\pm1.05^{a,b,c,d,e}$
Hr-AQx-AAEH	$46.43\pm0.98^{a,b,c,d,e,f}$
Hr-ETHx-AAEH	53.55 ± 1.04^{a}

The mean (X)±SD of six (n=6) determinations. Means in the column with the same letter are not significantly different at $P>0.05$,
SD: Standard deviation, HyGR: Hyperglycemic rats,

CONCLUSION

In that regard, results of the present study showed that the combination of the various herbal extracts synergistically improved the therapeutic potentials of the individual herbal extracts as agents of glycemic control. Furthermore, the study showed that ETHx of the herbal samples was comparatively more potent than the corresponding AQx as agents of glycemic control and for the management of hyperglycemia. Nevertheless, the aqueous and ethanolic leaf extracts of the four medicinal plants and their combinatorial formulations, in the present crude form, did not restore full therapeutic benefits to the HyGR within the experimental time.

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REFERENCES

1. Nagappa AN, Thakurdesai PA, Venkat Rao N, Singh J. Antidiabetic activity of *Terminalia catappa* Linn fruits. J Ethnopharmacol 2003;88:45-50.
2. Prabakaran D, Ashokkumar N. Protective effect of esculetin on hyperglycemia-mediated oxidative damage in the hepatic and renal tissues of experimental diabetic rats. Biochimie 2013;95:366-73.
3. Ugochukwu NH, Babady NE. Antihyperglycemic effect of aqueous and ethanolic extracts of *Gongronema latifolium* leaves on glucose and glycogen metabolism in livers of normal and streptozotocin-induced diabetic rats. Life Sci 2003;73:1925-38.
4. Gül N, Cebesoy S, Özsoy N. Lectins binding during alloxan-induced diabetes in rat soleus muscle. Afr J Biotechnol 2008;7:926-30.
5. Jung UJ, Park YB, Kim SR, Choi MS. Supplementation of persimmon leaf ameliorates hyperglycemia, dyslipidemia and hepatic fat accumulation in type 2 diabetic mice. PLoS One 2012;7:e49030.
6. Patel DK, Prasad SK, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pac J Trop Biomed 2012;2:320-30.
7. Mungle AN, Bodhankar NM, Chandak KK. Antidiabetic potential of *Dolichandrone falcata* leaves in alloxan induced diabetic rats. Int J Res Pharm Biomed Sci 2012;3:319-24.
8. Ibegbulem CO, Chikezie PC. Hypoglycemic properties of ethanolic extracts of *Gongronema latifolium*, *Aloe perryi, Viscum album* and *Allium sativum* administered to alloxan-induced diabetic albino rats (*Rattus norvegicus*). Pharmacogn Commun 2013;3:12-6.
9. Ojiako AO, Chikezie PC. Comparative proximate composition and hypoglycemic properties of three medicinal plants (*Veronia amygdalina, Azadirachta indica and Moringa oleifera*). Pharmacogn Commun 2014;4:40-8.
10. Available from: http://www.who.int/medcentre/factsheets/2003/fs134/en/. [Last accessed on 2015 Jan 01].
11. Tung CW, Lin YC, Chang HS, Wang CC, Chen IS, Jheng JL, *et al.* TIPdb-3D: The three-dimensional structure database of phytochemicals from Taiwan indigenous plants. Database (Oxford) 2014;2014.
12. Okoli CO, Akah PA, Onuoha NJ, Okoye TC, Nwoye AC, Nworu CS. *Acanthus montanus:* An experimental evaluation of the antimicrobial, anti-inflammatory and immunological properties of a traditional remedy for furuncles. BMC Complement Altern Med 2008;8:27.
13. Kumar V, Singh P, Chander R, Mahdi F, Singh S, Singh R, *et al.* Hypolipidemic activity of *Hibiscus rosa sinensis* root in rats. Indian J Biochem Biophys 2009;46:507-10.
14. Kumar S, Narwal S, Kumar V, Prakash O. A-glucosidase inhibitors from plants: A natural approach to treat diabetes. Pharmacogn Rev 2011;5:19-29.
15. Rotimi SO, Omotosho OE, Rotimi OA. Persistence of acidosis in alloxan-induced diabetic rats treated with the juice of *Asystasia gangetica* leaves. Pharmacogn Mag 2011;7:25-30.
16. Kensa VM. Studies on phytochemical profile and antimicrobial activity on *Asystasia gangetica* (L.) T. Anderson. Plant Sci Feed 2011;1:112-7.
17. Ukwe VC, Ubaka CM. Hypoglycemic activity of leaves of *Acanthus montanus* T. Anderson (Acanthaceae) in rats. Int J Diabetes Dev Ctries 2011;31:32-6.
18. Gopal TK, Megha G, Chamundeeswari D, Reddy CU. Phytochemical and pharmacological studies on whole plant of *Asystasia gangetica*. Indian J Res Pharm Biotechnol 2013;1:365-70.
19. Ojiako AO, Chikezie PC, Ogbuji CA. Radical scavenging potentials of single and combinatorial herbal formulations *in vitro*. J Tradit Complement Med 2015;DOI:10.1016/j.jtcme.2014.11.037. [In press].
20. Ahmed MF, Kazim SM, Ghori SS, Mehjabeen SS, Ahmed SR, Ali SM, *et al.* Antidiabetic Activity of *Vinca rosea* Extracts in Alloxan-Induced Diabetic Rats. Int J Endocrinol 2010;2010:841090.
21. Yeap SK, Mohd Ali N, Mohd Yusof H, Alitheen NB, Beh BK, Ho WY, *et al.* Antihyperglycemic effects of fermented and nonfermented mung bean extracts on alloxan-induced-diabetic mice. J Biomed Biotechnol 2012;2012:285430.
22. Chikezie PC. Sodium metabisulfite-induced polymerization of sickle cell hemoglobin incubated in the extracts of three medicinal plants (Anacardium occidentale, Psidium guajava, and *Terminalia catappa*). Pharmacogn Mag 2011;7:126-32.
23. Elekofehintia OO, Kamdemb JP, Kadec IJ, Rochab JB, Adanlawod IG. Hypoglycemic, antiperoxidative and antihyperlipidemic effects of saponins from *Solanum anguivi* Lam. fruits in alloxan-induced diabetic rats. South Afr J Bot 2013;88:56-61.
24. Koneri RB, Samaddar S, Ramaiah CT. Antidiabetic activity of a triterpenoid saponin isolated from *Momordica cymbalaria* Fenzl. Indian J Exp Biol 2014;52:46-52.
25. Chikezie PC, Iheanacho KM. Comparative hypoglycemic property of aqueous and ethanolic extracts of *Viscum album* (Mistletoe) and their effects on body and organ weights of diabetic rats (*Rattus norvegicus*). Pharmacogn Commun 2014;4:13-9.
26. Pierre W, Gildas AJ, Ulrich MC, Modeste WN, Benoît NT, Albert K. Hypoglycemic and hypolipidemic effects of *Bersama engleriana* leaves in nicotinamide/streptozotocin-induced type 2 diabetic rats. BMC Complement Altern Med 2012;12:264.
27. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am J Clin Nutr 2003;78:517S-20S.
28. Jain DP, Pancholi SS, Patel R. Synergistic antioxidant activity of green tea with some herbs. J Adv Pharm Technol Res 2011;2:177-83.

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Original Research

ScopeMed

Effects of *Gingko biloba* extract on tissue distribution of fluoxetine and venlafaxine in rats

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ABSTRACT

Objective: There are many concerns about the interactions of herbal products with conventional drugs, which are mostly used as multiple drug treatment approach. The present study was designed to evaluate the effect of long-term use of *Ginkgo biloba* extract (GK) on the absorption and tissue distribution of fluoxetine and venlafaxine. **Materials and Methods:** 46 Wistar rats are utilized and allocated into 8 groups; 2 groups administered the vehicle and saved as control; 4 groups are treated with 100 and 200 mg/kg of GK extract for 30 days; 2 groups are treated with 40 mg/kg verapamil for 10 days. The liver, kidney, and brain distribution of fluoxetine and venlafaxine were evaluated after single oral doses using high performance liquid chromatographic method. **Results:** 200 mg/kg GK increases fluoxetine concentrations in all studied organs, while GK 100 mg/kg increases venlafaxine levels in kidney tissue and not affected in the other two organs. **Conclusion:** Thirty days treatment with GK (100 mg/kg) increases kidney availability of venlafaxine, while 200 mg GK dose increases fluoxetine availability in the liver, kidney, and brain tissues after single oral doses.

KEY WORDS: Absorption, fluoxetine, *Ginkgo biloba*, tissue distribution, venlafaxine

INTRODUCTION

Absorption of drug molecules from oral dosage forms represents one of the important functions of the gastrointestinal tract (GIT); however, its barrier functions are also responsible for restricting absorption of many xenobiotics including drugs [1]. Availability of metabolizing enzyme systems and effective transporters, especially P-glycoprotein (P-gp), in the GIT epithelium protects many sensitive tissues and organs against invasion by many xenobiotics [2], in addition to the secretion of various metabolites into the GI lumen [3,4]. The safety and efficacy of many drugs depend on many variables involved in their interactions with the components of the biological system; such interactions may influence pharmacokinetic properties of introduced drugs, including their affinity for the available membrane transporters, with consequent changes in absorption, distribution, and elimination processes [5]. After absorption, distribution of drug molecules between tissues and organs depends on many variables; among them are regional blood flow, epithelial permeability, and protein binding. Moreover, the extent of transporter's expression plays a major role in this respect, especially for specific selective ligands [6]. The barrier and transport functions of these membrane transporters can be negatively or positively influenced by many ligands, including natural herb preparations like *G. biloba* extract (GK) [7,8]. Although the influence of GK on the absorption and distribution of many drugs is not extensively evaluated, the overall capacity of small doses of this extract to modify the functions of drug transporters shows conflicting results [9,10]. Such type of interference, when occurs, may change the pharmacokinetic behavior of many therapeutic agents, which may predispose to fatal consequences, especially in those with low therapeutic index or those used for treatment of disorders in the central nervous system. The present study aims to evaluate the effects of long-term administration of GK on the absorption and tissue distribution of fluoxetine and venlafaxine after single oral doses in rats.

MATERIALS AND METHODS

A total of 46 adult male of Wistar rats (150-200 g) were used in the study. The animals were breaded in the Animal House, College of Pharmacy, University of Baghdad, and housed in the Animal House of the College of Medicine, University of Babylon. They were kept in a polypropylene cages at controlled temperature (25 ± 2°C), and were allowed to acclimatize for at least 1-week prior to the experiment. They had free access to commercial pellet diet and water *ad libitum*. The research protocol was approved by the Local Research Ethics Committee of the College of Pharmacy, University of Baghdad, and in accordance with International Requirements of the Experimental Animal Research. The rats were randomly allocated into 8 Groups, 6 rats in each group (except verapamil - treated group), and treated as follow [Figure 1]: Two groups were given 5% carboxymethylcellulose (CMC; Beijing Shouke Hongtai Chem Technol Co, China) (the vehicle) orally by oral gavage tube for 30 days; and then given a single oral doses of fluoxetine (20 mg/kg) or venlafaxine (20 mg/kg) (Hefei Joye Import Export Co., China) which served as control groups; 2 Groups were given GK (100 mg/kg/day) (premier-health, UK) suspended in 5% CMC orally by gavage tube for 30 days, and then given single doses of fluoxetine (20 mg/kg) or venlafaxine (20 mg/kg) orally, while other two groups of rats were treated similarly except for the GK dose, which was 200 mg/kg/day. In the last two groups (5 rats each), the rats were given 40 mg/kg/day of verapamil (Abbott, UK) orally by gavage tube for 10 days [11], and then given single doses of fluoxetine (20 mg/kg) or venlafaxine (20 mg/kg) orally. All animals were sacrificed after 5.5 and 2.0 h after administration of the fluoxetine [12] and venlafaxine doses [13], respectively. Blood samples were aspirated directly from the heart into polyethylene tube, and left to clot, centrifuged at 10000 rpm for 20 min. The resulted serum was kept frozen (−40°C) until the time of drugs analysis. Livers and both kidneys were quickly removed, and perfused with ice-cooled saline (4°C) to remove the blood. After decapitation, the whole brain was carefully removed, rinsed in saline at 4°C, and the arachnoid membrane was carefully removed. 500 mg of liver tissue and 300 mg of kidney and brain tissues were excised, and homogenized with Teflon-head homogenizer. The tissue homogenates were stored in deep freeze (−80°C) to be used for drugs analysis.

Determination of Serum Fluoxetine and Venlafaxine

A volume of 50 μl aliquot of serum was deproteinized with a 100 μl aliquot of acetonitrile. After vortex mixing and centrifugation (16,000 rpm, 10 min), 30 μl of the supernatant was injected directly into a reversed-phase (C18) high performance liquid chromatographic (HPLC) column.

Measurements of Fluoxetine and Venlafaxine Levels in Brain, Kidney, and Liver Tissues

The tissue homogenate of each organ was deproteinized with 400 μl of acetonitrile and 500 μl of methanol, centrifuged, filtered, and evaporated to dryness at 45°C using vacuum oven. The resulted dry material was reconstituted with 100 μl of the mobile phase, and centrifuged again. Then 30 μl of the obtained supernatant was directly injected into the HPLC (Knauer, Germany). The samples were not diluted before HPLC measurement. Unknown concentrations of fluoxetine and venlafaxine were interpolated from a respective standard curves prepared previously [14].

Statistical Analysis

All values, which represent the tissue/serum ratio of drug concentrations, were expressed as mean ± standard deviation; the results were statistically evaluated using unpaired Student's *t*-test and one-way Analysis of Variance, supported by Bonferroni's *post hoc* analysis. Values with $P < 0.05$ were considered significantly different. Analysis was performed using graph pad prism software for Windows, version 5.0 (Graph Pad Software, Inc., San Diego, CA).

RESULTS

Figure 2 shows that the effect of long-term administration of GK (200 mg/kg) on the distribution of fluoxetine in the liver tissue was significantly higher than that produced by the vehicle, verapamil (40 mg/kg) and GK (100 mg/kg), respectively. Meanwhile, there were no significant differences between the effects of verapamil and 100 mg/kg GK on the distribution of fluoxetine in the liver tissue ($P > 0.05$). Figure 3 shows that

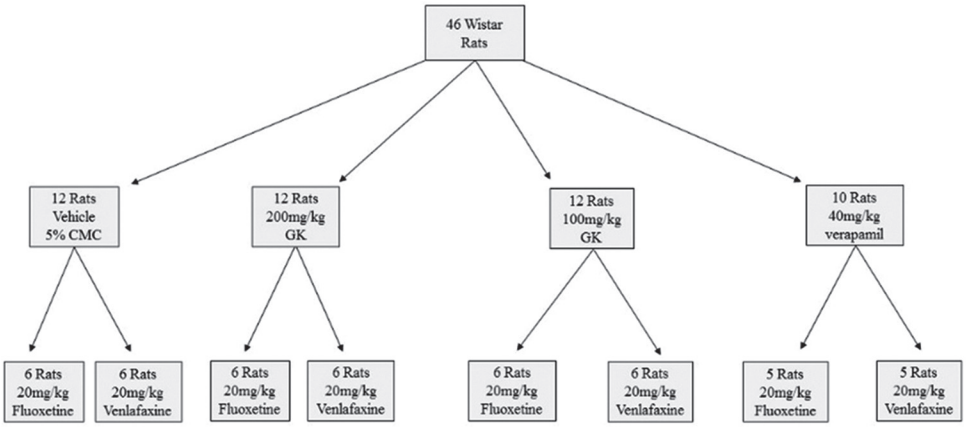

Figure 1: Flow chart of study design and animal groups

the effects of GK (200 mg/kg) and verapamil (40 mg/kg) on the distribution of fluoxetine in kidney tissue were significantly higher than that produced by the vehicle and 100 mg/kg GK, respectively, and no significant differences reported between the effects of GK (100 mg/kg) and the vehicle in this organ ($P > 0.05$). Moreover, Figure 4 shows that the effect of GK (200 mg/kg) on the distribution of fluoxetine in the brain tissue was significantly higher than that produced by the administration of the vehicle alone or 100 mg/kg GK. Meanwhile, the effect of verapamil was significantly higher than that reported in the vehicle-treated animals or 100 mg/kg GK. In Figure 5, no significant difference reported between the effect of GK (100 and 200 mg/kg) and verapamil on the distribution of venlafaxine in the liver tissue ($P > 0.05$). In addition, Figure 6 shows that the effects of GK (100 mg/kg) on the distribution of venlafaxine in the kidney tissue was significantly higher than that produced by either the vehicle alone, verapamil (40 mg/kg) or GK (200 mg/kg). Meanwhile, both verapamil (40 mg/kg) and GK (200 mg/kg) significantly decreased the extent of venlafaxine distribution, compared with that produced by the vehicle alone. There was no significant difference ($P > 0.05$) between the effect of verapamil (40 mg/kg) and GK (200 mg/kg) in this respect. Figure 7 shows that the effect of verapamil (40 mg/kg) on venlafaxine distribution in the brain tissue was significantly higher than that produced by either the vehicle alone or the two doses of GK. Meanwhile, there was no significant difference between the effects of the two doses of GK on the distribution of venlafaxine in brain tissue.

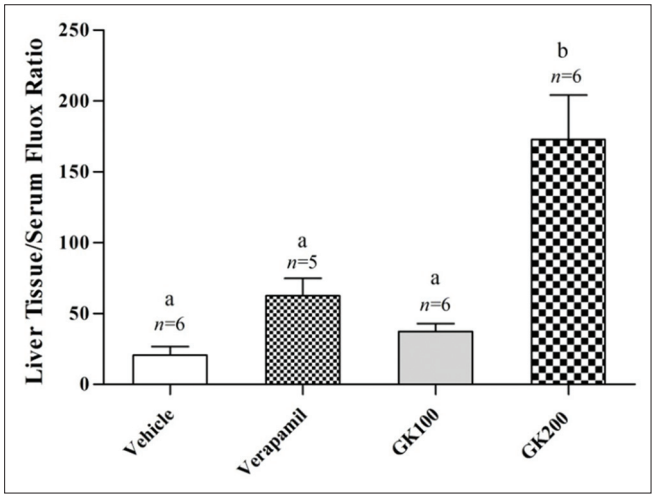

Figure 2: Effect of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the liver tissue/serum ratio of fluoxetine in rats challenged with single oral dose of fluoxetine (20 mg/kg); *n*: Number of animals; values with non-identical letters (a,b) are significantly different ($P < 0.05$)

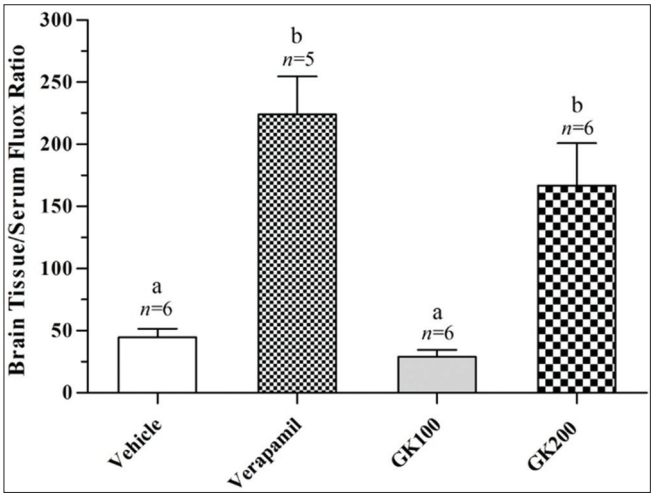

Figure 4: Effect of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the brain tissue/serum ratio of fluoxetine in rats challenged with single oral dose of fluoxetine (20 mg/kg); *n*: Number of animals; values with non-identical letters (a,b) are significantly different ($P < 0.05$)

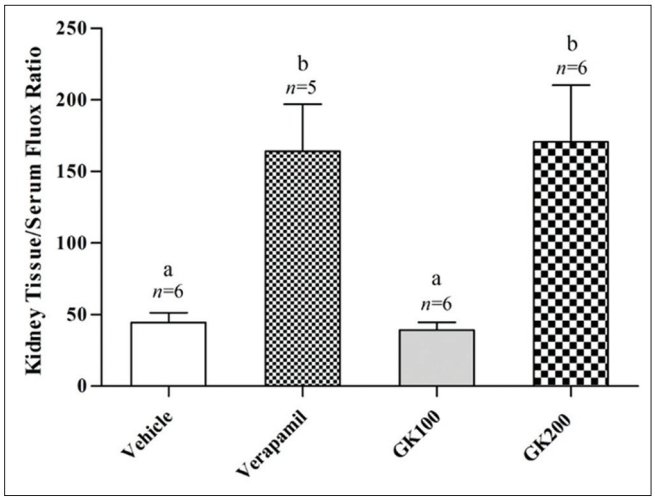

Figure 3: Effect of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the kidney tissue/serum ratio of fluoxetine in rats challenged with single oral dose of fluoxetine (20 mg/kg); *n*: Number of animals; values with non-identical letters (a,b) are significantly different ($P < 0.05$)

