Journal of Medicinal Plant Research

Volume 8 Number 20, 25 May, 2014

ISSN 2009-9723

ABOUT JMPR

The Journal of Medicinal Plant Research is published weekly (one volume per year) by Academic Journals.

The Journal of Medicinal Plants Research (JMPR) is an open access journal that provides rapid publication (weekly) of articles in all areas of Medicinal Plants research, Ethnopharmacology, Fitoterapia, Phytomedicine etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in JMPR are peerreviewed. Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: jmpr@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The Journal of Medicinal Plant Research will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Prof. Akah Peter Achunike
Editor-in-chief
Department of Pharmacology & Toxicology
University of Nigeria, Nsukka
Nigeria

Associate Editors

Dr. Ugur Cakilcioglu
Elazıg Directorate of National Education
Turkey.

Dr. Jianxin Chen
Information Center,
Beijing University of Chinese Medicine,
Beijing, China
100029,
China.

Dr. Hassan Sher
Department of Botany and Microbiology,
College of Science,
King Saud University, Riyadh
Kingdom of Saudi Arabia.

Dr. Jin Tao
Professor and Dong-Wu Scholar,
Department of Neurobiology,
Medical College of Soochow University,
199 Ren-Ai Road, Dushu Lake Campus,
Suzhou Industrial Park,
Suzhou 215123,
P.R.China.

Dr. Pongsak Rattanachaikunsopon
Department of Biological Science,
Faculty of Science,
Ubon Ratchathani University,
Ubon Ratchathani 34190,
Thailand.

Prof. Parveen Bansal
Department of Biochemistry
Postgraduate Institute of Medical Education and Research
Chandigarh
India.

Dr. Ravichandran Veerasamy
AIMST University
Faculty of Pharmacy, AIMST University, Semeling - 08100,
Kedah, Malaysia.

Dr. Sayeed Ahmad
Herbal Medicine Laboratory, Department of Pharmacognosy and Phytochemistry,
Faculty of Pharmacy, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi, 110062,
India.

Dr. Cheng Tan
Department of Dermatology, first Affiliated Hospital of Nanjing Univeristy of
Traditional Chinese Medicine.
155 Hanzhong Road, Nanjing, Jiangsu Province,
China. 210029

Dr. Naseem Ahmad
Young Scientist (DST, FAST TRACK Scheme)
Plant Biotechnology Laboratory
Department of Botany
Aligarh Muslim University
Aligarh- 202 002,(UP)
India.

Dr. Isiaka A. Ogunwande
Dept. Of Chemistry,
Lagos State University, Ojo, Lagos,
Nigeria.

Editorial Board

Prof Hatil Hashim EL-Kamali
Omdurman Islamic University, Botany Department,
Sudan.

Prof. Dr. Muradiye Nacak
Department of Pharmacology, Faculty of Medicine,
Gaziantep University,
Turkey.

Dr. Sadiq Azam
Department of Biotechnology,
Abdul Wali Khan University Mardan,
Pakistan.

Kongyun Wu
Department of Biology and Environment Engineering,
Guiyang College,
China.

Prof Swati Sen Mandi
Division of plant Biology,
Bose Institute
India.

Dr. Ujjwal Kumar De
Indian Vetreinary Research Institute,
Izatnagar, Bareilly, UP-243122
Veterinary Medicine,
India.

Dr. Arash Kheradmand
Lorestan University,
Iran.

Prof Dr Cemşit Karakurt
Pediatrics and Pediatric Cardiology
Inonu University Faculty of Medicine,
Turkey.

Samuel Adelani Babarinde
Department of Crop and Environmental Protection,
Ladoke Akintola University of Technology,
Ogbomoso
Nigeria.

Dr.Wafaa Ibrahim Rasheed
Professor of Medical Biochemistry National Research Center
Cairo
Egypt.

Journal of Medicinal Plants Research

Table of Contents: Volume 8 Number 20 25 May, 2014

ARTICLES

Research Articles

Gastroprotective and immunoadjuvant activities of butanolic extract of Calliandra haematocephala 703
Antony de Paula Barbosa

Biological screening of Albizia lebbek L. and Mimosa himalayana Gamble (Mimosaceae) 731
Abdul Shakoor, Amjad ur Rahman, Gul Zaib, Uzma Khan, Yasir Ihtesham, Abdul Aziz Napar and Muhammad Naveed

Preliminary evaluation of ethanol leaf extract of Borreria verticillata Linn (Rubiaceae) for analgesic and anti-inflammatory effects 736
Halima Sadiya Abdullahi-Gero, Abubakar Ahmed, Abdulkadir Umar Zezi and Isa Marte Hussaini

Chemical and biological study of Mentha suaveolens Ehrh. cultivated in Egypt 747
El-Sayeda A. El-Kashoury, Hesham I. El-Askary, Zeinab A. Kandil, Shahira M. Ezzat, Mohamed A. Salem and Amany A. Sleem

Rapid and easy molecular authentication of medicinal plant Zingiber officinale Roscoe by loop-mediated isothermal amplification (LAMP)-based marker 756
Anis Ahmad Chaudhary, Mohsin Khan, Waleed M. Al-Shaqha, Mohammad Alharbi and Osama A. Al-Khamees

academicJournals

Vol. 8(20), pp. 727-730, 25 May, 2014
DOI: 10.5897/JMPR2014.5359
Article Number: 145202F44838
ISSN 1996-0875
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Short Communication

Gastroprotective and immunoadjuvant activities of butanolic extract of *Calliandra haematocephala*

Antony de Paula Barbosa

Instituto de Pesquisas de Produtos Naturais, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, CEP 21941-971 Rio de Janeiro, Brazil.

Received 12 January, 2014; Accepted 16 May, 2014

Calliandra haematocephala **(Leguminosae) is a native species found in tropical America. This plant is widespread and cultivated with ornamental purposes in gardens and parks. Previous studies with other species of this genus confirmed some pharmacological properties, such as antiinflammatory, anticonvulsant, immunomodulatory, and mainly antiulcerogenic activity. Phytochemical investigations have been carried out on the constituents of this genus and demonstrated the presence of tannins, flavonoids and saponins. In order to confirm the ethnopharmacological use of this species, a phytochemical screening was performed with a butanolic extract and its gastroprotective and immunoadjuvant properties were evaluated. The gastroprotective effects were analyzed by measuring acute gastric lesions induced by acidified ethanol, using cimetidine as reference compound. The immunoadjuvant activity was evaluated against ovalbumin antigen, since the delayed type hypersensitivity reaction was measured as an** ***in vivo*** **assay of cellular immune response. In both experiments, the results of the biological properties were corroborated, which justifies the use of this plant in traditional medicine as stomach protector and immunomodulatory.**

Key words: *Calliandra haematocephala*, gastroprotective effects, immunoadjuvant activity.

INTRODUCTION

The genus *Calliandra* (Fabaceae) contains 132 species. Most of them are native of America, but few are of Asia and Africa (Tani et al., 1998). *Calliandra haematocephala* (Leguminosae) is native from tropical Americans, usually cultivated in gardens for ornamental purposes. Previous studies with other species of this genus confirmed some pharmacological properties, such as antiinflammatory, anticonvulsant, immunomodulatory, and mainly anti-ulcerogenic activity. Aqueous extracts of the branches of *Calliandra anomala* are used as an antimalarial and antifebrile agent on Mexico (Zeid et al., 2006). *Calliandra pulcherrima*, *Calliandra brevifolia* and *C. haematocephala* are related native species found in Tropical America. These evergreen plants are non invasive, but widespread ornamental species cultivated in gardens and parks. In Brazil, the aqueous extract of the aerial parts of *C. pulcherrima* is used as a remedy for malaria and leishmaniasis (Da Silva et al., 2005). Phytochemical investigations have been carried out on the constituents of this genus and demonstrated the presence of tannins,

*Corresponding author. E-mail: antonybarbosa@hotmail.com.
Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

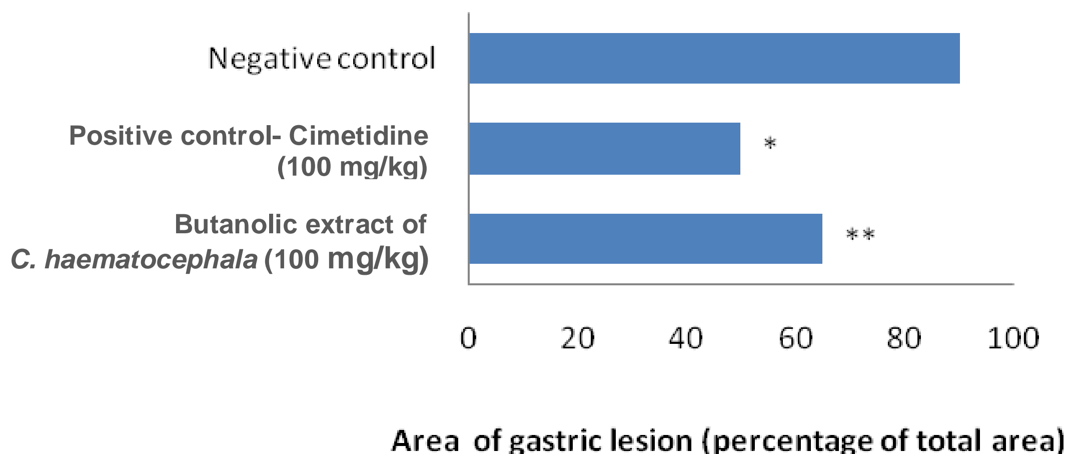

Figure 1. Area of gastric lesion (percentage of total area) in Swiss mice using cimetidine as positive control in comparison with the butanolic extract of *C. haematocephala*. Results are mean ± standard error of mean (SEM) (n=5); *p<0.05, **p<0.01 significantly different to the saline control. Student's t-test.

flavonoids and saponins (Zeid et al., 2007). In this work, the gastroprotective effects and the immunoadjuvant activity of the butanolic extract from the aerial parts of *C. haematocephala* were reported.

MATERIALS AND METHODS

Extraction

The aerial parts of the plant (200 g) were extracted with MeOH (1 L) for 72 h. The extract was concentrated under reduced pressure, and the resulting aqueous phase was shaked with *n*-BuOH (water/*n*-BuOH (1:1) v/v). The resulting organic phase was evaporated in vacuum to give a crude material (15 mg), which was used in both experimental models as the following.

Gastroprotective effects

Antiulcerogenic activity was evaluated by measuring acute gastric lesions induced by acidified ethanol. Male Swiss mice (three months old, 25 to 35 g) in groups of five were fasted for 24 h before the experiment and administered orally with 1 ml of pure water as the negative control, or butanolic extract (100 mg/kg), or the reference compound cimetidine (100 mg/kg) dissolved in vehicle as positive control. One hour after the treatments, all animals received orally 200 µl of acidified ethanol solution (0.3 M HCl/EtOH) to induce gastric lesions. The animals were killed after 1 h treatment with the ulcerogenic agent and the stomachs removed, opened along the greater curvature and rinsed with physiological saline to determine the lesion damage. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system. The percentage of the total lesion area (hemorrhagic lesions) to the total surface area of the stomach was defined as the ulcer index (Hamauzu et al., 2008).

Immunoadjuvant activity

Male Swiss mice (three months old) were subcutaneously immunized twice at weekly intervals with 100 µl saline (SAL) as the control group or 100 µg ovalbumin (OVA) mixed with 100 µg of each adjuvant dissolved in 100 µl of saline as vehicle. Delayed type hypersensitivity (DTH) responses were assessed by measuring the increment in the right footpad; thicknesses were after subcutaneous challenge with 100 µg OVA in 100 µl saline a week after the second immunization. The footpad thickness was measured with a spring-loaded dial gauge before and 24, 48 and 72 h after injection. Injecting each animal with 100 µl saline in the left hind footpad served as controls. The ovalbumin specific responses were obtained by subtracting the response to OVA challenge in unimmunized control mice (Mowat et al., 1991).