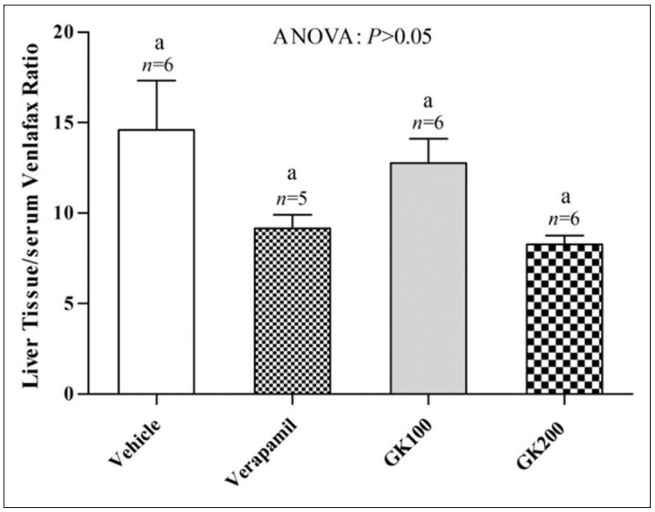

Figure 5: Effect of long-term administration of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the liver tissue/serum ratio of venlafaxine in rats challenged with single oral dose of venlafaxine (20 mg/kg); *n*: Number of animals; values with non-identical letters are significantly different ($P < 0.05$)

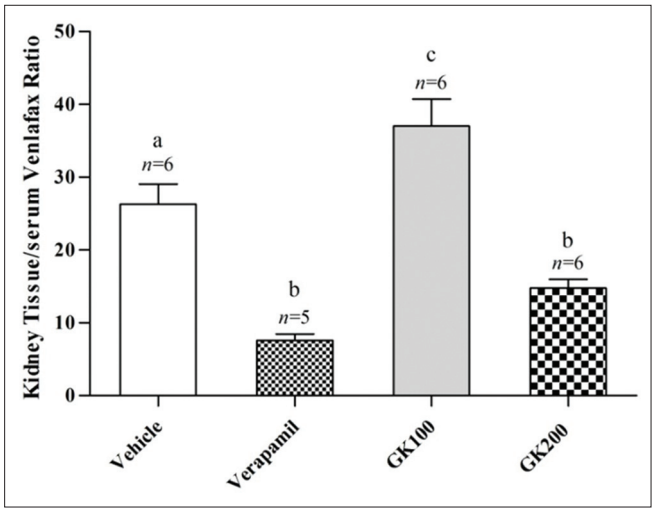

Figure 6: Effect of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the kidney tissue/serum ratio of venlafaxine in rats challenged with single oral dose of venlafaxine (20 mg/kg); *n*: Number of animals; values with non-identical letters (a-c) are significantly different ($P < 0.05$)

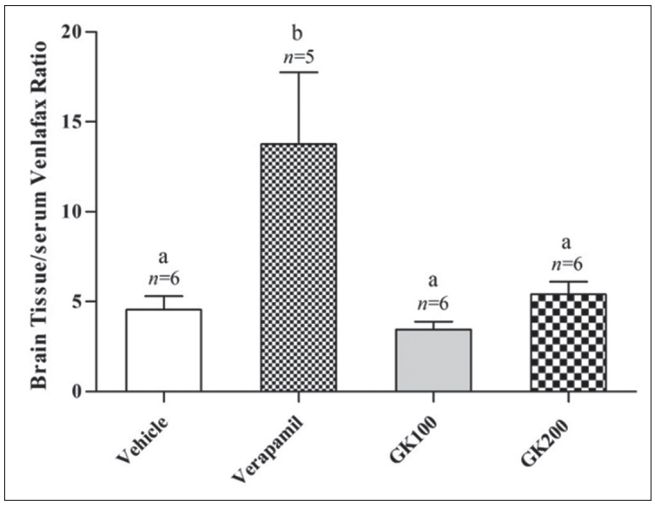

Figure 7: Effect of long-term administration of *Ginkgo biloba* extract (100 and 200 mg/kg) and verapamil (40 mg/kg) on the brain tissue/serum ratio of venlafaxine in rats challenged with single oral dose of venlafaxine (20 mg/kg); *n*: Number of animals; values with non-identical letters (a,b) are significantly different ($P < 0.05$)

DISCUSSION

Many animal studies, which investigate the expected interactions of GK with synthetic drugs, recognized the importance of justifying whether interference with tissue transporters by GK is relevant to be considered during clinical trials or not; particularly in drugs such as fluoxetine and venlafaxine, which are potential ligands for many affected transporters such as P-gp or others. The inhibition of transport activities *in vitro* can be extrapolated to the herb-drugs interaction potential *in vivo*, with effective high serum concentration, high tissue/serum fraction, and may be accompanied by exaggerated pharmacodynamic activity [15]. However, extracts obtained from *G. biloba* consist of many compounds, and this make the identification of a single effective constituent of these extracts and their plasma concentration a little bit difficult. Therefore, we compare the *in vivo* GK-drugs interaction potential in rats with that produced by verapamil, a simultaneous substrate for the expected drug transporters. In the present study, GK significantly increases liver tissue concentration of fluoxetine, while the tissue/serum ratio in brain and kidney are not significantly changed. Elevated hepatic level of fluoxetine may be attributed to the inhibitory effect of GK on P-gp in the liver tissue that increases its hepatic uptake. This finding was in tune with that reported by Hellum and Nilsen (2007), who indicated that GK is the most potent inhibitor of P-gp activity, among the tested herbs in their investigation [8]. Meanwhile, other finding contradicts the results of the present study. Li *et al*. (2009) found that GK increases P-gp gene expression in primary human hepatocytes, where the systemic availability of simvastatin is reduced secondarily to GK-mediated P-gp induction [16]. The reported data in the present study indicate that the low dose of *G. biloba* extract may have a mild inhibitory effect on the P-gp transporter activity. Moreover, increasing GK dose significantly increases in the tissue/serum ratio of fluoxetine in all the studied organs. These data indicate that GK may have a potent inhibitory effect on P-gp activity at high dose, which may lead to enhance fluoxetine uptake in all the studied organs. Other researchers studied the effect of acute and chronic use of GK on the P-gp function, without specific emphasis on the dose-response relationship in this respect. Yang *et al*. (2006) reported that GK decreases cyclosporine oral bioavailability in rats, due to increased expression of P-gp in the small intestine after long-term exposure [17]. This finding apparently contradicts the results obtained in our study. However, the effect of *G. biloba* might be similar to that reported for St. John's Wort. It reflects inhibition at short-term (acute exposure) *in vitro* and *in vivo*, and induction *in vivo*, only after a long-term administration (repeated exposure) [9,18]. Similar finding was reported for the interaction of many pure polyphenols, some of them are constituents of GK, with the absorption and distribution of metformin (P-gp ligand), while atenolol (not P-gp ligand) was not affected [19]. In the present study, tissue/serum ratios of venlafaxine were increased significantly in the kidney tissue, while not significantly changed in the liver and brain tissues. Since venlafaxine is a known P-gp ligand, its elevated concentration in the kidney tissue might be due to the inhibitory effect of GK on this efflux transporter (in the apical regions of the vascular epithelium). This finding comes in tune with that reported by Bent (2008), who indicates that GK is the most potent inhibitor of P-gp activity among the natural products that are tested in his investigation [8]. In addition, we previously reported that 200 mg/kg of GK significantly increases venlafaxine concentrations in the serum [20], and associated with significant decrease in the tissue/serum ratio in the liver and kidney tissues, as shown in the present study. This can be explained on the bases that the elevated serum levels might be attributed to the potent inhibitory effect of GK on the intestinal P-gp, which leads to increased venlafaxine absorption. Although the present study demonstrates well the significant influence of GK on absorption and tissue distribution of fluoxetine and venlafaxine in rats, such activity may not be necessarily observed in human. This idea is in accordance with many reports in this respect, and

human studies in this field are highly suggested [21,22]. The inhibitory effect of GK on the activity of P-gp, which expel out drug molecules, may enhance liver and kidney uptake of venlafaxine, and elevates its concentration significantly in the studied organs. The elevation in serum concentration of venlafaxine is higher than that reported in the liver and kidney tissues, and this may explain the significant decrease in tissue/serum ratio in these organs. Since drug transporters can play pivotal roles in drug handling within the biological system, many efforts during drug discovery and development process are directed to synthesize or isolate new chemical compounds, which interfere or not with P-gp, depending on the desired type of interaction. Accordingly, P-gp inhibitors are developed to improve penetration of drug molecules into neoplastic cells and optimize chemotherapy. Similarly, this approach is also followed to facilitate penetration of CNS-active drugs into the brain tissue to overcome the pharmacoresistance associated with many CNS disorders (e.g., Parkinson's disease). Meanwhile, many chemicals that are P-gp ligands should be identified early during the stage of development. At this level, it is important to consider the suggested idea that expression of drug transporters and CYP enzymes might be functionally linked to limit oral drug bioavailability [23]. In the intestine most of the interactions that involve drug transporters, and responsible for the efflux of drugs, often result in poor absorption and low oral bioavailability. Since the majority of drugs are developed as oral dosage forms, the use of animal models to evaluate their interactions at that site have become critical tools, for assessing a drug's potential *in vivo* absorption properties in the presence of expected modulators of membrane transporters.

CONCLUSION

30 days treatment with *G. biloba* extract (100 mg/kg) increases kidney availability of venlafaxine, while 200 mg dose increases fluoxetine availability in the liver, kidney, and brain tissues after single oral doses.

REFERENCES

1. Terada T, Hira D. Intestinal and hepatic drug transporters: Pharmacokinetic, pathophysiological, and pharmacogenetic roles. J Gastroenterol 2015;50:508-19.
2. Laksitorini M, Prasasty VD, Kiptoo PK, Siahaan TJ. Pathways and progress in improving drug delivery through the intestinal mucosa and blood-brain barriers. Ther Deliv 2014;5:1143-63.
3. Sharom FJ. The P-glycoprotein multidrug transporter. Essays Biochem 2011;50:161-78.
4. Ma JD, Tsunoda SM, Bertino JS Jr, Trivedi M, Beale KK, Nafziger AN. Evaluation of *in vivo* P-glycoprotein phenotyping probes: A need for validation. Clin Pharmacokinet 2010;49:223-37.
5. Drozdzik M, Gröer C, Penski J, Lapczuk J, Ostrowski M, Lai Y, *et al.* Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine. Mol Pharm 2014;11:3547-55.
6. Chillistone S, Hardman J. Factors affecting drug absorption and distribution. Anaesth Intensive Care Med 2011;12:151-5.
7. Abad MJ, Bedoya LM, Bermejo P. An update on drug interactions with the herbal medicine *G. biloba*. Curr Drug Metab 2010;11:171-81.
8. Hellum BH, Nilsen OG. *In vitro* inhibition of CYP3A4 metabolism and P-glycoprotein-mediated transport by trade herbal products. Basic Clin Pharmacol Toxicol 2008;102:466-75.
9. Pal D, Mitra AK. MDR- and CYP3A4-mediated drug-herbal interactions. Life Sci 2006;78:2131-45.
10. Fan L, Tao GY, Wang G, Chen Y, Zhang W, He YJ, *et al.* Effects of *G. biloba* extract ingestion on the pharmacokinetics of talinolol in healthy Chinese volunteers. Ann Pharmacother 2009;43:944-9.
11. Kirchengast M. Preclinical considerations and results with the combination of verapamil and trandolapril: Blood pressure reduction and beyond. J Hypertens 1997;15:S27-33.
12. Keller T, Cambon N, Genevray M, Crivelli F, Crivelli M, Dal BL, *et al.* Bioequivalence study of fluoxetine hydrochloride in healthy volunteers. Arzneimittelforschung 2005;55:491-7.
13. Goodnick PJ. Pharmacokinetic optimisation of therapy with newer antidepressants. Clin Pharmacokinet 1994;27:307-30.
14. Eswarudu MM, Anitha M, Gayathri N, Chaithanya T. A validated RP-HPLC method for the simultaneous estimation of fluoxetine hydrochloride and olanzapine in pharmaceutical dosage form. Int Res J Pharm 2012;3:310-3.
15. Dresser MJ, Gray AT, Giacomini KM. Kinetic and selectivity differences between rodent, rabbit, and human organic cation transporters (OCT1). J Pharmacol Exp Ther 2000;292:1146-52.
16. Li L, Stanton JD, Tolson AH, Luo Y, Wang H. Bioactive terpenoids and flavonoids from *G. biloba* extract induce the expression of hepatic drug-metabolizing enzymes through pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor-mediated pathways. Pharm Res 2009;26:872-82.
17. Yang CY, Chao PD, Hou YC, Tsai SY, Wen KC, Hsiu SL. Marked decrease of cyclosporin bioavailability caused by coadministration of ginkgo and onion in rats. Food Chem Toxicol 2006;44:1572-8.
18. Dürr D, Stieger B, Kullak-Ublick GA, Rentsch KM, Steinert HC, Meier PJ, *et al.* St John's Wort induces intestinal P-glycoprotein/MDR1 and intestinal and hepatic CYP3A4. Clin Pharmacol Ther 2000;68:598-604.
19. Hussain SA, Jaccob AA. Effects of long-term use of polyphenols on the absorption and tissue distribution of orally administered metformin and atenolol in rats. J Intercult Ethnopharmacol 2013;2:147-54.
20. AlzubaidiFA, Hussain SA. Effects of *G. biloba* extract on the oral bioavailability of fluoxetine and venlafaxine in rats. Am J Pharm Sci 2015;3:7-12.
21. Song IS, Kong TY, Jeong HU, Kim EN, Kwon SS, Kang HE, *et al.* Evaluation of the transporter-mediated herb-drug interaction potential of DA-9801, a standardized dioscorea extract for diabetic neuropathy, in human *in vitro* and rat *in vivo*. BMC Complement Altern Med 2014;14:251.
22. Ji HY, Liu KH, Kong TY, Jeong HU, Choi SZ, Son M, *et al.* Evaluation of DA-9801, a new herbal drug for diabetic neuropathy, on metabolism-mediated interaction. Arch Pharm Res 2013;36:1-5.
23. Kivistö KT, Niemi M, Fromm MF. Functional interaction of intestinal CYP3A4 and P-glycoprotein. Fundam Clin Pharmacol 2004;18:621-6.

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Original Research

ScopeMed

Antimicrobial and antioxidant effect of methanolic *Crinum jagus* bulb extract in wound healing

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ABSTRACT

Aim: The aim of this study was to evaluate the antimicrobial and antioxidant effects of *Crinum jagus* (J. Thomps.) Dandy methanolic bulb extract in wound healing. **Materials and Methods:** Phytochemical screening revealed the presence of alkaloids, glycosides, tannins, and saponins in the extract. *In vitro* antimicrobial activity of the extract was determined by agar well diffusion method. *In vivo* antimicrobial activity of the extract was determined by microbial assay of excision wound in rats contaminated with *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas areuginosa,* and *Candida albicans* and treated with 300 mg/kg body weight (bw) of 10 and 5% methanolic *C. jagus* bulb extract ointment (MCJBEO), respectively. Enzymatic antioxidant effect of the extract was determined *in vivo* by assaying superoxide dismutase (SOD) and catalase (CAT) activity, and malondialdehyde (MDA) level in excision wound biopsies of rats treated with 10 and 5% MCJBEO, respectively, following standard methods. Non-enzymatic antioxidant effect of the extract was determined *in vitro* using diphenylpicrylhydrazyl (DPPH) method following standard procedure. **Results:** The extract exhibited *in vitro* antimicrobial effect in a concentration-dependent manner with one hundred (100) mg/ml concentration of the extract having the highest inhibitory zone diameter for *B. subtilis* (25 mm), *S. aureus* (21 mm), and *C. albicans* (14 mm) followed by the 50, 25 and 12.5 mg/ml concentrations, respectively. *B. subtilis*, *S. aureus,* and *C. albicans* were not isolated from wounds of animals treated with both extract concentrations 10% and 5% MCJBEO, and reference drug (framycetin sulfate/clotrimazole). Activities of the enzymatic antioxidants SOD and CAT in wound biopsies treated with 10% MCJBEO were significantly ($P < 0.05$) higher when compared with those treated with 5% MCJBEO. Significantly ($P < 0.05$) decreased MDA level of wound biopsies from extract-treated rats was observed. The extract exhibited non-enzymatic antioxidant (DPPH) effect in a concentration-dependent manner. **Conclusion:** This study has shown that an anti-microbial and antioxidant effects could possibly be part of mechanism by which *C. jagus* bulb extract promote wound healing process.

KEY WORDS: Antimicrobial, antioxidant, *Crinum jagus*, *in vivo*, wound healing

INTRODUCTION

Wound healing is the process of re-establishing the integrity of damaged skin [1-3]. It is an orderly intricate process initiated by a damaged tissue itself, and it involves complex mechanisms which include: Hemostasis, inflammation, proliferation, and remodeling [1,4,5]. Each of these mechanisms requires several biochemical substances to occur [4,6-8]. Thromboxane A2 and plasminogen activator inhibitor Type 1 ensures hemostasis, heme and heme proteins trigger expression of adhesion molecules, leukocytic infiltration, and release of reactive oxygen species (ROS) also called toxic free radicals or oxidants. The oxidants are detrimental to wound contaminating microorganisms and to the skin tissue itself especially when in excess [4,6,8]. Therefore, hemeoxygenase-1 elicit antioxidant effect and scavenge (mop-up) the toxic free radicals, while matrix metalloproteinase ensures remodeling of the extracellular matrix [9]. The length of time it takes for wound healing to be optimum and complete is determined by factors such as availability of the needed biochemical substances, presence or absence of contaminating microorganism(s), and the toxic free radicals in the wound bed [10,11].

A myriad of wound contaminating organisms including bacteria (both Gram-positive e.g., *Staphylococcus* spp., Streptococci, *Bacillus* spp., etc. and Gram-negative e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus*, etc.) and fungi (both yeast e.g., *Candida albicans*, and mold e.g., *Aspergillus*) impede natural wound healing process [12-15]. They produce substances (enzymes, toxins, and free toxic radicals such as hydrogen peroxide [H_2O_2]) that degrade biochemical substances and destroy cellular components needed for wound healing [13-15]. Majority of

wound contaminating microorganisms have developed resistance to most of the commonly used orthodox antimicrobial agents and this has garnered public attention [16-20].

Toxic free radicals (oxidants) such as superoxide (SO_2^-) and hyrdroxyl (OH^-) anions, and hydrogen peroxide (H_2O_2) causes oxidative stress [21]. These oxidants are produced by infiltrating phagocytes (during inflammation stage of healing), contaminating microbes in wound bed, and the skin itself following ultraviolet exposure [21,22]. Oxidative stress further damages wounded skin tissue by lipid peroxidation (caused by lipid peroxidase and evidenced by increased malondialdehyde [MDA] level in wound tissue), and destruction of proteins and extracellular matrix [23] - this further impedes natural wound healing process. Substances that elicit antimicrobial and antioxidant effects are often used in wound management in orthodox medicine [24,25]. These substances are used to control the growth of contaminating organisms and mop-up (scavenge) toxic free radicals to achieve optimum healing [11,16]. The radical scavenging activity of natural enzymatic antioxidants (such as superoxide dismutase [SOD], catalase [CAT], glutathione dismutase, thioredoxin reductase, etc.) produced by the skin is augmented by the orthodox medicines to promote wound healing process [21]. Reports have shown that these orthodox agents are often costly [17], and often times elicit side effects which are detrimental to the recipient [11]. Therefore, there is need for cheaper and safe alternative or complementary substances that could elicit both antimicrobial and antioxidant effects to enhance natural wound healing process.

Crinum jagus (J. Thomps.) Dandy popularly called St. Christopher or Harmattan lily, Frest crinum or Poison bulb is widely used in form of decoction by traditional practitioners in Africa, including Southeastern Nigeria, for treatment of skin wounds and several other ailments [26,27,28-32] some of which have been scientifically validated [33,34,35]. Chemical investigations revealed that it contained high amount of phenolic compounds including crinamine, lycorine, psuedolycorine, 6-hydroxycrinamine, hamayne, tetrahydro-1, 4-oxazine (morpholine), bowdensine, and demethoxy-bowdensine [36,37,38,39]. It also contained saponins, tannins, calcium oxalate, and calcium tetrata [37,38]. Phenolic compounds in plant extracts exhibited enzymatic and non-enzymatic antioxidant effect [40,41]. Plant extracts containing flavonoids, triterpernoids, and tannins exhibited antimicrobial and antioxidant effects [40,42]. Therefore, *C. jagus* which contains some of these bioactive substances could possibly promote wound healing process by eliciting antimicrobial and/or antioxidant effects.