RESULTS AND DISCUSSION

Gastroprotective effects

The butanolic extract exhibited a moderate control of gastric lesions (35% of inhibition at 100 mg/kg), provoking an inhibition of the development of the hemorrhage and necrotic aspects of tissue injury; how-ever, showing lesser activity than the reference compound at the same dosage (50% of inhibition at 100 mg/kg) (Figure 1). The intensity of gastric ulcers was quantified by the percentage of the injury area in relation to the control group. The results obtained confirm the gastroprotective activity of the butanolic extract of *C. haematocephala*, which probably interfere with the ulcerogenic mechanism, through the synthesis or degradation reduction of prostaglandins responsible, along with other factors, for the gastric cytoprotection.

Immunoadjuvant activity

Several important biological properties have been attributed to saponins. Since the original observation that certain saponins cause substantial enhancement of immune responses when given together with an antigen in a vaccine, their use as adjuvants received special attention.

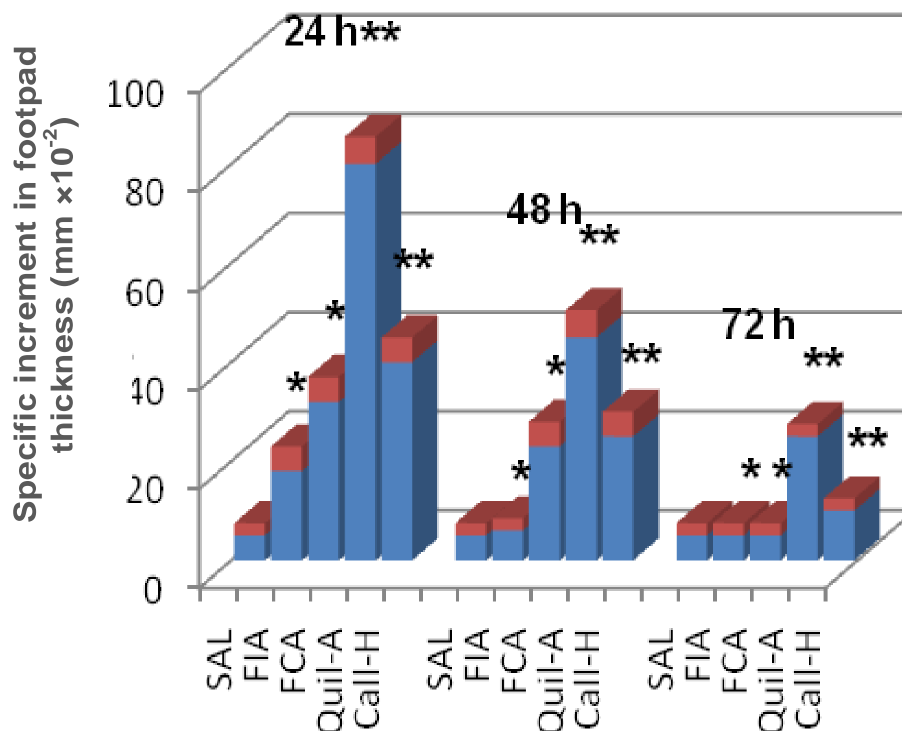

Figure 2. Delayed type hypersensitivity responses after two subcutaneous immnunizations with 100 µg of ovalbumin and 100 µg of each adjuvant. Results are mean ± standard error of mean (SEM) (n=5); *p<0.05, **p<0.01 significantly different to the saline control. Student's t-test.
Abbreviations: SAL, saline solution; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; Quill-A, commercial extract of *Quillaja saponaria*; Call-H, butanolic extract of *C. haematocephala.*

fixation of complement factors and the release of certain cytokines such as interleukin (IL)-2 and interferon (IFN), and also the humoral response, resulting in increased circulation and secretion of antibodies and cytokines such as IL-4, IL-5, IL-6 and IL-10. In order to investigate the biological properties of the butanolic extract from the aerial parts of *C. haematocephala*, it was evaluated for immunoadjuvant activity and compared with adjuvants commonly used in experimental models. The immunodadjuvant property was evaluated against ovalbumin antigen, since the delayed type hypersensitivity reaction was measured as an *in vivo* assay of cellular immune response. Mice immunized with ovalbumin conjugated with extracts showed remarkable responses greater than those when the antigen was combined with commercial adjuvants. This response developed rapidly after immunization and persisted at lower levels for at least three days. The results obtained suggest the relevant adjuvant potential of the butanolic extract from *C. haematocephala* in comparison with the commercial extract of *Quillaja saponaria*, a commonly used adjuvant for experimental vaccine formulations (Figure 2) (Marciani et al., 2000).

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Da Silva BP, Soares JBRC, De Souza EP, Palatnik M, De Sousa CBP, Parente JP (2005). Pulcherrimasaponin, from the leaves of *Calliandra pulcherrima*, as adjuvant for immunization in the murine model of visceral leishmaniasis. Vaccine 23:1061-1071.

Hamauzu Y, Irie M, Kondo M, Fujita T (2008). Antiulcerative properties of crude polyphenols and juice of apple, and *Chinese quince* extracts. Food Chem. 108:488-495.

Marciani DJ, Press JB, Reynolds RC, Pathak AK, Pathak V, Gundy LE, Farmer JT, Koratich MS, May RD (2000). Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. Vaccine 18:3141-3151.

Mowat AM, Donachie G, Reid, Jarrett O (1991). Immune-stimulating complexes containing Quil A and protein antigen prime class I MHC-restricted T lymphocytes in vivo and are immunogenic by the oral route. Immunology 72:317-322.

Tani C, Ogihara Y, Takeda T (1998). Studies on the constituents of

Calliandra anomala (Kunth) Macbr. IV. Structure analysis by HPLC retention time and FAB-MS spectrum. Chem. Pharm. Bull. 46:723-725.
Zeid AA, Hifnawy M, Saleh M, Sleem A, Mohamed R (2006). Flavonoids, volatiles and biological activities of the aerial parts of *Calliandra haematocephala* Hassk. Planta Med. 72(11): 1080-1080.
Zeid AHS, Hifnawy MS, Sleem AA, Mohamed RS (2007). Chemical constituents and anti-inflammatory activity of the aerial parts of *Calliandra haematocephala* Hassk. Planta Med. 73(9): P_009.

academicJournals

Vol. 8(20), pp. 731-735, 25 May, 2014
DOI: 10.5897/JMPR12.428
Article Number: D44247B44839
ISSN 1996-0875
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Biological screening of *Albizia lebbek* L. and *Mimosa himalayana* Gamble (Mimosaceae)

Abdul Shakoor[1]*, Amjad ur Rahman[1], Gul Zaib[2], Uzma Khan[3], Yasir Ihtesham[3], Abdul Aziz Napar[1] and Muhammad Naveed[1]

[1]Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan.
[2]Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan.
[3]Department of Botany, Hazara University, Mansehra, Pakistan.

Received 7 March, 2012; Accepted 12 May, 2014

Methanolic leaf extracts of two medicinal plant species of family Mimosaceae: *Albizia lebbek* L. and *Mimosa himalayana* Gamble, were used to evaluate their antibacterial and antifungal activity using agar diffusion method. Extractions from leaves of selected plants were carried out by simple maceration process. The methanolic extracts of these plants were tested against four strains of bacteria (one strain was gram positive that is, *Bacillus subtilis* and three were gram negative that is, *Ecscherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) and two strains of fungi (*Aspergillus niger* and *Aspergillus flavus*). At 15 mg/ml extract concentration, the maximum inhibitory zones observed in *A. lebbek* L. *and M. himalayana* Gamble were12.5 and 27 mm, respectively. *A. lebbek* L. and *M. himalayana* gave response against *A. niger* by producing 36.2 and 0.86% inhibition, respectively. No antifungal activity was reported by *A. lebbeck* L. and *M. himalayana* Gamble against *A. flavus*.

Key words: Mimosaceae, antibacterial, antifungal, methanol extracts, medicinal plants.

INTRODUCTION

It is estimated that there are 250,000 to 500,500 species of plants on earth (Borris, 1996). A relatively small percentage (1 to 10%) of these is used as food by both humans and other animal species. It is possible that even more are used for medicinal purposes. Hippocrates (in the late fifth century B.C) mentioned 300 to 400 medicinal plants. An estimate suggests that about 13,000 plant species worldwide are known to have use as drugs. The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries and for the presence of antimicrobials (Das et al., 1999). Most of these plants are being used for therapeutic purposes without specific knowledge of their active ingredients. In fact, Pakistani medicinal plants, for the purpose of drug development, are one of the least investigated sources of natural compounds (Satyavati et

*Corresponding author. E-mail: abdul_shakoor954@yahoo.com. Tel: +923335427591.
Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

al., 1976).

About 80% of the developing world population depends on traditional medicines for their primary health needs and 85% of these traditional medicines involve the use of plant extracts. This means that about four billion people depend on natural products as their primary source of medication. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times (Farombi, 2003). It has been reported that the higher plants have shown to be a potential source for the new antimicrobial agents. Natural antimicrobials can be derived from plants, animal tissues or microorganisms. To determine the potential and promote the use of herbal medicine, it is essential to investigate plants that find place in folklore medicine (Nair et al., 2005).

The aim of the present work was to determine whether the selected plant species of family Mimosaceae have antibacterial and antifungal activities using an agar diffusion method.

MATERIALS AND METHODS

Study site

The present research work was carried out in the Department of Plant Sciences, Quaid-i-Azam University (QAU) Islamabad. Brief accounts of materials as well as procedures used are described.

Plant materials and their handling

Plant materials were collected from vicinity of Quaid-i-Azam University, Islamabad-Pakistan. The plants were identified in the Herbarium, Department of Plant Sciences QAU, Islamabad and voucher specimens were deposited. Fresh leaves of *Albizia lebbek* L. and *Mimosa himalayana* were picked, rinsed with distilled water and kept under shade to dry before being weighed.

Preparation of leaf extracts

Extracts were prepared from the air-dried leaves using simple maceration process. The leaves were grounded in methanol using kitchen blender. This mixture was kept in extraction bottles at room temperature (25°C) for two weeks. After two weeks, the mixtures were filtered twice, using Whatman-41 filter paper. Methanol was then evaporated completely using rotary evaporator to obtain the extracts. The extract (15 mg) was dissolved in 1 ml of dimethyl sulfoxide. This stock solution 15 mg/ml was again diluted, thus 8 concentrations of the extract were prepared that is, 15.0, 12.5, 10.0, 7.5, 5.0, 3.0, 2.0 and 1.00 mg/ml. Along with these solutions, the standard antibiotic (DOX) was also prepared at a concentration of 2 mg/ml.

Preparation of leaf extracts

The extract (15 mg) was dissolved in 1 ml of dimethyl sulfoxide. This stock solution 15 mg/ml was again diluted, thus 8 concentrations of the extract were prepared that is, 15.0, 12.5, 10.0, 7.5, 5.0, 3.0, 2.0 and 1.0 mg/ml. Along with these solutions, the standard antibiotic (DOX) was also prepared at a concentration of 2 mg/ml.

Antimicrobial activity

Antibacterial activity was carried out by agar diffusion method and antifungal activity was carried out by using agar tube dilution as reported by Choudhary et al. (1995). Micro organisms used in this study were *Bacillus subtilis Ecscherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Aspergillus niger* and *Aspergillus flavus*. Antifungal activity was determined by using following formula.