Although Adesanya *et al*. [37] reported anti-staphylococcal activity of *C. jagus* bulb, the study was not conducted in a wound healing model; and *Staphylococcus* is one of the numerous potential wound contaminants that impede wound healing process. Ode *et al*. [35] and Nwaehujor *et al*. [36] reported non-enzymatic *in vitro* free radical scavenging of diphenylpicrylhydrazyl (DPPH) by *C. jagus* bulb extract which is not related to wound healing. The antimicrobial effect of *C. jagus* bulb extract on common potential wound contaminating microorganisms and its enzymatic antioxidant effect in wound healing have not been evaluated. The objective of this study was to determine if *C. jagus* methanolic bulb extract (CJMBE) could possibly exhibit antimicrobial and/or antioxidant effect in wound healing.

MATERIALS AND METHODS

The experimental protocols used in this study was approved by the Ethics Committee of the University of Nigeria, Nsukka and conforms with the guide to the care and use of animals in research and teaching of University of Nigeria, Nsukka, Enugu State Nigeria.

Animals

A total of 97, 8-week-old male albino Wistar rats weighing between 220 and 229 g were obtained from the laboratory animal unit, Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were fed on commercial growers mash (Top feeds®) and water was provided *ad libitum*. These rats were acclimatized for 2 weeks in the animal house at the Department of Veterinary Surgery, University of Nigeria, Nsukka.

Plant Collection and Identification

Fresh *C. jagus* bulbs were collected from Amokwe town in Udi Local Government Area Enugu State, Nigeria, in the month of May, 2014 and were identified at the International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, by a plant taxonomist, Mr. A. Ozioko. A voucher specimen (number FRMPC/05/14) was deposited in the center's herbarium.

Extraction

A kg of the *C. jagus* (J. Thomps.) Dandy bulbs were sliced into smaller pieces; air dried at room temperature for 2 weeks and then pulverized using the laboratory grinding machine at the Department of Crop Science, University of Nigeria, Nsukka. The pulverized bulbs were macerated in 80% methanol for 48 h with intermittent vigorous shaking at every 2 h. After 48 h, the mixture was filtered and the extract concentrated using a rotary evaporator set at 40°C. The dried CJMBE was weighed and the percentage yield calculated. The extract was then stored at 4°C in a refrigerator until needed.

Acute Toxicity Test

Totally, 25 adult rats were randomly divided into five groups of five animals per group. The animals were deprived water for 16 h before administration of the extract. The increasing doses of the extract 250, 500, 1000, 2000, and 5000 mg/kg body weight (bw) suspended in dimethyl sulfoxide (DMSO) was administered orally to the test groups, respectively, using a ball-tipped intubation needle fitted onto a syringe. The last group received 1 ml/kg bw of sterile distilled water and served as the control. The rats were allowed access to food and water *ad libitum* and were observed for 48 h for behavioral changes

and death. The time of onset, intensity, and duration of these symptoms, if any, was recorded.

Phytochemical Analysis for Bioactive Substances

The extract was screened for the presence of bioactive components tannins, saponins, glycosides, flavonoids, and alkaloids following the methods of Trease and Evans [43].

Preparation of Ointments

The method of Okore *et al*. [44] was adapted in preparation of two herbal ointments containing 10 and 5% w/w of the extract in sterile soft white paraffin. Immediately after preparation, the ointments were aseptically transferred into sterile cream tubes and sealed until further needed.

Pathogens and Preparation of Inocula

The bacterial (*Pseudomonas aeruginosa*, *Stapylococcus aureus*, and *Bacillus subtilis*) and fungal (*C. albicans*) organisms used in this study were collected from the Department of Pharmaceutics, University of Nigeria, Nsukka. They were clinical wound isolates from patients in Nsukka, Nigeria, fully identified and maintained on nutrient agar slope at 4°C at the Department of Pharmaceutical Microbiology Laboratory, University of Nigeria, Nsukka. Prior to use, the bacterial organisms were sub-cultured on sterile nutrient agar plate, incubated aerobically at 37°C for 24 h, while the *C. albicans* was sub-cultured on sterile Sabouraud dextrose agar, incubated at 25°C for 48 h. Colonies of each organism were homogenized in sterile phosphate buffered saline (PBS) and the turbidity adjusted to correspond to 0.5 McFarland's turbidity standard (equivalent to 1×10^8 cfu/ml). The standardized broth cultures were kept at 4°C until needed.

Preparation of Extract Concentrations

A 100 mg/ml stock concentration was prepared by dissolving 1 g of the extract in 10 ml of DMSO. Then, 2-fold dilutions were made from the stock concentration to obtain concentrations of 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml.

Determination of *In vitro* Antimicrobial Effect of *C. jagus* Bulb Extract

The inhibitory zone diameter (IZD) of each of the extract concentrations for each test organism was determined by agar-well diffusion method [45]. Briefly, the standardized broth cultures of the bacterial organisms were incubated at 37°C for 10 min, and then inoculated on sterile Mueller-Hinton agar plates using sterile swab sticks. Five holes of 6 mm diameter were bored into the agar plates at strategic points using sterile cork borer and labeled to correspond to the extract concentrations. Then each of the holes was filled with 50 μl of the extract concentration. The plates were allowed on the bench to ensure complete diffusion of the extract into the agar and then incubated accordingly as above. For the fungal organism, broth culture was incubated at 25°C for 10 min before inoculating sterile Sabouraud dextrose agar. Same procedure as above was undertaken and then the plates were incubated at 25°C for 48 h. After incubation, the zone of inhibition around each well was measured with a meter rule. Each test for each organism was performed in triplicate and the mean IZD calculated to the nearest whole millimeters.

Determination of *In vivo* Antimicrobial Effect of *C. jagus* Bulb Extract

Creation and contamination of excision wound with test microorganisms

Thirty six rats were anesthetized with 10 and 50 mg/kg bw of xylazine hydrochloride and ketamine hydrochloride, respectively. Their dorsum was shaved and disinfected with 70% alcohol. Then, full thickness (480 mm^2) circular excision wounds were created following the method described by Morton and Malone [46]. Post-wounding, the rats were randomly assigned into 4 groups of 9 animals per group. Then, using sterile Pasteur pipettes, wound on each animal was contaminated by flooding with 1 ml of standardized broth culture of each test organism. To minimize further microbial contamination of wound, each animal was carefully placed individually in disinfected cages kept in a disinfected, clean and dust-free animal house in the Department of Veterinary Surgery, University of Nigeria, Nsukka. The wounds were not treated for 24 h post-contamination to ensure colonization and establishment of infection [11].

Treatment of Infected Excision Wound

Treatment of contaminated animal wound commenced 48 h post-contamination. Four treatment groups consisting of 9 animals each were treated as follows: Groups A and B were treated topically with 10% and 5% w/w methanolic CJMBE ointment (MCJBEO), respectively, Group C was treated with sterile normal saline (negative control), while Group D was treated with framycetin sulfate/clotrimazole (Sofradex-f®) (positive control), respectively. Treatment of the animals continued until complete healing occurred.

Isolation of Infective Pathogen from Contaminated Excision Wound

At days 3, 7, and 14 post-infection (p.i.), wound swabs from 3 animals in each treatment group was taken in duplicate using sterile swab sticks. The swabs were inoculated into sterile nutrient and Sabouraud dextrose broths, incubated at 37°C for 24 h and 25°C for 48 h, for bacterial and fungal isolation, respectively. The broth cultures were observed for microbial growth (cloudiness/turbidity) and if any, a loopful of the broth culture was sub-cultured on appropriate sterile agar and incubated accordingly. Isolates of different colonial types, if any, were purified on appropriate fresh media and incubated accordingly. Morphological characteristics of pure colonies of the isolates were noted and appropriately described. Then, pure colonies of the isolates were gram stained and subjected to biochemical tests such as catalase, hemolysis and coagulase,

for identification following standard biochemical methods. The number of animals from which each organism was isolated was appropriately recorded and the percentage calculated.

Determination of Enzymatic Antioxidant Effect of *C. jagus* Bulb Extract in Wound Tissue

A total of 36 rats were anesthetized and full thickness (480 mm^2) circular excision wounds were created as described above. Four treatment groups consisting of 9 animals per group were treated as follows: Groups I and II were treated topically with 10 and 5% w/w MCJBEO, respectively, while Groups III and IV were treated with sterile white soft paraffin (negative control) and framycetin sulfate/clotrimazole (positive control), respectively. Then, the wound on each of the animal was carefully bandaged using sterile gauze and adhesive tape was placed over the gauze. The animals were placed individually in a clean disinfected metal cage after grouping to avoid them biting each other's wound. At days 3, 7, and 14 post-treatment, wound biopsy specimen was taken from 3 animals in each group and the bandages were changed. Immediately after collection of wound biopsy, specimen was placed in 10% PBS and used for biochemical assay of SOD and CAT activities, and MDA level. The SOD activity was determined following the method described by Sun *et al.* [47], catalase activity was determined following the procedure described by Sinha [48], while the MDA level was determined according to the method described by Draper and Hardley [49].

Determination of *In vitro* Non-enzymatic Antioxidant Effect of *C. jagus* Bulb Extract

Free radical scavenging activity of the extract of *C. jagus* was determined using DPPH assay as described by Brand-Williams *et al.* [50]. Briefly, 2 ml of various concentrations (10, 50, 100, 200, and 400 μg/ml) of the *Crinum jagus* bulb extract was added to 1 ml of DPPH (0.5 mM in 95% methanol) in a cuvette. The mixture was shaken and incubated at 30°C for 30 min in the dark. Then, the absorbance was taken at 517 nm using a spectrophotometer. Ascorbic acid at doses lower than that which have been reported to act as pro-oxidant [51,52] were used as a standard compound (control) in this assay. For each extract concentration, the experiment was performed in triplicate and the mean absorbance calculated. The percentage scavenging activity was calculated as follows:

Scavenging effect (%) = (control absorbance − sample absorbance/[control absorbance]) × 100

Statistical Analysis

Data obtained for *in vivo* antimicrobial effect were expressed in percentages, while data obtained for *in vivo* and *in vitro* antioxidant effects were summarized as mean ± standard error of mean. Mean values of SOD and CAT activities and MDA levels for different groups were compared using one-way Analysis of Variance. Duncan multiple range test was used to separate variant means. $P < 0.05$ was considered significant.

RESULTS

Extraction

The CJMBE had an aromatic smell and was brownish in color. The percentage yield was 14.7% w/w material.

Acute Toxicity Test

Administration of MCJBE extract in DMSO to rats even at the highest dose of 2000 mg/kg bw did not produce any death in the treated groups. No sign of acute toxicity was also observed.

Phytochemical Analysis

Preliminary phytochemical analysis of CJMBE MCJBE qualitatively revealed the presence of alkaloids, tannins, saponins, and glycosides [Table 1].

In vitro Antimicrobial Effect of *Crinum jagus* Bulb Extract

The 100 mg/ml concentration of the MCJBE gave the highest IZD for *B. subtilis* (25 mm), *S. aureus* (21 mm), and *C. albicans* (14 mm) [Table 2]. The 50 mg/ml concentration gave IZD for *B. subtilis* (21 mm), *S. aureus* (16 mm), and *C. albicans* (9 mm). The 25 mg/ml concentration gave IZD for *B. subtilis* (15 mm) and *S. aureus* (10 mm) while the 12.5 mg/ml concentration gave IZD for only *B. subtilis* (10 mm). None of the tested concentrations of the extract inhibited the growth of *P. aeruginosa*.

Table 1: Phytochemical analysis of methanolic *C. jagus* bulb extract

Phytoconstituent	Amount
Alkaloids	+++
Tannins	+++
Saponins	+
Glycosides	++
Flavonoids	-

+++: Appreciable amount, ++: Moderate amount, +: Trace amount, -: Completely absent, *C. jagus: Crinum jagus*

Table 2: IZD of test concentrations of extract to each tested microorganism

CJMBE MCJBE concentration (mg/ml)	IZD (mm) to each tested microorganism			
	B. subtilis	*S. aureus*	*C. albicans*	*P. aeruginosa*
100	25	21	14	0
50	21	16	9	0
25	15	11	0	0
12.5	10	0	0	0
6.5	0	0	0	0

MCJBE: Methanolic *Crinum jagus* methanolic bulb extract, *B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, C. albicans: Candida albicans, P. aeruginosa: Pseudomonas aeruginosa*, IZD: Inhibition zone diameter

In vivo Antimicrobial Effect of *Crinum jagus* Bulb Extract in Wound

The result of frequency of reisolation of infective microorganism from wounds of animals in Groups A (treated with 10% MCJBEO), B (treated with 5% MCJBEO), C (treated with sterile normal saline), and D (treated with framycetin sulfate/clotrimazole) are presented in Figures 1-4, respectively. *B. subtilis* and *S. aureus* were not reisolated from wound of any animal in Groups A, B, and D at days 3, 7, and 14 post-infection. *P. aeruginosa* was reisolated from wound of all the animals in all the groups throughout the experiment. *C. albicans* was not reisolated from wound of any animal in Groups A, B, and D throughout the experiment, but was reisolated from wound of all the animals in Group C throughout the experiment.

Antioxidant Effect of *C. jagus* Bulb Extract in Wound Tissue

At day 3 post-treatment, SOD activity of Group I (treated with 300 mg/kg of 10% MCJBEO) significantly ($P < 0.05$) increased when compared with Groups II (treated with

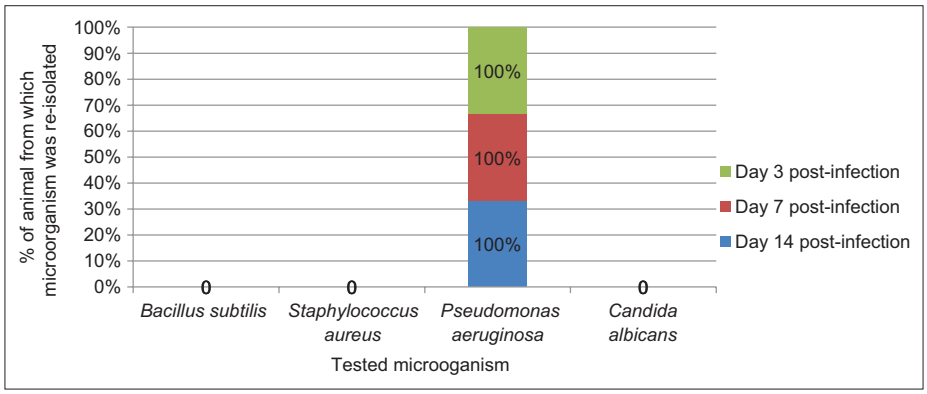

Figure 1: Frequency of reisolation of infective microorganism from animals in Group A treated with 10% methanolic *Crinum jagus* bulb methanolic extract ointment

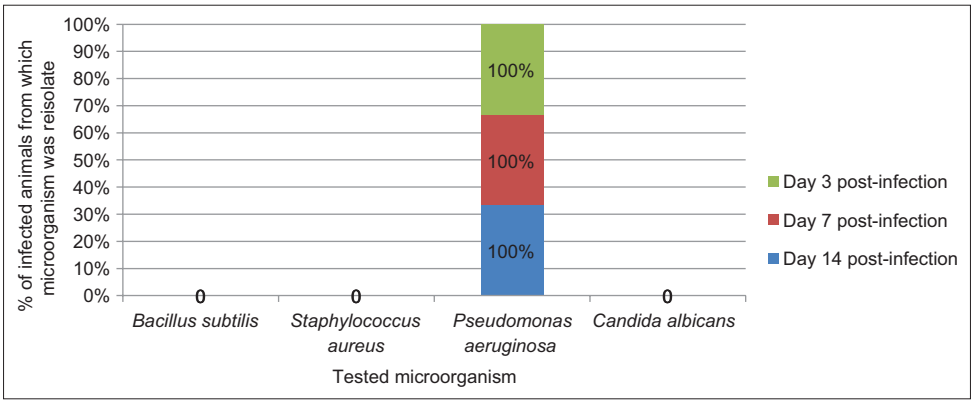

Figure 2: Frequency of reisolation of infective microorganism from animals in Group B treated with 5% methanolic *Crinum jagus* methanolic bulb extract ointment

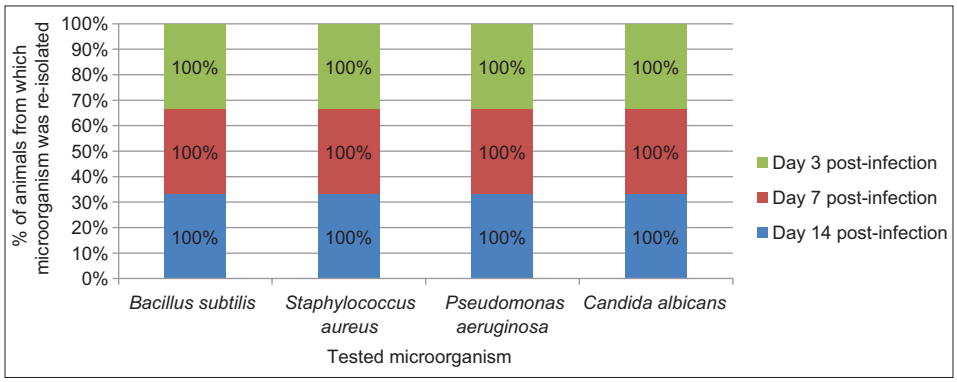

Figure 3: Frequency of reisolation of infective microorganism from animals in Group C treated with sterile normal saline (negative control)

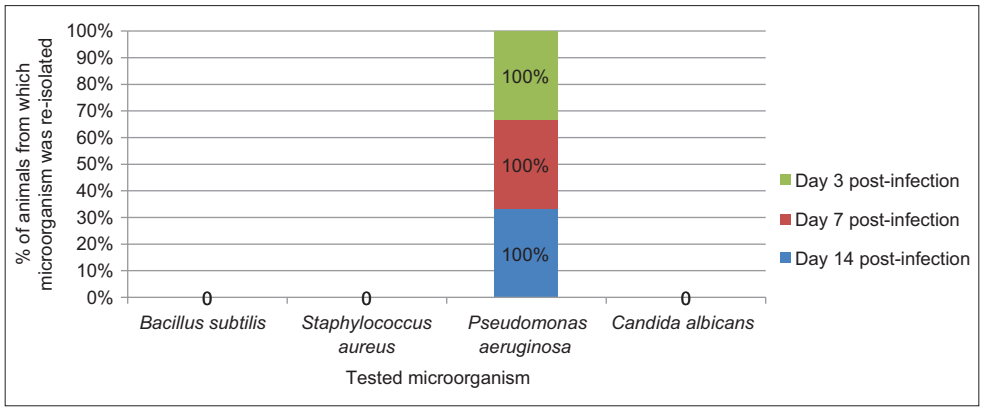

Figure 4: Frequency of reisolation of infective microorganisms from animals in Group D treated with framycetin sulfate/clotrimazole (Sofradex-f®)

300 mg/kg of 5% MCJBEO) and III (treated with sterile normal saline) [Table 3]. No significant ($P > 0.05$) difference existed in SOD activity between Groups I and IV (treated with framycetin sulfate/clotrimazole). SOD activity of Group II was significantly ($P < 0.05$) higher compared with Group III throughout the experiment but significantly lower compared with the Group IV. Similar trend was observed at day 7 post-treatment At day 14 post-treatment, SOD activity of Group I was significantly ($P < 0.05$) higher than those of the other groups.

There were significant differences ($P < 0.05$) in catalase activity of wound biopsy among all the groups at day 3 post-treatment [Table 4]. There was significant ($P < 0.05$) increase in CAT activity of Group III when compared with the other groups. Increased significant difference ($P < 0.05$) existed between CAT activity of Groups I, II and III. Similar trend was observed at days 7 and 14 post-treatment.

At day 3 post-treatment, MDA level in wound biopsies of animals in Group I significantly ($P < 0.05$) decreased when compared with the other groups [Table 5]. No significant difference ($P > 0.05$) occurred in MDA levels between Groups I and IV. Similar trends were observed at days 7 and 14 post-treatment At concentration of 10 μg/ml, the non-enzymatic free radical scavenging activity of CJMBE decreased significantly ($P < 0.05$) when compared with the control (ascorbic acid) [Table 6]. Similar trend was observed at concentrations of 50, 100, 200, and 400 μg/ml. Mean values of the antioxidant activity of the extract showed increasing activity with an increase in concentration.