Percentage inhibition of fungal growth = 100 - Linear growth in test (mm) / Linear growth in control (mm) × 100

RESULTS

Methanolic extracts of two Mimosaceae species that is, *A. lebbeck L.* and *M. himalayana* were tested against four strains of bacteria. Methanolic leaves extract of *A. lebbeck* L. at the concentrations of 15, 12.50 and 10.00 mg/ml exhibited 10 mm inhibition zones against the *E. coli*, whereas at the 7.50 mg/ml it showed the 9 mm inhibitory zone. Minimum inhibitory concentration (MIC) value was 5.00 mg/ml. *A. lebbeck* showed 15 mm inhibition zone at the concentration of 15 mg/ml, and at 12.50 mg/L it yielded 14 mm inhibition zone against *P. aeruginosa*. At 10.00 and 7.50 mg/ml, it showed 13 and 11 mm inhibitory zones, respectively. *A. lebbeck* extract yielded 21 mm inhibitory zones at the 15 and 12.50 mg/ml concentrations against the *K. pneumonia*. 10.00 and 7.50 mg/ml showed 20 mm inhibition of zone. Methanolic extract gave 19 mm clear inhibition zone at the 5.00 mg/ml concentration, whereas 3.00, 2.00 and 1.00 mg/ml gave 18 inhibittion zones against the *K. pneumonia.* Antibiotic DOX (doxycycline) gave 47 mm inhibition zone (Table 1). Methanolic extract of *M. himalayana* showed 27 mm inhibitory zone against *E. coli* at the 15 mg/ml concentration, whereas it showed 25 mm clear inhibition zone at 12.50 mg/ml concentration. Here MIC value was 1.00 mg/ml and it gave 13 mm inhibitory zone (Table 2, Figure 1).

Antifungal study of two Mimosaceae plants

This study was done to check antifungal activity of 2 species of Mimosaceae plants. Only one concentration of each plant extracts were prepared by dissolving 24 mg/ml in solvent dimethylsulfoxide (DMSO). The fungi used in this study were *A. niger* and *A. flavus.* After inoculation and incubation of the samples for about one week, antifungal assay gave the following results: *A. lebbeck* L. showed 36.20% and 74 mm growth inhibition; *M.*

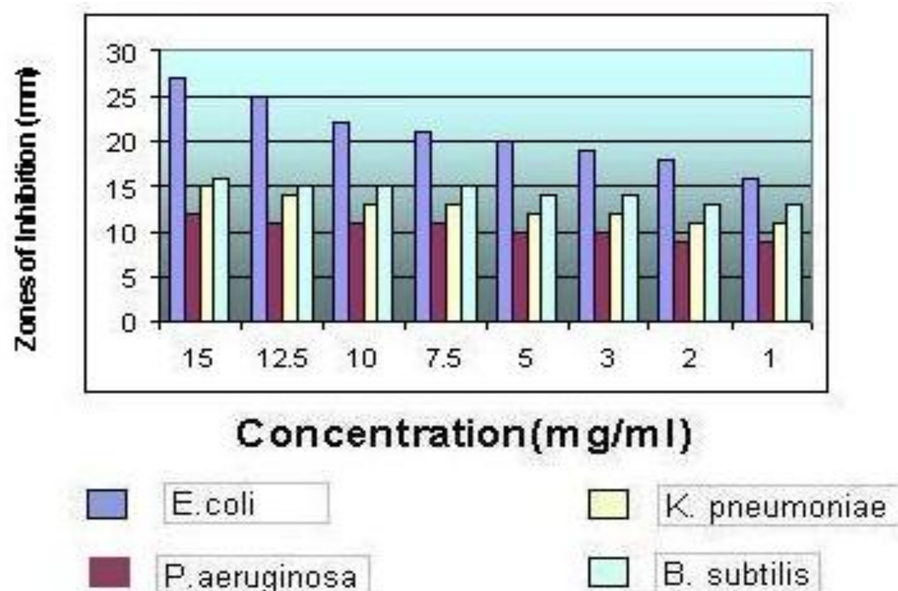

Figure 1. Zones of inhibition (mm) showing antibacterial activity of *M. himalayana*, against *E. coli, P., aeruginosa, K., pneumonia* and *B. subtilis.*

Table 1. Antibacterial activity of *A. lebbek* L. zones of inhibition (mm).

Concentration (mg/ml)	*E. coli* (mm)	*P. aeruginosa* (mm)	*K. pneumonia* (mm)	*B. subtilis* (mm)
15	10	15	21	14
12.5	10	14	21	14
10	10	13	20	14
7.5	9	11	20	13
5	8	9	19	12
3	0	0	18	11
2	0	0	18	11
1	0	0	18	11

Table 2. Antibacterial activity of *M. himalayana* L. zones of inhibition (mm).

Concentration (mg/ml)	*E. coli* (mm)	*P. aeruginosa* (mm)	*K. pneumonia* (mm)	*B. subtilis* (mm)
15	27	12	15	16
12.5	25	11	14	15
10	22	11	13	15
7.5	21	11	13	15
5	20	10	12	14
3	19	10	12	14
2	18	9	11	13
1	16	9	11	13

Table 3. *A. lebbek* L. against *A. niger* and *A. flavus.*

Fungi	LGC (mm)	LGT (mm)	% Inhibition
A. niger	116	74	36.20
A. flavus	122	122	No activity

LGC mm = linear growth in control (millimeter); LGT mm = linear growth in test (millimeter).

Table 4. *M. himalayana* against *A. niger* and *A. flavus.*

Fungi	LGC mm	LGT mm	% Inhibition
A. niger	116	115	0.86
A. flavus	122	122	No activity

LGC mm = linear growth in control (millimeter); LGT mm = linear growth in test (millimeter).

himalayana inhibited 0.86% and 115 mm growth against *A. niger*; *A. lebbeck* L. and *M. himalayana* did not show any activity against *A. flavus* as indicated in Tables 3 and 4.

DISCUSSION

Pakistan is rich in diversity of plants. People living in rural areas are interested in the use of plant-based drugs, because plant based drugs have no side effects and they are inexpensive. *A. lebbeck* L. and *M. himalayana* appear to have potential for testing as a plant of high medicinal values for various antimicrobial activities as well as other biological activities. These plants are abundantly found in Pakistan and easily accessible. In this study, MIC value of *A. lebbeck* L. ranged from 5.00 to 1.00 mg/ml for all bacteria, whereas *M. himalayana* MIC was 1.00 mg/ml. Srinivasan et al. (2001) showed that *A. lebbeck* L. has broadest spectra activity against all tested nine micro-organisms. *A. lebbeck* L. showed activity against *A. niger*.

Agyare et al. (2006) concluded that the *A. ferruginea* leaf and stem bark extracts contain tannins, sterols and saponins. They analyzed that the ethyl alcohol extract of *A. ferruginea* leaves and stem bark showed remarkable activity against all the test organisms. They showed that the extracts were less active against *P. aeruginosa* which is highly resistant to available orthodox antibiotics (Walker and Edwards, 1999). The petroleum ether extract was less active against organism. They described that the antimicrobial activity was more pronounced with leaf extract compared to stem bark.

In this study it was found that *A. lebbek* L. was active against all test microorganisms except *P. aeruginosa* below 5 mg/ml, whereas against fungus *A. flavus* it did not show any activity. Gandhirajan et al. (2009) found that the *M. pudica* methanolic extract exhibited anti-microbial activity against the tested microorganisms at three different concentrations of 50, 100 and 200 µg/disc. It gave 6 mm inhibition zone against *A. fumigatus* at the concentration of 50 µl and at the same concentration it gave 12 mm inhibition zone against *K. pneumonia*. They found that highest inhibition zone of 20 mm was observed against *K. pneumonia* at 200 µl.

In this work it was found that *Mimosa himalayana* showed highest inhibition zone of 27 mm against *E. coli* at 15 mg/ml concentration. It can be concluded from the study that *M. himalayana* showed significant activity against all test organism. Genest et al. (2008) found that none of extracts of *M. rubicaulis* was active against any of the test bacterial strains at test concentrations, while in this study *M. himalayana* gave lagre zone of 27 mm against *E. coli* and minimum of 9 mm against *P. aeruginosa* and it did not show any activity against *A. flavus*.

A number of explanations can be given for the difference in biological activity reports of some common extracts against same or similar microorganism, but the first logic is dissimilarities in phytochemicals of similar plants growing at different geographical locations (Olila et al., 2001). From the studies, it is concluded that the traditional plants may represent new sources of anti-microbials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine. These local ethnomedical preparations and prescriptions of plant sources should be scientifically evaluated and then disseminated properly and the knowledge about the botanical preparation of traditional sources of medicinal plants can be extended for future investigation into the field of pharmacology, phytoche-mistry, ethnobotany and other biological actions for drug discovery (Gandhirajan et al., 2009).

The results of the present study provide a scientific validation for the popular use of the medicinal plants studied and serve as a guide which may help in selection of plants with antimicrobial activities for further phyto-chemical work on the isolation and the identification of the active compounds.

From this study a conclusion can be drawn that two Mimosaceae plants are efficient against all pathogens. It was observed that plants were highly effective even at low concentration. In this research work, it was shown that plants showed remarkable activity against gram negative bacteria. It was and even it is believed today that gram negative bacteria are more resistant than gram positive.

Conclusion

The herbal plants may be used as an alternative and potential and promising source of pharmaceutical agents against different pathogens. The results suggest the presence of high concentration of an active principle in all the extracts of the tested two plants which showed potential antimicrobial activity. There is need of developing drugs from plants as micro organisms are becoming resistant to antibiotics and creating health problems.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Agyare C, Koffuor GA, Mensah AY, Agyemang DO (2006). Antimicrobial and uterine smooth muscles activity of *A. ferruginea* extracts. Bol. Latinoam Caribe. Plant Med. Aromat. 5(2):31-35.

Borris RP (1996). Natural Products Research: Perspectives from a major Pharmaceutical Company. J. Ethanopharmacol. 51:29-38.

Choudhary MI, Dure-e-Shawar, Perveen Z, Jabbar A, Ali I, Rehman A (1995). Antifungal steroidal lactones from Withiana coagulance. Phytochemestry 440(4):1243-1246.

Das S, Pal S, Mujib A, Dey S (1999). Biotechnology of medicinal plants-Recent advances and potential. 1st Ed. Vol 2: 126-139. UK 992 Publications, Hydarabad.

Farombi EO (2003). African indigenous plants with chemotherapeutic potentials and biotechnological approach to the production of bioactive prophylactic agents. Afr. J. Biotechnol. 2:662-671.

Gandhirajan N, Sriram S, Meena V, Sri Lakshmi JK, Sasikumar C, Rajeswari R (2009). Phytochemical screening and antimicrobial Activity of the plant extracts of *Mimosa pudica* L. Against selected microbes. Ethnobot. Leaflets 13:618-624.

Genest S, Kerr C, Shah A, Rahman MM, Saif-E-Naser GMM, Nigam P, Nahar L, Sarkar SD (2008). Comparative bioactivity studies on two *Mimosa* species. Bol. Latinoam Caribe. Plant Med. Aromat. 7(1):38-43.

Nair R, Kalariya T, Chanda S (2005). Antibacterial activity of some selected Indian medicinal flora. Turk. J. Biol. 29:1-7.

Olila D, Odyek O, Asibo OJ (2001). Antibacterial and antifungal activities of extracts of *Zanthoxylum chalybeum* and *Warburgia ugandensis*, Ugandan medicinal plants. Afr. Health Sci. 1(2):66-72.

Satyavati GV, Raina KK, Sharma M (1976). Medicinal Plants of India, Vol. I, A-G. Council of Medicinal Research, New Delhi, India. pp. 68-78.

Srinivasan D, Nathan S, Suresh T, Perumalsamy O (2001). Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine J. Ethnopharmacol. 74:217-220.

Walker R, Edwards C (1999). Clinical Pharmacy and Therapeutics, 2nd Edition. Churchill Livingstone, UK. p. 497-498.

academicJournals

Vol. 8(20), pp. 736-747, 25 May, 2014
DOI: 10.5897/JMPR2014.5450
Article Number: E56207D44841
ISSN 1996-0875
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Preliminary evaluation of ethanol leaf extract of *Borreria verticillata* Linn (Rubiaceae) for analgesic and anti-inflammatory effects

Halima Sadiya Abdullahi-Gero[1]*, Abubakar Ahmed[2], Abdulkadir Umar Zezi[1] and Isa Marte Hussaini[3]

[1]Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.
[2]Department of Pharmacognosy and Drug Development, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.
[3]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Maiduguri, Borno State, Nigeria.