Bacteriological Assay of Excision Wound Post-Contamination

Cultures of wound swabs from animals in Groups III yielded heavy growth of all the infective organisms (*S. aureus*, *B. subtitlis*, *P. aeruginosa*, and *C. albicans*) throughout the study period, whereas cultures of wound swabs from animals in Group I, II, and IV yielded scanty growth of only *P aeruginosa* at days 3, 7, and 14 post-treatment.

Table 3: SOD activity in wound biopsy of animals treated with MCJBEO

Group (treatment)	Mean±SEM SOD (µ/mg protein) in wound biopsy at days post-treatment		
	3	7	14
I (10% MCJBEO)	4.22±0.09[a]	4.57±0.02[a]	5.24±0.06[a]
II (5% MCJBEO)	2.58±0.04[b]	3.00±0.00[b]	2.85±0.00[b]
III (sterile soft white paraffin)	1.32±0.00[c]	1.38±0.07[c]	1.40±0.02[c]
IV (framycetin sulfate/ clotrimazole)	4.20±0.06[a]	4.68±0.07[a]	4.61±0.06[d]

Different superscript[abcd] across a column indicate significant difference in means at $P<0.05$, MCJBEO: Methanolic *Crinum jagus* methanolic bulb extract ointment, SEM: Standard error of mean, SOD: Superoxide dismutase

Table 4: CAT activity in wound biopsy of animals treated with MCJBEO

Group (treatment)	Mean±SEM CAT activity (µ/mg protein) in wound biopsy at days post-treatment.		
	3	7	14
I (10% MCJBEO)	2.58±0.04[a]	3.00±0.00[a]	2.85±0.00[a]
II (5% MCJBEO)	0.15±0.03[b]	0.11±0.00[b]	0.10±0.00[b]
III (sterile soft white paraffin)	4.62±0.52[c]	4.68±0.07[c]	4.61±0.06[c]
IV (framycetin sulfate/ clotrimazole)	0.62±0.00[d]	0.64±0.07[d]	0.64±0.06[d]

Different superscript[abcd] across a column indicate significant difference in means at $P<0.05$, MCJBEO: Methanolic *Crinum jagus* bulb extract ointment, SEM: Standard error of the mean, CAT: Catalase

DISCUSSION

In this study, the antimicrobial and antioxidant effects of CJMBE in wound healing was evaluated. Microbial wound contamination alters healing process and result in complication such as bacteremia and/or septicemia following wound invasion [53,54]. These complications are often common when resistant organisms constitute the contaminants. Ability of a substance to control the growth of a microorganism is ascertained by observing the extent to which the growth of the organism is inhibited by the substance. In the present study, *in vitro* antimicrobial study revealed that *C. jagus* bulb extract inhibited the growth of bacteria - *B. subtilis* and *S. aureus* and a yeast *C. albicans*. This indicates that the plant extract exhibited antibacterial and antifungal activities. The fact

that there was decreased antimicrobial activity with decrease in concentration of the extract, suggests that the antimicrobial effect of the *C. jagus* bulb extract is concentration-dependent. The 100 mg/ml concentration of the extract gave the highest IZD for the 3 inhibited organisms (*B. subtilis* [25 mm], *S. aureus* [21 mm], and *C. albicans* [14 mm]) which suggests that *C. jargus jagus* bulb extract exhibits the best antimicrobial effect at this (100 mg/ml) concentration. The 50 mg/ml concentration gave IZD for *B. subtilis* (21 mm), *S. aureus* (16 mm), and *C. albicans* (9 mm). The IZD for each organism at 50-6.25 mg/ml concentration is lower when compared with those of 100 mg/ml concentration. This result may suggest that the more the concentration of phytochemicals responsible for the antimicrobial activity, the better the effect. The fact that none of the tested concentrations of the extract inhibited the growth of *P. aeruginosa* indicates that *C. jagus* do not exhibit anti-pseudomonal effect. This suggests that the *P. aeruginosa* isolate used was resistant to the extract. The antimicrobial activity of *C. jagus* could be attributed to its alkaloids, tannins, and saponin content as revealed by the phytochemical analysis. Studies have reported antibacterial effect of plant extract containing tannins, alkaloids, and saponins [11,17,40]. Crinamine, a phenolic alkaloid contained in *C. jagus* bulb have been reported to exhibit antibacterial activity [37]. The anti-candidal effect of *C. jagus* bulb extract observe in this study may be related to its high tannin content (as revealed by the result of phytochemical screening). Plant extracts containing tannins have been widely reported to inhibit the growth of *Candida* [55,56,57]. Moreover, inhibition of growth of *S. aureus* by the *C. jagus* bulb extract in this study corroborates the report of Adesanya *et al*. [37]. Failure to inhibit the growth of *P. aeruginosa* could be attributed to its inherent resistance to most antibacterial agents [58]. It could also be that the *P. aeruginosa* strain is a highly-resistant isolate having being isolated from septic wound.

The result of the *in vitro* antimicrobial studies (highest IZD produced by 100 and 50 mg/ml) prompted us to prepare the 10 and 5% *C. jagus* extract ointments for the *in vivo* wound healing studies. Failure to re-isolate *B. subtilis*, *S. aureus*, and *C. albicans* from infected wound of any animal in Groups A (treated with 10% MCJBEO), B (treated with 5% MCJBEO) and D (treated with framycetin sulfate/clotrimazole) throughout the study period is attributable to the antimicrobial effect of both concentrations of the extract and of course the reference antimicrobial agent, framycetin sulfate/clotrimazole. Re-isolation of *P. aeruginosa* from all the animals in all the groups throughout the course of the experiment further suggests that the organism was resistant to the extract irrespective of the concentration and to the reference drug. The result of the *in vivo* antimicrobial studies further supports the antimicrobial effect of the extract on the tested microorganisms except *P. aeruginosa*.

In this study, assay of SOD and CAT activities, and MDA level was performed to determine the superoxide (O_2^-) and hydrogen peroxide (H_2O_2) radical scavenging activity of *Crinum jagus* extract, and degree of lipid peroxidase activity (cellular lipid peroxidation), respectively. Superoxide radical is considered a major biological source of ROS [59]. Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress and tissue damage [60,61]. This radical is scavenged and by SOD, an enzyme that dismutates superoxide anions (O_2^-) to generate hydrogen peroxide (H_2O_2) [61] which is then detoxified by catalase to water and oxygen which are non-toxic to tissues [61,62]. Therefore, the significantly increased SOD of the extract treated Groups (I and II) when compared with Group III (treated with sterile soft white paraffin), suggests that the extract increased SOD activity in the wound biopsies. However, the fact that SOD activity of Group I (treated with 10% MCJBEO) was significantly higher than the other groups, may suggest that the extract exhibited a better superoxide radical scavenging at this concentration. This suggests that *C. jagus* bulb extract elicited superoxide radical scavenging in a concentration-dependent manner. Interestingly, the SOD activity of wound biopsies from animals in Group I was significantly higher than all the other groups, including those treated with the reference drug, framycetin sulfate/clotrimazole, an antibiotic/antifungal combination drug. This finding suggests that at 10% concentration, superoxide radical scavenging activity of *C. jagus* bulb extract was better sustained than at the 5% concentration, and of course, the controls. This may explain the observed faster wound healing in animals in the group, in contrast to their counterparts in the other groups. Ability of the *C. jagus* extract to increase the SOD activity in the wound bed could be related to its high tannin content [63] as revealed by the result of the phytochemical screening.

Table 5: MDA level in wound biopsy of animals treated with MCJBEO

Group (treatment)	Mean±SEM CAT activity (µ/mg protein) in wound biopsy at days post-treatment		
	3	7	14
I (10% MCJBEO)	0.33±0.06[a]	0.32±0.00[a]	0.30±0.03[a]
II (5% MCJBEO)	0.54±0.04[b]	0.54±0.04[b]	0.53±0.00[b]
III (sterile soft white paraffin)	0.67±0.03[c]	0.67±0.02[c]	0.66±0.03[c]
IV (framycetin sulfate/clotrimazole)	0.41±0.00[a]	0.32±0.00[a]	0.31±0.00[a]

Different superscript[abcd] across a column indicate significant difference in means at $P<0.05$, MCJBEO: Methanolic *Crinum jagus* methanolic bulb extract ointment, SEM: Standard error of mean, MDA: Malondialdehyde

Table 6: Free radical scavenging activity of MCJBE

Test substance	Percentage antioxidant activity at different test concentrations (µg/ml)				
	10	50	100	200	400
MCJBE	6.82±0.05[b]	45.48±0.14[b]	53.08±0.43[b]	53.34±0.69[b]	58.19±1.51[b]
Ascorbic acid	73.65±0.85[a]	74.14±0.03[a]	74.63±1.16[a]	77.17±0.76[a]	79.18±0.16[a]

Different superscript[ab] in a column indicate significant difference in mean at $P<0.05$, MCJBE: Methanolic *Crinum jagus* methanolic bulb extract

Hydrogen peroxide, a non-reactive compound is converted to free hydroxyl radical (OH^-), which reacts with biomolecules to cause tissue damage and cell death [61]. It is scavenged by CAT which breaks it down into water and oxygen [62]. Significantly increased CAT activity in wound biopsies of animals in Group III (negative control) on comparison with their counterparts in the other groups may be attributed to the presence of the infective microorganisms present in the wound. Result of the post-contamination wound microbial assay revealed the presence of *S. aureus* and other infective contaminating microbes in wounds of animals in the negative control group. *S. aureus* is known to produce catalase which destroys phagocytic cells recruited to engulf them in the wound site [64]. Therefore, since the negative control group was neither treated with CJMBEO nor the reference drug, the isolation of microbes especially *S. aureus* in the wound of animals in the group was not surprising and hence, the observed significant increase in catalase activity. Nevertheless, significantly increased CAT activity of Group I as against Groups II, and IV suggests that *C. jagus* bulb extract exhibited H_2O_2 scavenging activity in a concentration-dependent manner. This result also suggests that H_2O_2 scavenging was better sustained by 10% concentration of the extract throughout the experiment. Free superoxide radical and H_2O_2 scavenging activity of plant phenolics especially tannins have been reported [61,63]. Interestingly, the significant increase in CAT activity of Group I on comparison with Group IV (treated with framycetin sulfate/clotrimazole) suggest that the extract enhanced catalase activity more than the reference drug. This finding is supported by the fact that infective pathogens were not isolated from wounds of animals in both groups, hence there was no microbe that could have produced catalase in Group I.

The consequence of decreased SOD and CAT activities is increased cellular lipid peroxidation and delayed wound healing [65]. Lipid peroxidation is caused by the activity of lipid peroxidase which is evidenced by the presence of MDA in tissues [65,66]. MDA is toxic and causes considerable changes in the structural organization and function of cell membrane making it to be porous [66]. In the present study, a significant decrease in MDA level in wound biopsies of animals in Group I compared with the other groups suggests that *C. jagus* exhibited anti-lipid peroxidation activity [65]. This finding also suggests that *C. jagus* bulb extract exhibited this activity in a concentration-dependent manner. None significant difference observed in MDA level between Groups I and IV (positive control), suggests that the extent at which the 10% MCJBEO prevented cellular lipid peroxidation is comparable to that of the reference drug, framycetin sulfate/clotrimazole. This finding conforms to Panneerselvam and Govindasamy [65] who reported that a significant increase in SOD and CAT activities results in decreased lipid peroxidase activity indicated by decreased concentration of MDA. Therefore, the results of this study suggests that 10% MCJBEO exhibited antioxidant activity, which resulted in low MDA level in the wound biopsies of animals in the group since increase in lipid peroxidation (MDA) level suggests increased generation of toxic free radicals [67].

In the present study, *in vitro* non-enzymatic free radical scavenging activity of *C. jagus* bulb extract was determined using DPPH method [41]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolorizes the DPPH solution. The degree of color change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [41,68]. In the present study, the reference antioxidant (ascorbic acid) used showed significantly higher free radical scavenging activity than all the *C. jagus* bulb extract concentrations tested. However interestingly, the mean values of the antioxidant activity of the extract revealed that the extract exhibited free radical scavenging activity in a concentration-dependent manner. Optimum wound healing occurs when ROS are reduced to a level where oxidative stress is minimal [25]. Moreover, reduced ROS in wound biopsies is evidenced by increased antioxidant activities [25]. Many studies have shown that tannins contained in plant extracts exhibits antioxidant activity [41,69,70]. Therefore, the non-enzymatic antioxidant effects exhibited by the *C. jagus* bulb extract in this study could also be attributed to its high tannin content.

CONCLUSION

This study has shown that methanolic CJMBE exhibited antioxidant and antimicrobial effects against some common wound contaminating microorganism both *in vitro* and *in vivo* in a wound healing model. These effects could possibly be part of its mechanism in promoting wound healing, forming the basis for its use in wound management in folkloric medicine. However, further studies that would involve cell biology are recommended.

REFERENCES

1. Nguyen DT, Orgill DP, Murphy GF. The pathophysiologic basis for wound healing and cutaneous regeneration. Biomaterials for Treating Skin Loss. Cambridge/Boca Raton: Wood Head Publishing (UK/Europe) and CRC Press (US); 2009. p. 25-57.
2. Omale J, Isaac AV. Excision and incision wound healing potential of *Saba Florida* (Benth) leaf extract in rattus novergicus *Rattus novergicus*. Int J Pharm Biochem Res 2010;1:101-7.
3. Al-Henhena N, Mahmood AA, Al-Magrami A, Nor Syuhada AR, Zahra AA, Summaya MD, *et al*. Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats. J Med Plants Res 2011;5:3660-6.
4. Pandith H, Zhang X, Liggett J, Min KW, Gritsanapan W, Baek SJ. Hemostatic and Wound Healing Properties of *Chromolaena odorata* Leaf Extract. ISRN Dermatol 2013;2013:168269.
5. Tsala DE, Nga N, Thiery BN, Bienvenue T, Theophile D. Evaluation of the antioxidant activity and the healing action of the ethanol extract of *Calotropis procera* bark against surgical wounds. J Intercult Ethnopharmacol 2014;4:64-9.
6. Vezza R, Mezzasoma AM, Venditti G, Gresele P. Prostaglandin endoperoxides and thromboxane A2 activate the same receptor isoforms in human platelets. Thromb Haemost 2002;87:114-21.
7. Martin P, Leibovich SJ. Inflammatory cells during wound repair: The good, the bad and the ugly. Trends Cell Biol 2005;15:599-607.
8. Aso Y. Plasminogen activator inhibitor (PAI)-1 in vascular inflammation and thrombosis. Front Biosci 2007;12:2957-66.
9. Stamenkovic I. Extracellular matrix remodelling: The role of matrix metalloproteinases. J Pathol 2003;200:448-64.
10. Houghton PJ, Hylands PJ, Mensahb AY, Hensel A, Deters AM. *In vitro* tests and ethnopharmacological investigations: Wound healing as an example. J Ethnopharmacol 2005;100:100-7.

11. Udegbunam SO, Udegbunam RI, Muogbo CC, Anyanwu MU, Nwaehujor CO. Wound healing and antibacterial properties of methanolic extract of Pupalia lappacea Juss in rats. BMC Complement Altern Med 2014;14:157.
12. Gyles CL. Pathogenesis of Bacteria Infections in Animals. Ames: Iowa University Press; 1997. p. 164.
13. Huycke MM, Sahm DF, Gilmore MS. Multiple-drug resistant enterococci: The nature of the problem and an agenda for the future. Emerg Infect Dis 1998;4:239-49.
14. Vogel PL, Komtebedde J, Hirsh DC, Kass PH. Wound contamination and antimicrobial susceptibility of bacteria cultured during total ear canal ablation and lateral bulla osteotomy in dogs. J Am Vet Med Assoc 1999;214:1641-3.
15. Gales AC, Ronald NJ, Michael A, Pfaller A, Helie S. Sentry Study Group. Two year assessment of the pathogen frequency and antimicrobial resistance patterns among organisms isolated from skin and soft tissue infections in Latin American hospitals. Int J Infect Dis 2000;4:75-6.
16. Abu-Al-Basal MA. Healing potential of *Rosmarinus officinalis* L. on full thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/C mice. J Ethnopharmacol 2010;131:443-50.
17. Udegbunam SO, Nnaji TO, Udegbunam RI, Okafor JC, Agbo I. Evaluation of herbal ointment formulation of *Milicia excelsa* (Welw) C.C berg for wound healing. Afr J Biotechnol 2013;12:3351-9.
18. Chah KF, Eze CA, Emuelosi CE, Esimone CO, Chah KF, Eze CA, *et al*. Antibacterial and Wound healing properties of Methanolic extracts of some Nigerian medicinal plants. J Ethnopharmacol 2005;104:164-7.
19. Mboto CL, Eja ME, Adegoke AA, Iwatt GD, Askong BE, Takon I, Udo SM, Akeh M. Phytochemical properties and antimicrobial activities of combined effect of extracts of the leaves of *Garcinia kola, Vernonia amygdalina* and honey on some medicinally important microorganisms. Afr J Microbiol Res 2009;3:557-9.
20. Mahin T, Masood K, Yahid N, Heidar M. Evaluation of antibacterial activity and wound healing of *Pistacia atlantica* and *Pistacia khinjuk*. J Med Plants Res 2011;5:4310-4.
21. Trenam CW, Blake DR, Morris CJ. Skin inflammation: Reactive oxygen species and the role of iron. J Invest Dermatol 1992;99:675-82.
22. Darr D, Fridovitch L. Free radicals in cutaneous biology. J Invest Dermatol 1994;102:671-5.
23. Calabrese V, Scapagnini G, Catalano C, Dinotta F, Geraci D, Morganti P. Biochemical studies of a natural antioxidant isolated from rosemary and its application in cosmetic dermatology. Int J Tissue React 2000;22:5-13.
24. Udupa AL, Kulkarni DR, Udupa SL. Effect of *Tridax procumbans* extracts on wound healing. Int J Pharmacogn 1995;33:37-40.
25. Fitzmaurice SD, Sivamani RK, Isseroff RR. Antioxidant therapies for wound healing: A clinical guide to currently commercially available products. J Pharmacol Biophysic Res 2011;24:113-26.
26. Olorode O. Taxonomy of West African Flowering Plants. London: Longman Publishing Company; 1984. p. 121.
27. Houghton PJ, Agbedahuns JM, Adegbulugbe A. Choline esterase inhibitory properties of alkaloids from two Nigerian *Crinum* species. Sci Direct 2004;65:2893-6.
28. Kokwaro JO. Medicinal Plants of East Africa. Kenya: General Printers; 1976. p. 230.
29. Gill LS. Ethnomedicinal Uses of Plants in Nigeria. Benin City, Nigeria: Uniben Press; 1992. p. 27.
30. Sonibare MA, Gbile ZO. Ethnobotanical survey of anti-asthmatic plants in South Western Nigeria. Afr J Tradit Complement Altern Med 2008;5:340-5.
31. Idu M, Obaruyi GO, Erhabor JO. Ethnobotanical uses of plants among the binis in the treatment of ophthalmic and ENT (ear, nose and throat) ailments. Ethnobot Leafl 2008;13:480.
32. Ogunkunle ATJ, Olopade OR. Studies on the asthma coughs plant *Crinum jagus* L. (Amaryllidaceae) in Nigeria. Afr J Plant Sci 2011;5:108-14.
33. Ode OJ, Asuzu IU. The anti-snake venom activities of the methanolic extract of the bulb of *Crinum jagus* (Amaryllidaceae). Toxicon 2006;48:331-42.
34. Ode OJ, Nwaehujor CO, Onakpa MM. Evaluation of antihaemorrhagic and antioxidant potentials of *Crinum jagus* bulb. Int J Appl Biol Pharm Tech 2010;1:1330-6.
35. Shorinwa OA, Ebong OO, Obianime AW, Siminialayi IM. The effect of acetone extracts of *Crinum jagus* bulbs on the histology of the kidney, liver and testis of albino rats. Peak J Med Plant Res 2013;2:38-44.
36. Nwaehujor CO, Nwinyi FC, Ode JO. Liver protective activity of the methanol extract of *Crinum jargus bulb* against acetaminophen-induced hepatic damage in rats. Asian J Biochem 2012;7:182-93.
37. Adesanya SA, Olugbade TA, Odebiyi OO, Aladesanmi AJ. Antibacterial alkaloids in *Crinum jagus*. Int J Pharmacol 1992;30:303-7.
38. Edema MD, Okieimen FE. Chemical and anticonvulsant screening of *Crinum jagus*. Nig J Chem Res 2002;7:25-8.
39. Tram NT, Titorenkova TV, Bankova VS, Handjieva NV, Popov S. *Crinum* L. (Amaryllidaceae). Fitoterapia 2002;73:183-208.
40. Goncalves, RS, Battistin A, Pauletti G, Rota L, Serafini LA. Antioxidant properties of essential oils from *Mentha* species evidenced by electrochemical methods. Rev Bras Pl Med Botucatu 2009;11:372-82.
41. Saeed N, Khan MR, Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. BMC Complement Altern Med 2012;12:221.
42. Ekren S, Yerlikaya O, Tokul HE, Akpınar A, Açu M. Chemical composition, antimicrobial activity and antioxidant capacity of some medicinal and aromatic plant extracts. Afr J Microbiol Res 2013;7:383-8.
43. Trease EC, Evans WC. Pharmacognosy. 12th ed. London: Bailliere and Tindall; 1983. p. 115-625.
44. Okore VC, Ibezim EC, Adikwu MU, Attama AA, Esimone CO, Uzuegbu BD, *et al*. Laboratory Techniques in Pharmaceutics and Pharmaceutical Microbiology. 2nd ed. Nigeria: El'Demark Publishers; 2004. p. 1-20.
45. Perez C, Pauli M, Bazerque P. An antibiotic assay by the agar-well diffusion method. Acta Biol Med 1990;15:113-5.
46. Morton JJ, Malone MM. Evaluation of vulnerary activity by open wound procedure in rats. J Trauma 1992;20:323-4.
47. Sun Y, Oberley LW, Li Y. A simple method for clinical assay of superoxide dismutase. Clin Chem 1988;34:497-500.
48. Sinha KA. Colorimetric assay of catalase. Anal Biochem 1972;47:389-94.
49. Draper HH, Hadley M. Malondialdehyde determination as index of lipid peroxidation. Methods Enzymol 1990;186:421-31.
50. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebenson Wiss Technol 1995;28:25-30.
51. Yen GC, Duh PD, Tsai UL. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. Food Chem 2002;79:307-13.
52. Ling LT, Palanisamy UD, Cheng HM. Prooxidant/antioxidant ratio (ProAntidex) as a better index of net free radical scavenging potential. Molecules 2010;15:7884-92.
53. Bowler PG, Duerden BI, Armstrong DG. Wound microbiology and associated approaches to wound management. Clin Microbiol Rev 2001;14:244-69.
54. Mohan H. Inflammation and healing. Textbook of Pathology. 5th ed. New Delhi: Ed Jaypee Brothers; 2005. p. 51-92.
55. Vasconcelos LC, Sampaio MC, Sampaio FC, Higino JS. Use of *Punica granatum* as an antifungal agent against candidosis associated with denture stomatitis. Mycoses 2003;46:192-6.
56. Vasconcelos LC, Sampaio FC, Sampaio MC, Pereira Mdo S, Higino JS, Peixoto MH. Minimum inhibitory concentration of adherence of *Punica granatum* Linn (pomegranate) gel against *S. mutans, S. mitis* and *C. albicans*. Braz Dent J 2006;17:223-7.
57. Anibal PC, Peixoto IT, Foglio MA, Höfling JF. Antifungal activity of the ethanolic extracts of *Punica granatum* L. and evaluation of the morphological and structural modifications of its compounds upon the cells of Candida spp. Braz J Microbiol 2013;44:839-48.
58. Li XZ, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: Resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob Agents Chemother 1994;38:1732-41.
59. Alves CQ, David JM, David JP, Bahia MV, Aguiar RM. Methods for determination of *in vitro* antioxidant activity for extracts and organic compounds. Química Nova 2010;33:2202-10.
60. Meyer AS, Isaksen A. Application of enzymes as food antioxidants. Trends Food Sci Tech 1995;6:300-4.
61. Khan RA, Khan MR, Sahreen S, Ahmed M. Evaluation of phenolic contents and antioxidant activity of various solvent extracts of *Sonchus asper* (L.) Hill. Chem Cent J 2012;6:12.
62. Singleton P. Bacteria in Microbiology, Biotechnology and Medicine. 5th ed. Cheichester Sussex England: John Wiley and Sons; 2005. p. 2-60.

63. Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J Agri Food Chem 1998;46:1887-92.
64. Quinn PJ, Markey BK. Concise Review of Veterinary Microbiology. 2nd ed. Oxford: Blackwell Publishing Limited; 2003. p. 15-7.
65. Panneerselvan SR, Govindasamy S. Effect of sodium molybdate on the status of lipids, lipid peroxidation and antioxidant systems in alloxan-induced diabetic rats. Clin Chim Acta 2004;345:93-8.
66. Wall RK, Jaffe S, Kumar D, Songenete N, Kalra VK. Increased adherence of oxidant treated human and bovine erythrocyte to culture endothelial cells. J Cell Physiol 1987;113:25-36.
67. Woeff SP, Dean RT. Glucose anti oxidation and protein modification. The potential role of anti-oxidative glycosylation in diabetes. Biochem J 1987;247:243-50.
68. Krishnaiah D, Sarbatly R, Nithyanandam RR. A review of the antioxidant potential of medicinal plant species. Food Bioprod Process 2011;89:217-33.
69. Zhang SJ, Lin YM, Zhou HC, Wei SD, Lin GH, Ye GF. Antioxidant tannins from stem bark and fine root of *Casuarina equisetifolia*. Molecules 2010;15:5658-70.
70. Zhao S, Liu JY, Chen SY, Shi LL, Liu YJ, Ma C. Antioxidant potential of polyphenols and tannins from burs of *Castanea mollissima* Blume. Molecules 2011;16:8590-600.

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Short Communication

Prophylactic effects of humic acid-glucan combination against experimental liver injury

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ABSTRACT

Aim: Despite intensive research, liver diseases represent a significant health problem and current medicine does not offer a substance able to significantly inhibit the hepatotoxicity leading to various stages of liver disease. Based on our previously published studies showing the protective effects of a glucan-humic acid (HA) combination, we focused on the hypothesis that the combination of these two natural molecules can offer prophylactic protection against experimentally induced hepatotoxicity. **Materials and Methods:** Lipopolysaccharide, carbon tetrachloride, and ethanol were used to experimentally damage the liver. Levels of aspartate aminotransferase, alanine transaminase, alkaline phosphatase, glutathione, superoxide dismutase, and malondialdehyde, known to correspond to the liver damage, were assayed. **Results:** Using three different hepatotoxins, we found that in all cases, some samples of HA and most of all the glucan-HA combination, offer strong protection against liver damage. **Conclusion:** Glucan-HA combination is a promising agent for use in liver protection.

KEY WORDS: Enzymes, humic acid, glucan, liver, protection

INTRODUCTION

Liver disease can be inherited or caused by a variety of factors that damage the liver, such as viruses and alcohol use. In addition, various environmental factors can cause liver damage. With over 36,000 deaths from chronic liver diseases, it is clear that the search for a cure or preventive treatment currently represents one of the main focuses of medicine.

As the current medicine does not offer a substance able to significantly ameliorate the hepatotoxicity leading to liver disease, it is not surprising that the attention is more and more focused on various natural molecules, including immunomodulators. In most of these studies, the described effects of various natural molecules on liver damage were positive [1-5].

The long-term focus of our laboratories is on the synergistic effects of two natural molecules, humic acids (HA), and β-glucan. HA are ubiquitous molecules, which can be found wherever organic matter is being decomposed. Despite long knowledge of HA, with significant studies going back approximately 100 years, as during World War I, peat extracts were used to prevent infections [6]. Some of their health related effects are still unclear. Some studies showed stimulation of lymphocytes [7], some antiviral properties [8].

Biological properties of polysaccharides have been researched since the 1940s [9] and are currently subject of over 10,000 scientific studies. Glucans are treated as pathogens by pattern recognition receptors on macrophages, neutrophils, monocytes, and natural killer cells [10]. Glucans from different sources possess differential receptor affinities, mostly for Complement Receptor 3 (CD11b/CD18) on macrophages and neutrophils, and Dectin-1 receptor on macrophages [11,12]. The significant effects of glucans have been established in anti-infection and anti-cancer immunity [13], lowering cholesterol [14], suppression of stress [15], and stimulating immunity of chronically ill children [16].

Our previous studies revealed that glucan's effects can be further improved by adding HA [17]. Further studies showed that the administration of glucan had beneficial effects on hepatocytes [18] and hepatoprotective effects on experimentally-induced liver damage [19]. In addition, our own data showed strong synergistic effects of HA-glucan combination in hepatoprotection against lipopolysaccharide (LPS) injury [20].

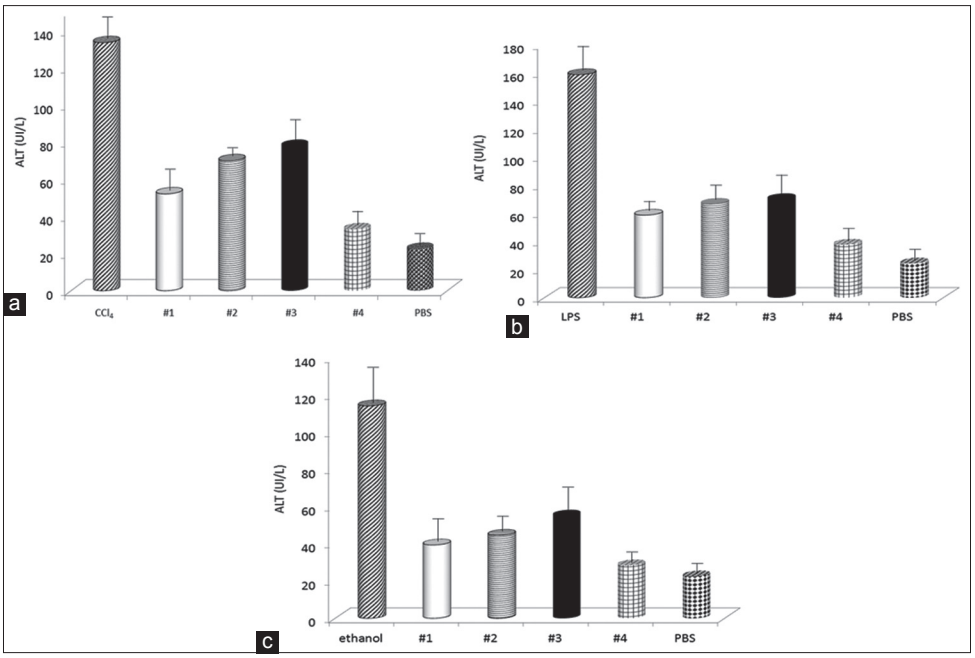

Figure 1: (a, b and c) The alanine aminotransferase activity in the serum after carbon tetrachloride, Lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

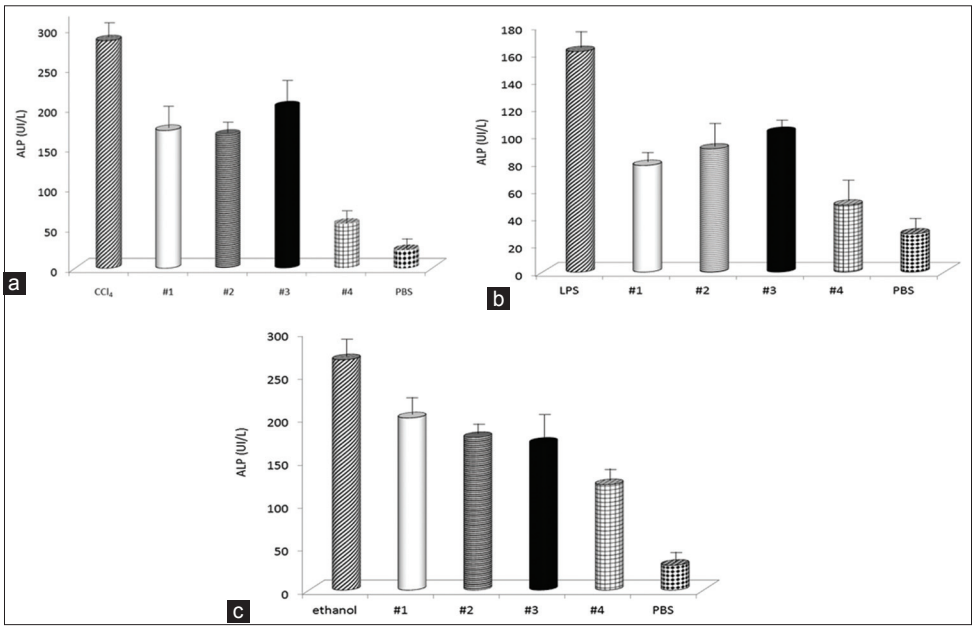

Figure 2: (a, b, and c) The alkaline phosphatase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

However, these data confirmed that the HA-glucan combination can offer healing properties, but offer no information about the effects when used before the liver damage. This shortcoming led us to the current study evaluating the possible prophylactic effects of a HA-glucan combination of experimentally-induced hepatotoxicity. In order to be sure our findings have general reach, we used three different, but well-established, models of hepatotoxicity: LPS induced [21], ethanol-induced [22], and carbon tetrachloride (CCl_4)-induced [23]. The main reason for the fact we decided to use these three models is their widespread in research, combined they represent more than 90% of experimentally-induced hepatotoxic studies.

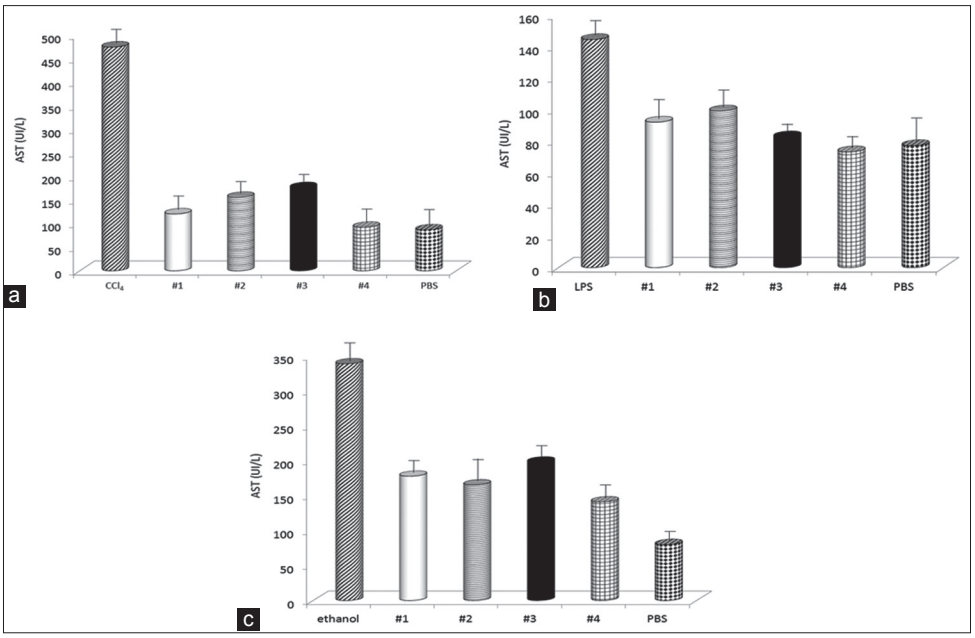

Figure 3: (a, b, and c) The aspartate aminotransferase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

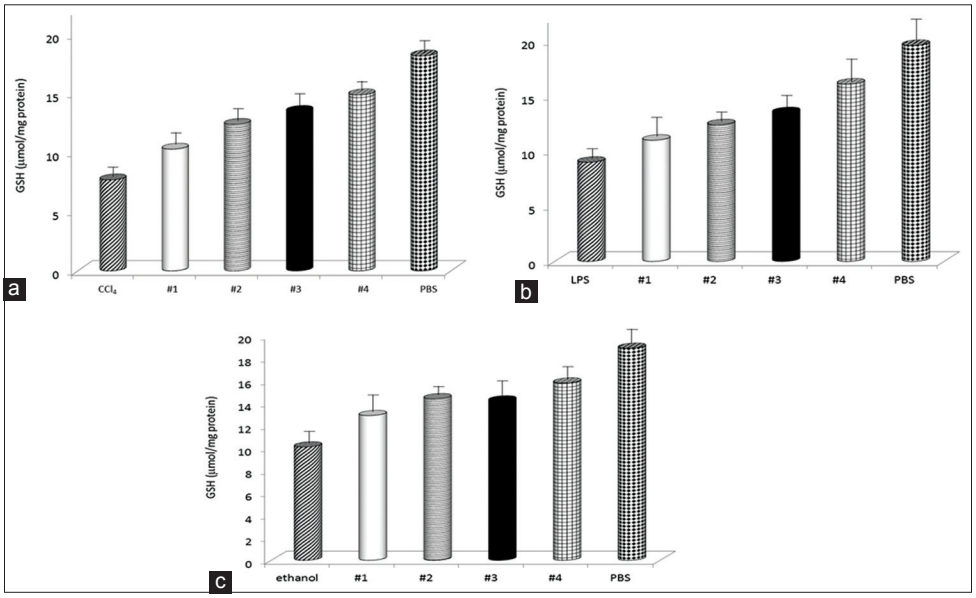

Figure 4: (a, b, and c) The glutathione activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

MATERIALS AND METHODS

Animals

Female, 6-10 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO2 asphyxiation.

Materials

Ethanol, LPS (from *Escherichia coli*), and carbon tetrachloride were purchased from Sigma (St. Louis, MO, USA).

β - 1, 3 Glucan

We used a combination of mannooligosaccharides and β-glucan extracted from saccharomyces cerevisiae by autolysis at high

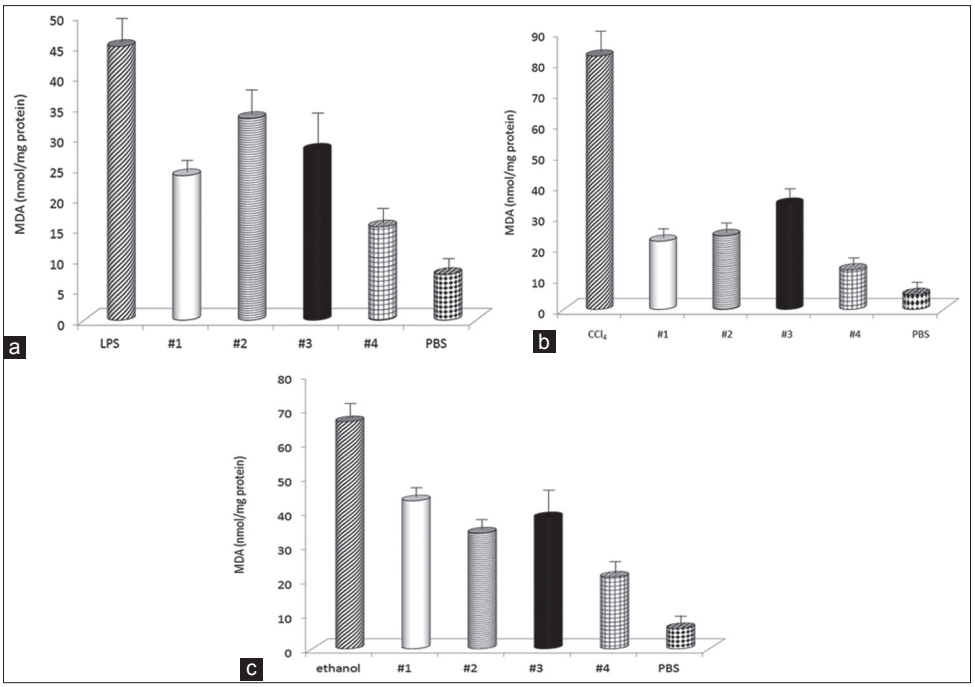

Figure 5: (a, b and c) The lipid peroxidation (malondialdehyde) activity in the serum after carbon tetrachloride, Lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-humic acid combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

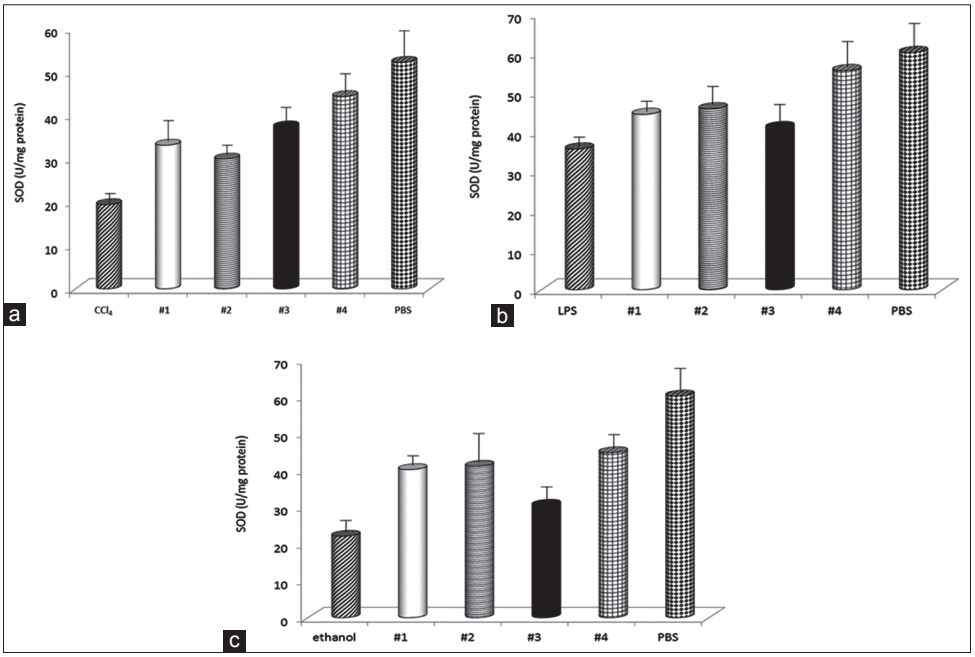

Figure 6: (a, b, and c) The superoxide dismutase activity in the serum after carbon tetrachloride, lipopolysaccharide or ethanol challenge in mice fed a control diet (control) or supplemented with humic acid (HA) or glucan-HA combo. Individual samples were named #1 (glucan), #2 (AH8), #3 (AH10), and #4 (AH8+AH10+glucan). Values represent a mean of 15 mice. All supplemented groups showed significant difference from the challenged group at $P < 0.05$ level

temperature at controlled pH. When completed, the cell walls and extracts are separated by centrifugation and cell wall is spray dried. The glycosidic composition is 21% mannan, 24% β-glucan (Lallermand Animal Nutrition, Montreal, Canada).

Humic Acid

Two lignin-derived organic systems were obtained from diverse organic materials using the methodology described by the

International Humic Substances Society (IHSS) to extract humic substances and HA, as described in [24]. A first HA was extracted from black peat (Galicia, Spain) (HA8) and the other one from red Quebracho (*Schinopsis* spp.) barks (HA10) [20].

Hepatoprotective Activity

Hepatotoxicity was induced by oral feeding of ethanol (1 g/kg of body weight) for 10 days as described by Park *et al.* [22], by CCl_4 (0.5 ml/kg body weight in olive oil, injected ip.) according to Prasanna and Purnima [25] or by an ip. injection of 100 ng/kg body weight of (LPS) as described by Olleros *et al.* [21]. Alcohol was diluted in water, LPS in phosphate-buffered saline (PBS). Mice were randomly divided into several groups and administered orally by gavage during 14 days as follows: Group 1 - treated with glucan; Group 2 - treated with AH8; Group 3 - treated with AH10; Group 4 - treated with a combination of glucan, AH8, and AH10; and Group 5 - control group treated with PBS. At the end of the study, blood was collected and serum prepared. After that, mice were sacrificed and livers were immediately excised and used for homogenates.

Biochemical Markers

The enzymatic activities of aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were assayed spectrophotometrically by (Antech Diagnostics, Louisville, KY, USA). Liver homogenate was prepared by the following technique: Livers were excised and rinsed in saline. A small section from each liver was placed in 10% PBS-formalin solution to be used in histological slides. The rest was frozen in liquid nitrogen. Frozen liver was ground to a fine powder and 20-25 mg of powder was solubilized. The glutathione (GSH) levels were measured by the GSH test kit (Dojindo Labs, Kumamoto, Japan), superoxide dismutase (SOD) as described by Prasanna and Purnima [25] and malondialdehyde (MDA) [26].

Statistical Analysis

Data were expressed as means ± standard deviation. Statistical analysis was performed by a Pair *t*-test using a GraphPad Prism 502 software (GraphPad Software, USA). Values of $P \leq 0.05$ were considered statistically significant.

RESULTS

All three materials used in our study represent widely used experimental models of liver damage. All animals were randomly selected into individual groups. In one group, the liver damage was induced by applying one of the three treatments (LPS, CCl_4 or ethanol). Four other groups were treated with either glucan, two different types of HA, or a combination of glucan and both HA. These four groups were pretreated with these materials for 14 days before the liver-damaging treatment.

Use of CCl_4, LPS or ethanol caused significant stimulation of serum levels of AST, ALT, and ALP [Figures 1-3]. Pretreatment of tested material showed that all four tested groups significantly decreased the levels of these enzymes, and the glucan-HA combination was always the most active. Similar data were found when we focused on hepatic enzymes. Our experimental treatment caused a strong decrease in the levels of GSH [Figure 4], stimulated the level of MDA [Figure 5] and decreased the levels of SOD [Figure 6]. Again, all our prophylactic treatments are active and significantly improved the liver damage tested by enzymatic levels. As in the first part of the study, the glucan-HA combination showed the strongest effects.

DISCUSSION

Liver damage caused by chemotherapeutic agents is of intense interest to both researchers and clinicians. Ethanol, LPS, and CCl_4 represent well-established models of hemotoxic damage of the liver tissue. Ethanol works via live metabolic processes changing over 80% of ethanol to the highly toxic acetaldehyde which is further oxidized into various oxygen species [27]. CCl_4 is an extremely potent liver toxin, as a single exposure to CCl_4 causes a rapid increase in the levels of numerous enzymes, necrosis, and steatosis [28]. In the case of CCl_4, this type of liver injury is the most intensively studied model for xenobiotic-induced oxidative hepatotoxicity [29], making this type of liver damage to be the model of choice for screening efficacy of various possible hepatoprotective drugs. Most of the toxic effects are caused by trichloromethyl free radicals [4].

More and more natural molecules are gaining attraction in the fight against liver damage. Among those, glucans are considered to be the most promising. Mushroom glucan was found to have both hepato- and nephroprotective effects in rats [30], similar effects were described for glycoprotein Antrodan [31]. Oat-derived glucan was found to inhibit LPS-induced liver damage [32] and a glucan-melatonin combination had protective effects against liver injury [33]. Our own data showed the palliative effect of a glucan-HA combination of liver damage caused by LPS, ethanol of CCl_4 [20,34].

All of these studies evaluated the effects of glucans or other natural molecules after the liver injury, which means they were used either simultaneously or after the liver toxic treatment. This study focused on the question of whether the glucan-HA combination, which so strongly reversed the experimentally induced liver damage, also offers a protection. Therefore, we treated the animals with our samples before we used the liver damaging agent.

Many authors have repeated that lipid peroxidation is closely associated with liver pathogenesis. MDA is a byproduct of oxidant-induced liver protein and lipid oxidation, GSH is a component of the antioxidant system. SOD represents endogenous antioxidant and acts via dysmutation of superoxide anions.

The mechanisms of the liver protections are still unknown despite intensive research. Glucans are well established free

radical scavengers wit antioxidant effects [35]. Therefore, we hypothesized that it might have positive effects on liver damage. Similarly, antioxidant effects were described in HA [36], because of the sharp increase in the level of free radicals was described in liver damage [37].

The efficacy of any hepatoprotective molecule strongly depends on its ability to suppress damaging effects. The results of our study showed that daily oral supplementation helped to reduce the levels of ALT and AST in the serum of tested animals. HA alone and the combination in particular were very active, offering a significant decrease in liver damage. The prophylactic effects might be caused by strong potentiation of the antioxidant protective system, supported by protection of the GSH levels depressed by the liver damage by hepatotoxins. CCl_4 is metabolized by the cytochrome 450 to the trichloromethyl free radical [38], which subsequently forms trichloromethyl peroxyl radical attacking lipids on the endoplasmic reticulum leading to cellular necrosis [39]. It is possible that the glucan-HA combination acts by mopping up these free radicals and therefore limiting their damaging effects.

In summary, our findings showed that the inflammatory response to liver toxic agents was significantly decreased by pretreating the animals with orally-supplemented humic-acid combination. These effects were in agreement with inhibiting of the changes in AST, ALT, ALP, GSH, SOD, and MDA, caused by experimentally-induced liver damage. Therefore, we propose that the HA-glucan combination might offer a good possibility of natural molecules helping to reduce damage to the liver.

REFERENCES

1. Sun ZL, Gao GL, Xia YF, Feng J, Qiao ZY. A new hepoprotective saponin from Semen Celosia cristatae. Fitoterapia 2011;82:591-4.
2. Wang Y, Lou Z, Wu QB, Guo ML. A novel hepatoprotective saponin from Celosia cristata L. Fitoterapia 2010;81:1246-52.
3. Yang J, Li Y, Wang F, Wu C. Hepatoprotective effects of apple polyphenols on CCl4-induced acute liver damage in mice. J Agric Food Chem 2010 26;58:6525-31.
4. Brent JA, Rumack BH. Role of free radicals in toxic hepatic injury. II. Are free radicals the cause of toxin-induced liver injury? J Toxicol Clin Toxicol 1993;31:173-96.
5. Jadeja RN, Thounaojam MC, Ansarullah, Jadav SV, Patel MD, Patel DK, *et al.* Toxicological evaluation and hepatoprotective potential of Clerodendron glandulosum.Coleb leaf extract. Hum Exp Toxicol 2011;30:63-70.
6. Haanen BF. Facts about Peat. Ottawa: Mines Brand Publ Can Dept Mines; 1924. p. 614-56.
7. Jooné GK, Dekker J, van Rensburg CE. Investigation of the immunostimulatory properties of oxihumate. Z Naturforsch C 2003;58:263-7.
8. Klocking R, Sprossing M, Witzer P, Thiel KD, Helbig B. Anti-viral wirks same huminsaureahnliche Polymere. Z Physiother 2003;33:95-101.
9. Novak M, Vetvicka V. Beta-glucans, history, and the present: Immunomodulatory aspects and mechanisms of action. J Immunotoxicol 2008;5:47-57.
10. Novak M, Vetvicka V. Glucans as biological response modifiers. Endocr Metab Immune Disord Drug Targets 2009;9:67-75.
11. Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, *et al.* Mechanism by which orally administered beta-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. J Immunol 2004;173:797-806.
12. Brown GD, Herre J, Williams DL, Willment JA, Marshall AS, Gordon S. Dectin-1 mediates the biological effects of beta-glucans. J Exp Med 2003;197:1119-24.
13. Vetvicka V. β-Glucans as Natural Biological Response Modifiers. New York: Nova Biomedical; 2013.
14. Vetvicka V, Vetvickova J. Effects of yeast-derived beta-glucans on blood cholesterol and macrophage functionality. J Immunotoxicol 2009;6:30-5.
15. Vetvicka V, Vancikova Z. Anti-stress action of several orally-given β-glucans. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2010;154:235-8.
16. Richter J, Svozil V, Král V, Rajnohová Dobiášová L, Stiborová I, Vetvicka V. Clinical trials of yeast-derived ß-(1,3) glucan in children: Effects on innate immunity. Ann Transl Med 2014;2:15.
17. Vetvicka V, Baigorri R, Zamarreño AM, Garcia-Mina JM, Yvin JC. Glucan and humic acid: Synergistic effects on the immune system. J Med Food 2010;13:863-9.
18. Uskokovic A, Mihailovic M, Dinic S, Jovanovic AJ, Grdovic N, Markovic J, *et al*. Administration of a β-glucan-enriched extract activates beneficial hepatic antioxidant and anti-inflammatory mechanisms in streptozotocin-induced diabetic rats. J Funct Foods 2013;5:1966-74.
19. Keles ON, Can S, Cigsar G, Colak S, Erol HS, Arakas N, *et al*. Hepatoprotective effects of βef,3-(D)-glucan on bortezomib-induced liver damage in rats. Kafkas Univ Vet Fakul Derg 2014;20:929-38.
20. Vetvicka V, Garcia-Mina JM, Proctor M, Yvin J-C. Synergistic effects of humic acid and glucan in hepatoprotection against experimental liver injury. Austin J Clin Pathol 2014;1:4.
21. Olleros ML, Vesin D, Fotio AL, Santiago-Raber ML, Tauzin S, Szymkowski DE, *et al.* Soluble TNF, but not membrane TNF, is critical in LPS-induced hepatitis. J Hepatol 2010;53:1059-68.
22. Park HJ, Lee SJ, Song Y, Jang SH, Ko YG, Kang SN, *et al.* Schisandra chinensis prevents alcohol-induced fatty liver disease in rats. J Med Food 2014;17:103-10.
23. Ferreira EA, Gris EF, Felipe KB, Correia JF, Cargnin-Ferreira E, Wilhelm Filho D, *et al.* Potent hepatoprotective effect in CCl(4)-induced hepatic injury in mice of phloroacetophenone from *Myrcia multiflora*. Libyan J Med 2010 8;5.
24. Fuentes M, Olaetxea M, Baigorri R, Zamarreño MA, Etienne P, Laineb P, *et al*. Main binding sites involved in Fe(III) and Cu(II) complexation in humic-based structures. J Geochem Explor 2013;129:14-7.
25. Prasanna GS, Purnima A. Protective effect of leaf extract of *Trichilla connaroides* on hypercholesterolemia induced oxidative stress. Int J Pharmacol 2011;6:106-12.
26. Yadav D, Hertan HI, Schweitzer P, Norkus EP, Pitchumoni CS. Serum and liver micronutrient antioxidants and serum oxidative stress in patients with chronic hepatitis C. Am J Gastroenterol 2002;97:2634-9.
27. Tuma DJ, Casey CA. Dangerous byproducts of alcohol breakdown – focus on adducts. Alcohol Res Health 2003;27:285-90.
28. Zimmerman, H. Chemical hepatic injury and its detection. In: Plaa RO, Hewitt W, editors. Toxicology of the Liver. New York: Raven Press; 1982. p. 1-45.
29. Brautbar N, Williams J. Industrial solvents and liver toxicity: Risk assessment, risk factors and mechanisms. Int J Hyg Environ Health 2002;205:479-91.
30. Nworu CS, Ihim SA, Ugwu LE, Laiyemo KA, Akah PA. Hepato- and nephroprotective activities of a Nigerian local king tuber oyster mushroom, *Pleurotus tuberregium* (higher Basidiomycetes), in chemically induced organ toxicities in rats. Int J Med Mushrooms 2014;16:305-18.
31. Ker YB, Peng CC, Chang WL, Chyau CC, Peng RY. Hepatoprotective bioactivity of the glycoprotein, antrodan, isolated from *Antrodia cinnamomea* mycelia. PLoS One 2014;9:e93191.
32. You S, Hu X, Zhao Q, Chen X, Xu C. Oat ß-glucan inhibits lipopolysaccharide-induced nonalcoholic steatohepatitis in mice. Food Funct 2013;4:1360-8.
33. Aydogan MS, Erdogan MA, Polat A, Yücel A, Ozgül U, Parlakpinar H, *et al.* Protective effects of melatonin and ß-d-glucan against liver injury in rats - a comparative study. Adv Clin Exp Med 2013;22:621-7.
34. Vetvicka V, Garcia-Mina JM, Proctor M, Yvin JC. Humic Acid and glucan: Protection against liver injury induced by carbon tetrachloride. J Med Food 2015;18:572-7.
35. Kofuji K, Aoki A, Tsubaki K, Konishi M, Isobe T, Murata Y. Antioxidant activity of ß-Glucan. ISRN Pharm 2012;2012:125864.
36. Graf E. Antioxidant potential of ferulic acid. Free Radic Biol Med 1992;13:435-48.

37. Lukivskaya OY, Naruta E, Sadovnichy V, Kirko S, Buko VU. Reversal of experimental ethanol-induced liver steatosis by borage oil. Phytother Res 2012;26:1626-31.
38. Clawson GA. Mechanisms of carbon tetrachloride hepatotoxicity. Pathol Immunopathol Res 1989;8:104-12.
39. Rees KR, Spector WG. Reversible nature of liver cell damage due to carbon tetrachloride as demonstrated by the use of phenergan. Nature 1961;190:821-2.

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Review Article

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Biomedical properties and potentiality of *Lippia microphylla* Cham. and its essential oils

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ABSTRACT

Lippia microphylla Cham. (Verbenaceae) is an endemic underexploited Brazilian vegetal. This work reviewed the biological potentialities of *Lippia microphylla*, emphasizing the properties of essential oils (EOs) and analyzed scientific indicators about genus *Lippia* and *L. microphylla*. Databases from 1948 to the present were searched and a software (vantage point 7.1) associated with Derwent Innovation Index was used to identify the indicators of the genus *Lippia*, and biological activities and compounds in the *L. macrophylla* species. Ethnopharmacological records report use of *L. microphylla* leaves to treat gastrointestinal disorders, influenza, bronchitis, cough, nasal congestion, and sinusitis during vaporization, whose aromatic volatile oils are rich in monoterpenes, especially cineole, terpineol, and thymol. Other EOs have larvicidal activity on *Aedes aegypti* larvae, and antifungal, antibacterial and cytotoxic and antitumor action on human and murine cancer cells. Brazil is the country with more articles about *Lippia* species, but it deposited only 9 patents since 1993. Most of the publications about *L. microphylla* are concentrated in food and chemical sciences. This bioprospection helps to choice areas of interest for capital investment and to give support for Brazilian Institutions to establish cooperation and improve technological impact at the point of view of creation and innovation.

KEY WORDS: Antitumor action, *Lippia microphylla*, pharmacological activity, scientific indicators, terpenes

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INTRODUCTION

About 80% of the worldwide population use herbal products for their basic health care (primary care), such as extracts, teas and their active principles, a market estimated at US$ 50 billion per year [1]. Despite the interest in molecular modeling, combinatorial chemistry and other chemical synthesis techniques by institutions and pharmaceutical industries, the natural products, particularly medicinal plants, persist as an important source of new therapeutic agents against infectious (fungal or bacterial) and cardiovascular diseases, insects, cancer, and immunomodulation [2-6].

Genus *Lippia* (Verbenaceae, Lamiales/Magnoliopsida) includes about 200 species of herbs, shrubs and small trees mainly distributed in Central and South Americas and in Africa Tropical [7-8]. Some *Lippia* species are prevalent at "Caatinga" biome, a region with approximately 1,539,000 km^2, distributed in nine Brazilian Northeastern states, with warm and dry climate and where grows a peculiar xerophyte vegetation. In "Caatinga flora," there are almost 1 000 vascular plant species. Because of the extreme climate conditions most species are endemic and present particular morphological adaptations [3,4,9,10].

Among the *Lippia* species, *L. microphylla* Cham. (Syn.: *L. microphylla* Cham. and Schlecht.; *L. microphylla* Mart.; *Lantana microphylla* Mart. ex Uphof.) is recognized for its therapeutic usages [Figure 1] [11]. A few studies have explored specific therapeutic points of *L. microphylla*. Then, this

Figure 1: General aspects of *Lippia microphylla* Cham

work aimed to review its biological potentialities, emphasizing the properties of essential oils (EOs). Moreover, in order to suggest a strategic plan for genus *Lippia* and *L. microphylla*, main publication areas, its respective patents and institutions and authors were also analyzed to identify studies and orientate the development of pharmaceutical products.

For a complete and reliable review, primary and secondary resources were used, including original and review articles, books and government documents written in English, Portuguese or Spanish. Databases searched were Lilacs-Bireme (Databases on Latin American Health and Biological Sciences), MEDLINE/Index Medicus (Medical Literature Analysis and Retrieval System Online), SciELO (Scientific Electronic Library Online), Web of Science, PubMed (maintained by the National Library of Medicine) and Science Direct. A software (Vantage Point 7.1) associated with Derwent Innovation Index was used to performer bibliometric analyses, data generation, and identification of quantitative scientific indicators from 1948 to the present. Therefore, it was used the following keywords: *Lippia*, biological properties, cytotoxicity, folk use, *L. microphylla*, and EOs. Vantage Point version 7.1 is a powerful text-mining tool for discovering knowledge in search results from patent and literature databases, giving a better perspective about information and enabling to clarify relationships and find critical patterns in distinct areas of expertise.

L. microphylla Cham: Phytochemistry and Pharmacology

Lippia species have shown a large number of important usages in folk medicine for various diseases, particularly in the treatment of cough, bronchitis, indigestion, liver, hypertension, dysentery [12-14], worms, and skin diseases [15]. Many species have promising biological activities, including antiviral [16], antimalarial [17], anti-inflammatory, analgesic, antipyretic [18], molluscicidal against *Biomphalaria glabrata* [15], antimicrobial [19-25], insecticidal [26], and anticonvulsant [27] properties. Compounds isolated from *Lippia* also revealed *in vitro* antitumor activity on leukemia (K-562, HL-60, CEM), colon (HCT-116), breast (MCF-7), glioblastoma (U-251), and prostate (PC -3) cell lines [28-30]. Besides its medicinal properties, the leaves of the most *Lippia* species are used for food preparation. Moreover, it is interesting to note the importance of *Leptotyphlops dulcis*, whose main component of leaves and flowers is (+)-hernandulcine, a molecule 1000-fold sweeter than sucrose [14,31,32].