Received 15 April, 2014; Accepted 14 May, 2014

***Borreria verticillata* L is used for the treatment of some painful and inflammatory conditions by traditional medical practitioners in Nigeria and other countries. The ethanol leaf extract of *B. verticillata* (EEBV) was investigated for possible analgesic and anti-inflammatory effects in mice and rats. The models used for the analgesic study were acetic acid induced abdominal writhes, hot plate tests in mice and formalin induced pain in rats. Carrageenan and formalin induced rat paw oedema were used to investigate anti-inflammatory effects. The oral (p.o) median lethal dose (LD_{50}) was greater than 5000 mg/kg body weight in mice and rats, while the intraperitoneal (i.p) LD_{50} in mice was 3807.88 mg/kg and greater than 5000 mg/kg in rats. The results of the study showed that the extract to have significant ($p<0.001$) analgesic effect at dose range of 200 to 1000 mg/kg p.o/i.p in mice in the acetic acid induced writhes and hot plate tests. Significant ($p<0.05$) analgesic effect was observed at 500 and 1000 mg/kg p.o in both phases of formalin induced pain in rats. EEBV exhibited anti-inflammatory effects which were found to be significant ($p<0.001$, $p<0.05$) at doses of 200 to 1000 mg/kg p.o/i.p in the rats and in all models used.**

Key words: *Borreria verticillata,* analgesic, anti-inflammatory.

INTRODUCTION

The use of medicinal plants for the relief and treatment of disease can be traced back to five millennia in various civilizations. Medicinal plants have played a vital role in world health (Calixto 2000; Calixto et al., 2000a). Despite the recent developments recorded in modern medicine, medicinal plants still make important contributions to health care (Calixto, 2000). In some African countries such as Ghana, Mali, Nigeria and Zambia, the first line of treatment for fevers resulting from malaria in about 60% of children, is the use of herbal medicines (WHO, 2003). Pain is an unpleasant sensory and emotional experience, associated with actual or potential tissue damage (IASP). Most people will experience pain at some time in their lives, because pain is a symptom that accompanies many

*Corresponding author. E-mail: sadiyaabbashe@outlook.com.
Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

ailments. Inflammation is the pathophysiological response of a living tissue to injury that leads to local accumulation of plasma fluids and blood cells (Mohanbabu et al., 2011). Currently, available drugs for the management of pain are opioids, non-opioid and non-steroidal anti-inflammatory drugs (NSAIDS). Prolong use of steroids (Corticosteroids) lead to several side effects including depression of the immune system. NSAIDs are used to manage acute mild to moderate pain and they have the potentials to cause severe adverse effects which include gastric mucosa ulceration and bleeding. Opioids are used for chronic agonizing and post operative pain and they have the potentials for addiction, dependence and tolerance (Almeida et al., 2001). The problems associated with the use of these drugs demands for research into plants with antioxidant properties that have folkloric use for the management of pain and inflammation with a view to finding new, safe and efficacious drug. *Borreria verticillata* has long history of use for several health conditions including fever and pain. Studies have investigated the antimicrobial activity of *B. verticillata*, but to the best of our knowledge none have been cited for the analgesic or anti-inflammatory activity of the plant.

This study therefore aims at examining the analgesic and anti-inflammatory effect of the ethanol leaf extract of *B. verticillata* (EEBV). The outcome of this study may explain the rationale behind the use of *B. verticillata* in the management of painful and inflammatory conditions by traditional medical practitioners as well as provide a base line data on the safety or otherwise of the plant. *B. verticillata* is a shrub plant of the Rubiaceae family. It is known by the following common names; shrubby false button weed, shrubby false button wood (Burkill, 2000). In Nigeria, it is known as Karya garma (Hausa) Irawo-ile (Yoruba), Abia-ikana (Ibibio). The flower is used as antipyretic and analgesic (Vieira et al., 1999; Moreira et al., 2010), the roots as anti diarrhoea and for treatment of erysipelas and haemorrhoids (Lorenzi and Matos, 2002). In West India, the decoction of the plant is used for diabetes and dysmenorrhoea. It is used in combination with *Cuscuta* and *Zebrina schnizlein* for the treatment of amenorrhoea (Ayenzu, 1978; Conserva and Ferreira, 2012). *B. verticillata* is used to treat bacteria skin infections and leprosy in Senegal (Sofowora, 2008). The juice of fresh aerial part is used in Nigeria for the treatment of skin eczema (Benjamin, 1979; Ajibesin et al., 2008). Essential oils isolated from *B. verticillata* have been shown to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans* (Burkill, 2000; Ushie and Adamu, 2010).

MATERIALS AND METHODS

Drugs and chemicals

The following drugs and chemicals of analytical grade were used: Ethanol (Sigma Aldrich USA), N-hexane (Sigma Aldrich USA), Piroxicam (Hovid, Malaysia), Morphine (Martindale, Essex), Carrageenan (Sigma Aldrich U.S.A), Formaldehyde (BDH, Chemical Laboratory, England, UK).

Equipment

Hot plate (22/SS – 615,Gallenkamp, England), weighing balance (Mettler, P152), stop watch, needles and syringes, spatula, animal cages, pestle and mortar, test tubes, beakers, digital vernier calliper (Precision measuring, SR44), gloves and cotton wool.

Plant

The whole plant of *B. verticillata* was collected from Basawa in Zaria, Kaduna State, Nigeria. The plant was identified and authenticated by Mallam Umar Gallah of the Herbarium Section in the Department of Biological Sciences, ABU, Zaria, Nigeria. A voucher specimen number (672) was deposited at the herbarium for future reference. The leaves were then picked and dried under shade until constant weight was obtained. The dried leaves were then crushed into coarse powder using a pestle and mortar.

Extraction

The powdered leaves (200 g) were extracted with 1.2 L of N-hexane. The marc was extracted with 1.2 L of aqueous ethanol (70% ethanol and 30% water, v/v) for 24 h at room temperature using a percolator. The solvent was removed over a water bath at temperature 45°C. The extract was stored in a closed container and referred to as EEBV.

Phytochemical screening

Preliminary phytochemical screenings were carried out on EEBV in order to confirm the presence of phyto-constituents following the methods described by Sofowora (2008) and Evans (2009).