Popularly called as "alecrim-da-chapada," "alecrim-de-tabuleiro," "alecrim-pimenta" and "alecrim-do-mato" in the Northeast Brazilian, *L. microphylla* is a deciduous shrub with a thin and brittle stem (up to 2 m in height), white flowers and with simple and aromatic leaves, which presents serrate margins and evident nerves with no more than 1 cm in length [11,33,34].

Ethnopharmacological records report the use of *L. microphylla* leaves to treat gastrointestinal disorders and influenza, bronchitis and sinusitis during vaporization resulting from boiling water. Phytochemical studies revealed the presence of quinones and flavonoids from stem and roots' ethanol extracts [33,34]. Meanwhile, its aromatic volatile oils extracted by water vapor exhibit an EOs rich in monoterpenes, especially cineole, and terpineol, its more likely active principles.

Secondary metabolites of plants, many of them produced to protect against microorganisms and predator insects, are natural candidates for the discovery of new active products [5,6,25,35,36]. The cineole is responsible for the *Eucalyptus globulus* balsamic activity. The saturated antiseptic and balsamic vapors with cineole and other EOs found in *L. microphylla* lighten respiratory tract mucous membranes during congestion, which explains its folk use for the treatment of influenza, cough and nasal congestion. Its compounds are capable of fluidizing bronchial secretion, facilitating expectoration, and decreasing cough reflex and refreshing breath. Due to these balsamic properties, home practices of inhalation to alleviate symptoms of respiratory diseases are considered an easy way to treat them. To prepare the inhalation, leaves (50-60 g) are put in boiling water, and the person inhales the fumes through a resistant funnel, being careful to heat both parts of the face where sinus are positioned. If the EO is available, it should be used 1-2 mL per 1-2 L of boiling water [11].

Some works published previously have also highlighted the insecticidal importance of the EOs presented in *Lippia* species [37-40]. EOs of *L. microphylla*, in particular, showed significant larvicidal activity on *A. aegypti* larvae, with a LD_{50} of 75.6 ppm [39]. Gleiser *et al*. [41] showed that *Lippia integrifolia* and *Lippia junelliana* oils are potent repellents against *A. aegypti* adult insects. Both species contained similar quantities of limonene and camphor (20.7% and 26.5%), though they differ in others such as myrcene that was detected in *L. junelliana* (14.1%) and methylheptenone in *L. integrifolia* (24.9%). Limonene is a registered active element in pesticide products used as insecticide and repellent, and cotton fabrics treated with limonene have shown repellence against *Anopheles* mosquitoes. In addition, repellent properties against *A. aegypti* have been reported for limonene, camphor, and myrcene [42,43]. Thus, it is possible that the higher repellence of *L. junelliana* compared to *L. integrifolia* is due to the additional repellent effect of myrcene

and this compound can be responsible for the insecticidal activity found in *L. microphylla*, since myrcene is also present in its leaves [44].

Oils from *L. microphylla* also revealed antifungal (strains of *Aspergillus niger*, *Fusarium* spp., *Rizhopus* spp., and *Rhizoctonia solani*) and antibacterial activities (*Staphylococcus aureus*, *Shigella flexneri*, *Escherichia coli*, and *Streptococcus pyogenes*) using gel diffusion methods [20,45,46]. A tolerance of Gram-negative bacteria to EOs, such as *E. coli*, has been attributed to the existence of a hydrophilic outer layer, which may be blocks the infiltration of hydrophobic components throughout the cell membrane. On the other hand, the inhibitory action of natural products on mold cells involves cytoplasm granulation, plasmatic membrane disrupting and inactivation and/or synthesis inhibition of enzymes. These actions can appear isolate or simultaneously, leading to the mycelium propagation, and growth inhibition [47]. In fact, oils rich in monoterpenic compounds are reported to exhibit high levels of antimicrobial activity [48]. Moreover, they are probably responsible, at least in part by the antioxidant activity in *L. microphylla* extracts [49].

Volatile Components of *L. microphylla*

In the Verbenaceae family, genus *Lippia* products great quantities of volatile mixtures. Chemical studies by gas chromatographic techniques of volatile constituents from *L. microphylla* leaves resulted in the identification of α-pinene (1), sabinene (2), β-pinene (3), β-myrcene (4), p-cymene (5), 1,8-cineole (6), γ-terpinene (7), 4-terpineol (8), α-terpineol (9), anisole (10), thymol (11), and carvacrol (12) [Figure 2]. Among them, the main components are 1, 8-cineol (36%), β-pinene (11%), and thymol (11%) [44]. Silva *et al.* [50] identified the germacrene D (13) and bicyclogermacrene (14) [Figure 2] from EOs and hexanic fractions as the most common compounds in specimens collected in dry and rainy periods, while the major monoterpene was α-pinene.

Fixed Components of *L. microphylla*

Analyses of the fixed constituents of the ethanolic extracts of *L. microphylla* roots and stems result in isolation of a flavonol glycoside (15) and four quinones, two prenylated naphthoquinone dimers (16 and 17) and two furan naphthoquinones (18 and 19) [Figure 3]. Among these, microphyllaquinone (16) and a mixture of 6-methoxy- and 7-methoxy-naphtho[2,3-b]-furan-4,9-quinones (18 + 19, respectively) isolated from *L. microphylla* were evaluated for their cytotoxicity, using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method [51], which analyzes the ability of living cells to reduce the yellow dye MTT to a purple formazan product. According to Santos *et al.* [34], the molecules microphyllaquinone and the mixture demonstrated cytotoxic potential against a panel of different murine and human cancer cell lines (B-16 [murine melanoma], CEM [lymphocyte leukemia], HL-60 [promyelocyte leukemia], HCT-8 [colon adenocarcinoma,] and MCF-7 [breast adenocarcinoma]), with IC_{50} values ranging from 0.69 to 3.13 μg/mL [Table 1].

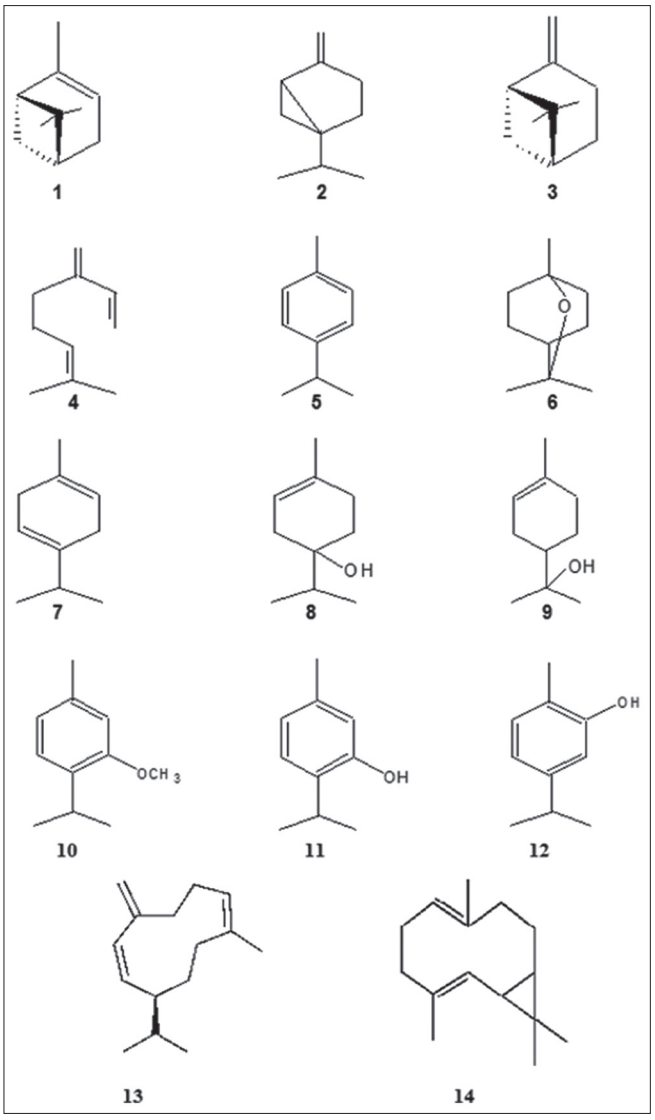

Figure 2: Volatile essential oils from *Lippia microphylla* Cham

The EO of *L. microphylla* leaves also showed *in vitro* cytotoxic action on Sarcoma 180 (IC_{50} of 100.1 [94.9-105.5] μg/mL) and human chronic myelocyte leukemia K-562 cells (IC_{50} of 51.9 [47.9-56.3] μg/mL) [Table 2]. Lytic activity was not detected in normal erythrocytes from Swiss mice at a concentration of 250 μg/mL. After 7 days of treatment, this same oil presented *in vivo* antitumor action in a dose-dependent way, and tumor growth inhibition of 38.2% and 59.8% (50 and 100 mg/kg, respectively) [52].

Interestingly, in the presence of cyclosporine A, an inhibitor of the pore formation in the mitochondrial permeability transition, it was detected reduction in the cytotoxicity on Sarcoma 180 cells with this EO (IC_{50} of 118.3 [113.7-123.1] μg/mL). Hence, declining in cytotoxicity in the presence of cyclosporine A is indicative of the intrinsic pathway involvement in the mechanism of death [Table 2], whereas after adding pore inhibitors, probably occurred the mitochondrial pores' opening blocking, which inhibited the release of pro-apoptotic

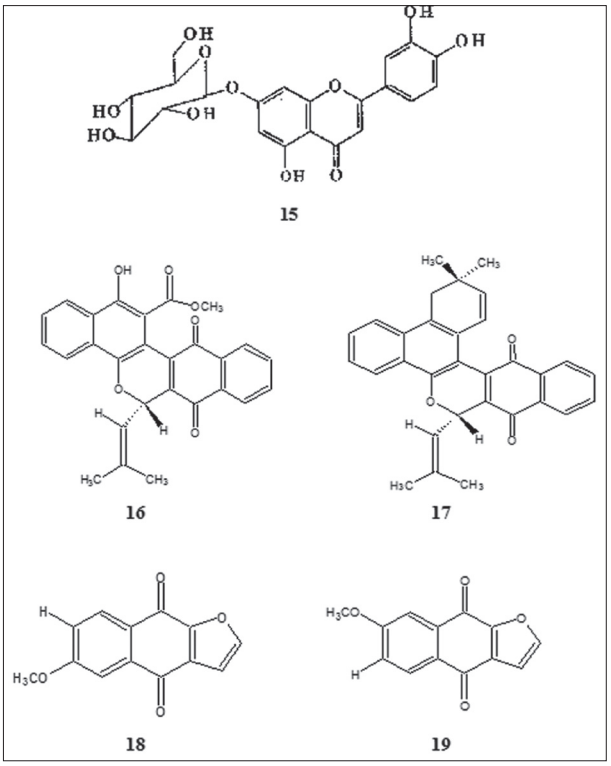

Figure 3: Fixed components of *Lippia microphylla* Cham

Table 1: Cytotoxicity of naphthoquinones isolated from *L. microphylla* and analyzed by MTT assay after 72 h exposure

Cancer cell line	IC_{50} (μg/mL)	
	16	18+19
B-16 (murine melanoma)	3.13±0.12	0.77±0.05
CEM (human lymphocyte leukemia)	2.57±0.33	1.61±0.25
HL-60 (human promyelocyte leukemia)	2.92±0.13	1.62±0.09
HCT-8 (human colon adenocarcinoma)	2.33±0.29	0.69±0.06
MCF-7 (human breast adenocarcinoma)	2.44±0.13	1.30±0.05

Data presented as IC_{50} values ± SEM from three independent experiments with cancer lines. 16, microphyllaquinone; 18+19, mixture of 6-methoxy- and 7-methoxy-naphtho [2,3-b]-furan-4,9-quinones. Adapted from Santos *et al.* (2003), *L. microphylla: Lippia microphylla*, SEM: standard error of measurement

proteins and reduced the cytotoxicity of the oil. Similarly, when K-562 cells were treated with the EO in the presence of N-acetylcysteine, an antioxidant molecule and scavenger of free radicals that stimulates the biosynthesis of reduced glutathione, cytotoxicity diminution of the oil was also evidenced [52,53]. Then, it was proposed that reactive oxygen species (ROS) production is involved, at least partially, in the mechanism of cytotoxicity on K-562 cells, and activation of apoptotic intrinsic pathways in Sarcoma 180 cells. In fact, mitochondrial membrane permeabilization culminates in cytochrome *c* release. Cytochrome *c* binds to Apaf-1 (apoptotic protease-activating factor 1) and generate the catalytically active form of caspase-9, which activates caspase-3, the most important effector caspase that acts as an effective DNase to slice the genomic DNA into nucleosomes, producing fragments of 180-200 base pairs, degradation of laminin and mitotic apparatus proteins and nuclear and cellular reduction and pyknosis [54,55].

Table 2: *In vitro* antitumor activity of the essential oil from *L. microphylla* leaves determined by MTT assay after 72 h exposure

Substance	IC_{50} (μg/mL)	
	Sarcoma 180 (experimental murine tumor)	K-562 (human chronic myelocyte leukemia)
EOs	100.1 (94.9-105.5)	51.9 (47.9-56.3)
EOs+GSH	107.3 (102.8-111.9)	55.4 (55.4-55.6)
EOs+NA	109.2 (103.8-114.9)	94.2 (85.8-103.4)
EOs+CA	118.3 (113.7-123.1)*	51.9 (47.9-56.3)

Data presented are IC_{50} values and 95% confidence intervals for K-562 and Sarcoma 180 cells. GSH: Reduced glutathione; NAC: *N*-acetylcysteine; CA: Cyclosporine A. *$P<0.05$ compared to control by ANOVA followed by *Turkey* test. Adapted from Xavier (2011), *L. microphylla: Lippia microphylla*, EOs: Essential oils

Releasing of the molecules due to alterations in mitochondrial permeability transition leads to the loss of cellular homeostasis, preventing ATP synthesis, and increasing production of ROS. Studies of certain antineoplasic agents in distinct cell lines have demonstrated that some substances execute their activity by oxidative stress caused by ROS, generally occurring when the homeostasis of oxidation and reduction is altered within cells. Since ROS possess strong chemical reactivity with biomolecules such as proteins and DNA, this may result in DNA denaturation, leading to changes in protein synthesis and cell duplication. Furthermore, it is known that ROS induce activation of caspases-3 and -9 [55-57].

SCIENTIFIC DATA FOR PHYTOTHERAPIC DEVELOPMENT

Before formulating the action plan for implementation of the herbal medicine, we suggest the prospection of main institutions, researchers and areas of knowledge in a defined timeline to direct the strategic planning [58,59]. From this perspective, the prospecting is a manner to anticipate advances and can influence the orientation of technological trajectories [60]. Within the context of herbal medicines, this tool allows to target search according to which has already been produced, and to establish partnerships or cooperation that leverage innovation as determined by the requirements of public and private institutions and government agencies.

Using *Lippia* as keyword to search in the Web of Science and Derwent Innovation Index, a total of 691 articles and 94 patents were found, respectively. When the investigation was refined for *L. microphylla*, the number of manuscripts decreased for 10 and patents were not found. The first article citing *Lippia* species was published in 1948. After that, there was a vertiginous growth of manuscripts, especially in the 2000s, as seen in the Figure 4. The countries that more published about *Lippia* were Brazil, Argentina, México and United States of America [Figure 5].

It is important to note that this rising of articles approaching *Lippia* species overlaps with the implantation of the National Program on Biodiversity, whose objectives include investments and management of funds to produce new medicines [58]. In fact, identifying which are the most important needs and

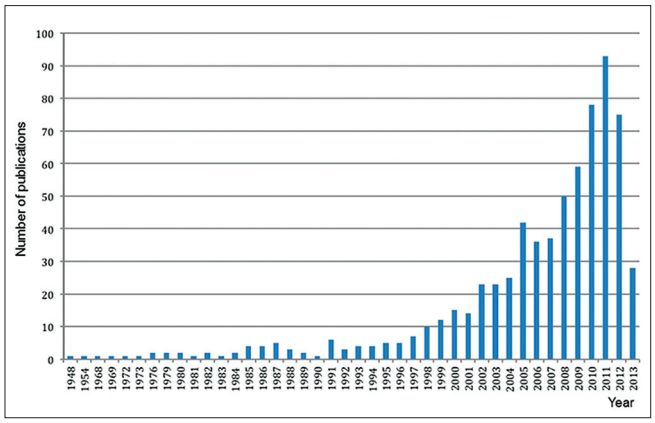

Figure 4: Temporal evolution on the number of published articles involving the genus *Lippia* (Vantage Point 7.1)

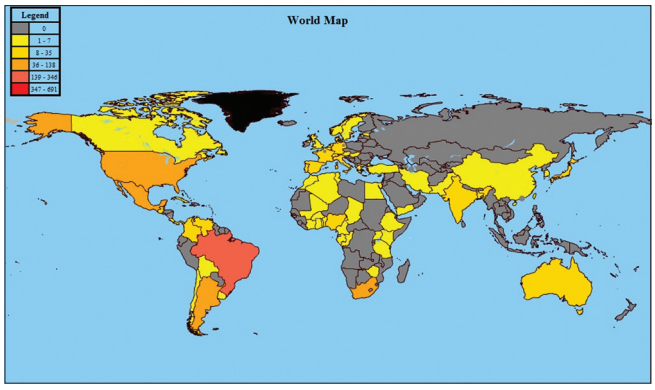

Figure 5: World participation in publications belonging to the genus *Lippia* (Vantage Point 7.1)

opportunities for Research and Development (R and D) in the future, from planned interventions in innovation systems can be an important step in established programs in Brazil as the National Program on Medicinal Plants and Phytotherapics. This program, approved in 2006, aims to ensure safe access and rational use of medicinal plants and phytotherapics by the population based on the list of regulated plants by ANVISA (National Agency of Sanitary Surveillance in Brazil) [61].

In relation to *Lippia* species, *L. alba* (142 articles) and *L. sidoides* (97 articles) are the species that have received more attention [Figure 6]. On the other hand, *L. microphylla* has a few reports about its biology and pharmacology as described above. These results are confirmed by the Figure 7 that presents the areas of publications on *L. microphylla*, most of them concentrated in food and chemical sciences.

Figure 8 shows the interaction between Brazilian and international institutions. This data is extremely important to identify universities involved in the study about *L. microphylla*, and their partnerships and cooperation in order to direct funding, define methods and technology to implement the appropriated findings. Research groups at the Federal University of Ceará and Federal University of Paraíba are the Brazilian institutions with more articles about *L. microphylla*. EMBRAPA Foundation works without partnerships or, at least, it did not

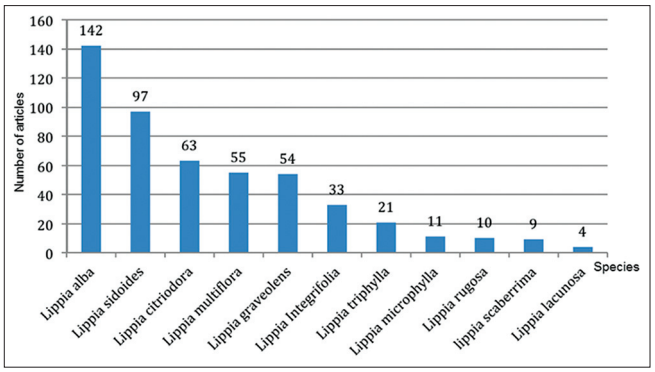

Figure 6: Number of articles about the major species belonging to the genus *Lippia* (Vantage Point 7.1)

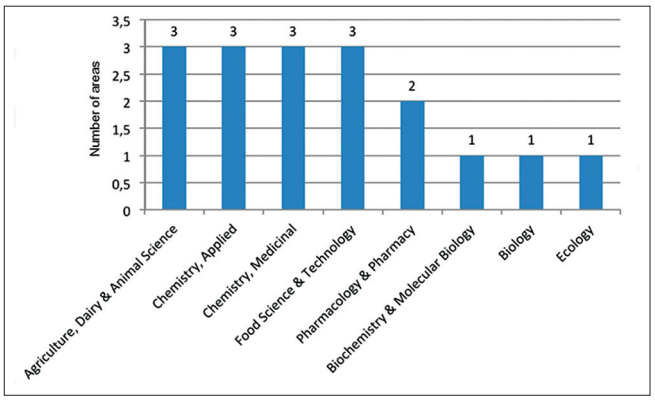

Figure 7: Areas of publications about *Lippia microphylla* (Vantage Point 7.1)

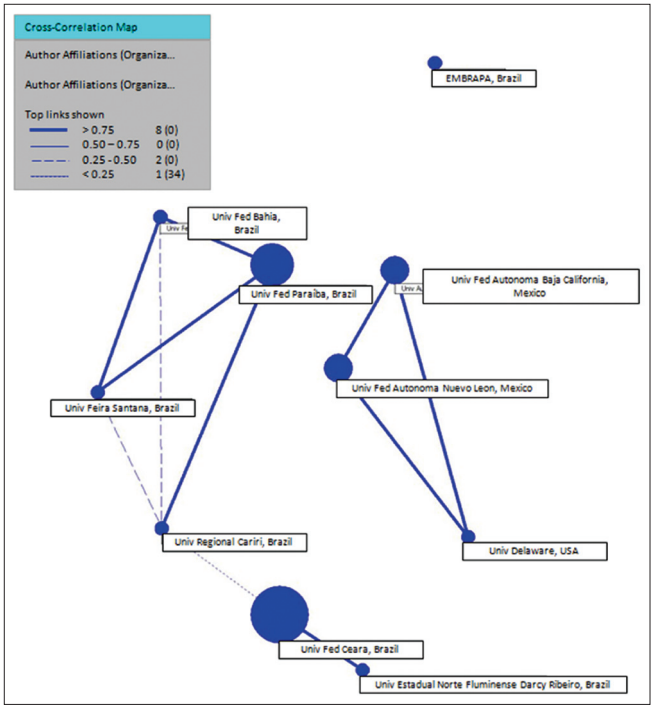

Figure 8: Institutions and their collaborations about *Lippia microphylla* publications (Vantage Point 7.1)

publish works with other institutions yet. Universities of USA (Delaware) and Mexico often work together. The academic

institutions around the world are the leading centers for generation of new patentable technologies, as observed in this study. However, Brazil has low competitivity and shows little effort to innovate in the area of technological inventions, probably due to some failures in the innovation system (cooperation between government, business, and institutions to promote an effective system of production and development of medicines). Then, Brazil does not have a valuable protecting structure of *Lippia* species, reflecting the lack of incentives to safeguard the technologies developed using industrial property.

Finally, in 2004, it was promulgated a Brazilian law about Technological Innovation (number 10,973), which was regulated in 2005 (Decree 5,563). This law normalizes the incentives for the involvement of Scientific and Technological Institutions (Institutos de Ciência e Tecnologia - ICT's) in the innovation process for innovation in companies, for the independent inventor and conception of investment funds for innovation. It is the first Brazilian law that deals with the relationship between Universities and/or Research Institutions and companies with the creation of Technological Innovation Centers (Núcleos de Inovação Tecnológica - NIT's), helping the institutional maturity to make strategic management of intellectual property in the Brazilian ICT's [62].

Despite a high Brazilian scientific production, these findings did not trigger large impacts on the economic development. Numbers of patents is far from the quantity of published articles, existing an abysmal between which is published which is patented, and which would become a product or service to generate work and wealth to the country [Figure 9]. Japan made its first deposit in 1990 and, currently, it is the largest holder of patents (36). Brazil arose in 1993 and deposited only 9 patents. First was recorded in 1987 [Figure 10]. Thus, these data are not consistent with the Brazilian scientific production about *Lippia* species and the number of published articles, suggesting a lack of encouragement in researches for the development of inventions involving *Lippia* species. Moreover, the scientific production in Brazil is recent (last 100 years), and is concentrated in public universities and research centers, and in honorable exceptions, in private institutions, as a result of the Brazilian public policies in Science and Education [63]. Certainly, this is the capital challenge in the Brazilian national innovation system for the transfer of technology generated in universities and research centers to industry, in a way that new processes and products may be generated from these institutions.

The growing interest in plant products with different purposes is linked, in part, to the low cost of drug production based on active principles isolated from natural products when compared to investments for synthesis in the laboratory [64]. In the context of the herbal market evolution, estimated at US$ 22 billion and corresponding to 3.7% of the global market, this growth accompanies the pharmaceutical industry, considered one of the most lucrative in the world. Brazil is considered the seventh largest market, and from 2006 to 2010 grew by 14%. In 2015, Brazil will be the sixth largest market in this sector [65]. Hence, Brazilian pharmaceutical and bio prospecting areas require constant and high investments in research and development

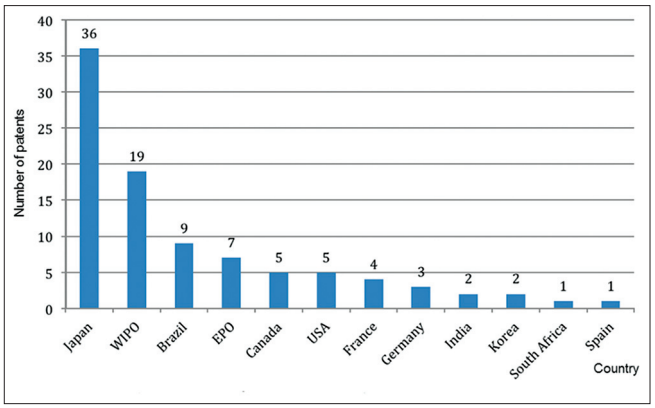

Figure 9: Countries with patents about *Lippia* species (Vantage Point 7.1). WIPO = World Intellectual Property Organization; EPO = European Organization; USA = United States of America

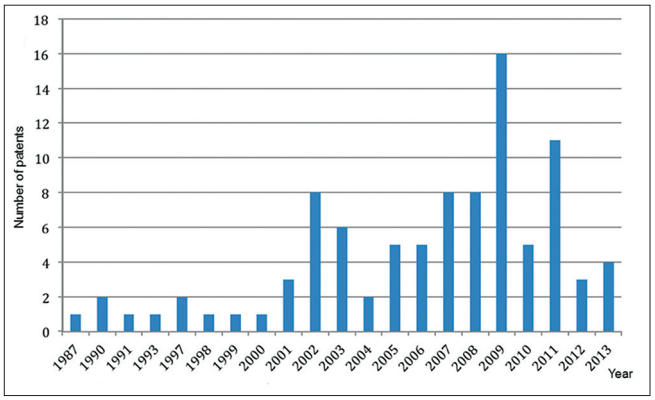

Figure 10: Number of patents about genus *Lippia* depositated from 1987 (Vantage Point 7.1)

of new products, since the natural resources have money-making value, attract investors and catch the attention to the preservation of endemic species.

CONCLUSION

Lippia species are a source of remarkable bioactive substances (such as EOs) with economic potential for local communities. Specifically, *L. microphylla* is an endemic underexploited Brazilian vegetal with great medicinal properties that has gained much attention in the general population and scientific community. Then, this bio prospection helps to build perspectives of the bioactivity areas of real interest for capital investment and to give support for Brazilian institutions to establish cooperation and partnerships in order to change the scientific reality, where basic and applied researches in pharmaceutical sectors are ineffective and without technological impact to create and innovate.

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REFERENCES

1. World Health Organization. The World medicines situation 2011. Traditional medicines: Global situation, issues and challenges. Geneva: WHO; 2011. p. 1-14.
2. Butler MS. The role of natural product chemistry in drug discovery. J Nat Prod 2004;67:2141-53.
3. Ferreira PM, Farias DF, Viana MP, Souza TM, Vasconcelos IM, Soares BM, *et al.* Study of the antiproliferative potential of seed extracts from Northeastern Brazilian plants. An Acad Bras Cienc 2011;83:1045-58.
4. Araújo EJF, Araújo DYML, Freitas RM, Ferreira PMP. Toxicology of the medicinal plant *Casearia sylvestris* Swartz: A literature review. Rev Cienc Farm Basica Apl 2014;35:355-61.
5. Militão GC, Dantas IN, Ferreira PM, Alves AP, Chaves DC, Monte FJ, *et al. In vitro* and *in vivo* anticancer properties of cucurbitacin isolated from *Cayaponia racemosa*. Pharm Biol 2012;50:1479-87.
6. Carvalho CC, Micheletti AC, Ferreira PM, Pessoa C, Fonseca TH, Gomes MA, *et al*. Biological screening of extracts of Brazilian *Asteraceae* plants. Afr J Pharm Pharmacol 2013;7:2000-5.
7. Terblanché FC, Kornelius G. Essential oil constituents of the genus *Lippia* (Verbenaceae) – A literature review. J Essent Oil Res 1996;8:471-85.
8. Santos JS, Melo JIM, Abreu MC, Sales MF. Verbenaceae sensu stricto in the region of Xingó: Alagoas e Sergipe, Brazil. Rodriguésia 2009;40:985-98.
9. Agra MF, Baracho GS, Nurit K, Basílio IJ, Coelho VP. Medicinal and poisonous diversity of the flora of "Cariri Paraibano", Brazil. J Ethnopharmacol 2007;111:383-95.
10. Santos JP, Araújo EL, Albuquerque UP. Richness and distribution of useful woody plants in the semi-arid region of Northeastern Brazil. J Arid Environ 2008;72:652-63.
11. Lorenzi H, Matos FJA. Medicinal Plants in Brazil: Natives e Exotics. Nova Odessa; 2002. p. 1-512.
12. Pham Huu Chanh, Koffi Y, Pham Huu Chanh A. Comparative hypotensive effects of compounds extracted from *Lippia multiflora* leaves. Planta Med 1988;54:294-6.
13. Souto-Bachiller FA, Jesus-Echevarria M, Cárdenas-Gonzalez OE, Acuña-Rodriguez MF, Meléndedez RA, Romero-Ramsey L. Terpenoid composition of *Lippia dulcis*. Phytochemistry 1997;44:1077-86.
14. Pascual ME, Slowing K, Carretero E, Sánchez Mata D, Villar A. Lippia: Traditional uses, chemistry and pharmacology: A review. J Ethnopharmacol 2001;76:201-14.
15. Matos FJA, Oliveira F. Lippia sidoides Cham. Pharmacognosy, chemistry and pharmacology. Rev Bras Farmacogn 1998;79:84-7.
16. Abad MJ, Bermejo P, Villar A, Palomino SS, Carraso L. Antiviral activity of medicinal plant extracts. Phytother Res 1997;11:198-202.
17. Valentin A, Pélissier Y, Benoit F, Marion C, Kone D, Mallie M, *et al.* Composition and antimalarial activity *in vitro* of volatile components of *Lippia multiflora*. Phytochemistry 1995;40:1439-42.
18. Forestieri AM, Monforte MT, Ragusa S, Trovato A. Antiinflammatory, analgesic and antipyretic activity in rodents of plant extracts used in African medicine. Phytother Re 1996;10:100-6.
19. Lemos TL, Matos FJ, Alencar JW, Craveiro AA. Antimicrobial activity of essential oils of Brazilian plants. Phytother Res 1990;4:82-4.
20. Souza EL, Lima EO, Freire KR, Sousa CP. Inhibitory action of some essential oils and phytochemicals on the growth of various moulds isolated from foods. Braz Arch Biol Technol 2005;48:245-50.
21. Botelho MA, Nogueira NAP, Bastos GM, Fonseca SGC, Lemos TLG, Matos FJA, *et al*. The antimicrobial The antimicrobial activity of essential oil of Lippia sidoides, carvacrol and thymol against oral pathogens. Braz J Med 2007;40:349-56.
22. Oliveira DR, Leitao GG, Bizzo HR, Lopes D, Alviano DS, Leitão SG. (2007). Chemical and antimicrobial analyses of the essential oil of *Lippia origanoides* H.B.K. Food Chem 2007;101:236-40.
23. Silva VA, Freitas AFR, Pereira MSV, Siqueira Júnior JP, Pereira AV, Higino JS. *In vitro* evaluation of the antimicrobial activity of Lippia sidoides Cham extract. on biological isolates of Staphylococcus aureus. Rev Bras Plantas Med 2010;12:452-5.
24. Funari CS, Gullo FP, Napolitano A, Carneiro RL, Mendes-Giannini MJ, Fusco-Almeida AM, *et al.* Chemical and antifungal investigations of six *Lippia* species (Verbenaceae) from Brazil. Food Chem 2012;135:2086-94.
25. Veras HN, Rodrigues FF, Colares AV, Menezes IR, Coutinho HD, Botelho MA, *et al.* Synergistic antibiotic activity of volatile compounds from the essential oil of *Lippia sidoides* and thymol. Fitoterapia 2012;83:508-12.
26. de Lima GP, de Souza TM, de Paula Freire G, Farias DF, Cunha AP, Ricardo NM, *et al.* Further insecticidal activities of essential oils from *Lippia sidoides* and *Croton* species against *Aedes aegypti* L. Parasitol Res 2013;112:1953-8.
27. Abena AA, Ngondzo-Kombeti GR, Bioka D. Psychopharmacologic properties of *Lippia multiflora*. Encephale 1998;24:449-54.
28. Costa SM, Lemos TL, Pessoa OD, Pessoa C, Montenegro RC, Braz-Filho R. Chemical constituents from *Lippia sidoides* and cytotoxic activity. J Nat Prod 2001;64:792-5.
29. Mesa-Arango AC, Montiel-Ramos J, Zapata B, Durán C, Betancur-Galvis L, Stashenko E. Citral and carvone chemotypes from the essential oils of Colombian *Lippia alba* (Mill.) N.E. Brown: Composition, cytotoxicity and antifungal activity. Mem Inst Oswaldo Cruz 2009;104:878-84.
30. Gonzalez-Guëreca MC, Soto-Hernandez M, Martinez-Vazquez M. Isolation of (-)(2S)-5,6,7,3',5'-pentahydroxyflavanone-7-O-ß-D-glucopyranoside, from *Lippia graveolens* H.B.K. var. berlandieri Schauer, a new anti-inflammatory and cytotoxic flavanone. Nat Prod Res 2010;24:1528-36.
31. Compadre CM, Robbins EF, Kinghorn AD. The intensely sweet herb, *Lippia dulcis* Trev: Historical uses, field inquiries, and constituents. J Ethnopharmacol 1986;15:89-106.
32. Kaneda N, Lee IS, Gupta MP, Soejarto DD, Kinghorn AD. (+) v-4 beta-hydroxyhernandulcin, a new sweet sesquiterpene from the leaves and flowers of *Lippia dulcis*. J Nat Prod 1992;55:1136-41.
33. Santos HS, Silveira ER, Costa SM, Lemos TL, Pessoa OD, Uchoa DE. 1H and 13C NMR application to structure elucidations of prenylated naphthoquinone dimers from *Lippia microphylla*. An Ressonância Magn Nucl 2002;1:32-6.
34. Santos HS, Costa SM, Pessoa OD, Moraes MO, Pessoa C, Fortier S, *et al.* Cytotoxic naphthoquinones from roots of *Lippia microphylla*. Z Naturforsch C 2003;58:517-20.
35. López-Alarcón C, Denicola A. Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. Anal Chim Acta 2013;763:1-10.
36. Farias DF, Souza TM, Viana MP, Soares BM, Cunha AP, Vasconcelos IM, *et al.* Antibacterial, antioxidant, and anticholinesterase activities of plant seed extracts from Brazilian semiarid region. Biomed Res Int 2013;2013:510736.
37. Carvalho AF, Melo VM, Craveiro AA, Machado MI, Bantim MB, Rabelo EF. Larvicidal activity of the essential oil from *Lippia sidoides* Cham. Against Aedes aegypti linn. Mem Inst Oswaldo Cruz 2003;98:569-71.
38. Cavalcanti ES, Morais SM, Lima MA, Santana EW. Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti* L. Mem Inst Oswaldo Cruz 2004;99:541-4.
39. Santiago GM, Lemos TL, Pessoa OD, Arriaga AM, Matos FJ, Lima MA, *et al*. Larvicidal activity against *Aedes aegypti* L. (Diptera: Culicidae) essential oils of *Lippia* species from Brazil. Nat Prod Commun 2006;1:573-6.
40. Folashade KO, Omoregie EH. Essential oil of *Lippia multiflora* Moldenke: A review. J App Pharm Sci 2012;2:15-23.
41. Gleiser RM, Bonino MA, Zygadlo JA. Repellence of essential oils of aromatic plants growing in Argentina against *Aedes aegypti* (Diptera: Culicidae). Parasitol Res 2011;108:69-78.
42. Environmental Protection Agency (EPA). Reregistration Eligibility Decision (RED) facts: Limonene. EPA-738-F-94-030. Washington: EPA 1994. p. 1-5.
43. Hebeish A, Fouda MM, Hamdy IA, El-Sawy SM, Abdel-Mohdy FA. Preparation of durable insect repellent cotton fabric: Limonene as insecticide. Carbohydr Polym 2008;74:268-73.
44. Costa SM, Santos HS, Lemos TL, Pessoa OD. Constituents of the essential oil of *Lippia microphylla* Cham. from Northeast of Brazil. J Essent Oil Res 2005;17:378-9.
45. Rodrigues FF, Henrique DM, Campos AR, Sidney GL, Costa JG. Antibacterial activity and chemical composition of essential oil of *Lippia microphylla* Cham. Acta Sci Bio Sci 2011;33:141-4.
46. Schurt DA, Trassato LB, Souza GR, Silva WL. Use of essential oil of *Lippia microphylla* on *in vitro* growth inhibition of *Rhizoctonia solani*. Trop Plant Pathol 2012;38:34-9.
47. Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564-82.

48. Lakusici B, Ristic M, Slavkovski V, Stankovic JA, Milenkovic M. Chemical composition and antimicrobial of the essential oil from Satureja horvatii Silic (Lamiaceae). J Serb Chem Soc 2008;73:703-11.
49. David JP, Meira M, David JM, Brandão HN, Branco A, de Fátima Agra M, *et al.* Radical scavenging, antioxidant and cytotoxic activity of Brazilian Caatinga plants. Fitoterapia 2007;78:215-8.
50. Silva PS, Viccini LF, Singulani JL, Siqueira EP, Zani CL, Alves TM. Chemical composition of the essential oil and hexanic fraction of *Lippia* and *Lantana* species. Rev Bras Farmacognosia 2010;20:843-9.
51. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
52. Xavier AL. Study of the potential antitumor the essential oil of Lippia microphylla leaves call (Verbenaceae) and its toxicity. Post-graduate Program in Natural Products and Synthetic Bioactives. João Pessoa: UFPB; 2011. p. 1-95.
53. Wu YJ, Muldoon LL, Neuwelt EA. The chemoprotective agent N-acetylcysteine blocks cisplatin-induced apoptosis through caspase signaling pathway. J Pharmacol Exp Ther 2005;312:424-31.
54. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770-6.
55. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011;144:646-74.
56. Cao DX, Qiao B, Ge ZQ, Yuan YJ. Comparison of burst of reactive oxygen species and activation of caspase-3 in apoptosis of K562 and HL-60 cells induced by docetaxel. Cancer Lett 2004;214:103-13.
57. Meshkini A, Yazdanparast R. Involvement of oxidative stress in taxol-induced apoptosis in chronic myelogenous leukemia K562 cells. Exp Toxicol Pathol 2012;64:357-65.
58. Santos MDM, Coelho GM, Santos DMD, Filho LF. Prospecting of Future Technologies: Methods, Techniques and Approaches. Brasília: CGEE; 2004. p. 198-229.
59. Marques LG, Simões ER, Ferreira PM, Santos MR, Pessoa C. Development of the technological potential of *Casearia sylvestris*. Rev Geintec 2013;3:70-85.
60. Zackiewicz M, Salles-Filho S. Technological foresight: A tool for science and technology policy. Parcerias Estratégicas 2001;10:144-61.
61. Brasil. Ministry of Health. Secretariat of Science, Technology and Strategic Inputs. Department of Pharmaceutical Services. National Policy of Medicinal Plants and Herbal Medicines. Brasília: Série B - Textos Básicos de Saúde; 2006. p. 1-60.
62. Brasil. Presidency, Civil House, deputy chef for Legal Affairs. Law No 10,973/2004. http://www.planalto.gov.br /ccivil03/ato2004-2006/2004/lei/l10.973.htm>. [Access date 21.02.2015.
63. Speziali MG, Guimarães PPG, Sinisterra RD. Demystifying patent protection in universities. Quim Nova 2012;35:1700-5.
64. Klein T, Longhini R, Bruschi ML, Mello JCP. Herbal Medicines: A Promising Market. Rev Ciênc Farm Básica Apl 2009;30:241-8.
65. Institute for Healthcare Informatics. 2011. The Global use of medicines: Outlook trough 2015. Available from: http://www.imshealth.com/deployedfiles/ims//Insights/IMS%20Institute20for%20Healthcare%20Informatics/Global_Use_of_Medicines_Report.pdf. [Last accessed on 2014 Jul 07].

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