Acute toxicity studies in rats and mice (LD_{50})

The determinations of LD_{50} were conducted following the method of Lorke (1983), in both rats and mice and by oral (p.o) and intraperitoneal (i.p) routes. The method was divided into two phases. In the first phase, 3 groups of three animals each received the plant extract of *B.verticillata* (EEBV) in doses of 10, 100 and 1000 mg/kg body weight and was observed for sign of toxicity and death for 24 h. In the second phase, 3 groups of one animal each received a more specific dose of the extract (1600, 2900 and 5000 mg/kg). The LD_{50} was determined by calculating the geometric mean of the lowest lethal dose and the highest non lethal dose (1/1 and 0/1), that is, LD50 = √ (Highest non lethal dose) × (Lowest lethal dose).

Analgesic activity

Hot plate test

The analgesic activity of the extract was measured by hot plate method as described by Eddy and Lambach (1953) and Vogel (2002). Thirty pre-screened (reaction time: 3 to 4 s) Swiss albino mice were randomly divided into 5 groups each composing of 6 mice. Group 1 received normal saline 10 ml/kg body weight, groups

2, 3 and 4 received EEBV of 200, 400 and 800 mg/kg body weight intraperitoneally, respectively. Group 5 received morphine 5 mg/kg intraperitoneally. The reaction time was taken as from the time the animal was placed on the hot plate to the time it licked its paw or jumped out of the hot plate. The reaction time for the mouse to lick its paw or jumped was taken as pain reaction time. The reaction time was recorded at 0, 30, 60, 120, and 180 min following treatments with extract, control or standard drug. A cut off time of time of 20 s was set to prevent injury to the animal. The percent analgesia was calculated, thus;

$$Analgesia\ (\%) = \frac{Latency\ test - Latency\ control}{Latency\ control} \times 100$$

Acetic acid writhes test

The method described by Koster et al. (1959) was adopted. Thirty Swiss albino mice were divided randomly into 5 groups of 6 animals each. Group 1 received normal saline of 10 ml/kg intraperitoneally, groups 2, 3 and 4 received EEBV at doses of 200, 400 and 800 mg/kg intraperitoneally, respectively. Group 5 received piroxicam 20 mg/kg intraperitoneally. After 30 min of drug administration, the mice were treated with 0.6% v/v acetic acid 10 ml/kg intraperitoneally (Koster et al., 1959). Mice were placed in individual cages. Five minutes after acetic acid was administered, the number of writhes was counted for a period of 10 min. The percentage inhibition of writhes was calculated using the formula:

$$Inhibition\ (\%) = \frac{Mean\ No.of\ writhes\ control - Mean\ No.of\ writhes\ test}{Mean\ No.of\ writhes\ control} \times 100$$

The same procedure was repeated with another set of animals, but animal received treatments orally.

Formalin induced pain in rats

Thirty six albino rats were divided into 6 groups of 6 rats each and were administered either normal saline of 10 ml/kg oral, EEBV (250, 500 and 1000 mg/kg), morphine 5 mg/kg or piroxicam 20 mg/kg oral. One hour after treatments, 0.05 ml of freshly prepared formalin of 2.5% was injected subcutaneously into the sub plantar region of the left hind paw of each rat. The rats were placed individually in an observation chamber and monitored for 1 h. The severity of pain response was recorded for each rat based on the following Likert scale. Rat walked or stood firmly on injected paw (0); the injected paw was partially elevated (1); the injected paw was clearly lifted off the floor (2); and the injected paw was licked, chewed or shook (3). The anti-nociceptive effect was determined in 2 phases. Phase 1 was recorded during the first 5 min and phase 2 during the last 45 min with 10 min lag period in between the two phases (Dubuisson and Dennis, 1977; Tjolsen et al., 1992).

Carrageenan induced paw oedema

Thirty albino rats were randomly divided into 5 groups of 6 rats each. Acute inflammation was induced by injecting 0.1 ml of 1% saline solution of carrageenan into the sub planter surface of each rat hind paw (Winter et al., 1962). Normal saline (10 ml/kg), EEBV (250, 500 and 1000 mg/kg) and piroxicam 20 mg/kg were administered to the animals 30 min before carrageenan injection (for intraperitoneally treatment) and 1 h before carrageenan injection (for oral treatment). The paw diameter was measured at 0, 1, 2, 3, 4 and 5 h following carrageenan injection. The differences between reading at 0 h and at different time intervals were taken as the diameter of oedema.

Formalin induced inflammation

Thirty albino rats were divided randomly into 5 groups of 6 rats. The first group served as negative control and received normal saline of 10 ml/kg, while groups 2, 3 and 4 received different doses of the extract (250, 500, 1000 mg/kg) body weight orally. The fifth group received piroxicam (20 mg/kg) body weight (intraperitoneally). One hour later, all groups were administered 50 µl of 2.5% solution of formalin subcutaneously into the sub planter region of the left hind paw of each rat. An increase in hind paw diameter induced by formalin was used to measure acute inflammation (Winter et al., 1963). The paw diameter was measured with the aid of a digital vernier calliper at 0, 1, 2, 3, 4 and 24 h after the injection of formalin. The differences between the readings at time 0 h and at different time intervals were taken as the diameter of oedema (Hess and Milong, 1972).

Statistical analysis

All data obtained were expressed a mean ± standard error of mean. Data were analysed by one way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) version 19 followed by Turkey's Post Hoc test for hot plate test, acetic acid induced writhes test, carrageenan and formalin induced paw oedema and independent samples Kruskal-Walis and Mann Whitney's test for formalin induced pain. Values of $p<0.05$ were considered significant. Data were presented in form of figures, graph and tables.

RESULTS

Preliminary phytochemical screening

The preliminary phytochemical screening revealed the presence of alkaloids, saponins, phenol, tannins, glycosides, sterols/terpenoids, carbohydrates and flavonoids.

Median lethal dose (LD50)

The intraperitoneal and oral median lethal doses (LD_{50}) of ethanol leaf extract of *B. verticillata* in rats were found to be greater than 5000 mg/kg body weight. The oral LD_{50} in mice was also above 5000 mg/kg, whereas the intraperitoneal LD_{50} in mice was 3807.88 mg/kg body weight.

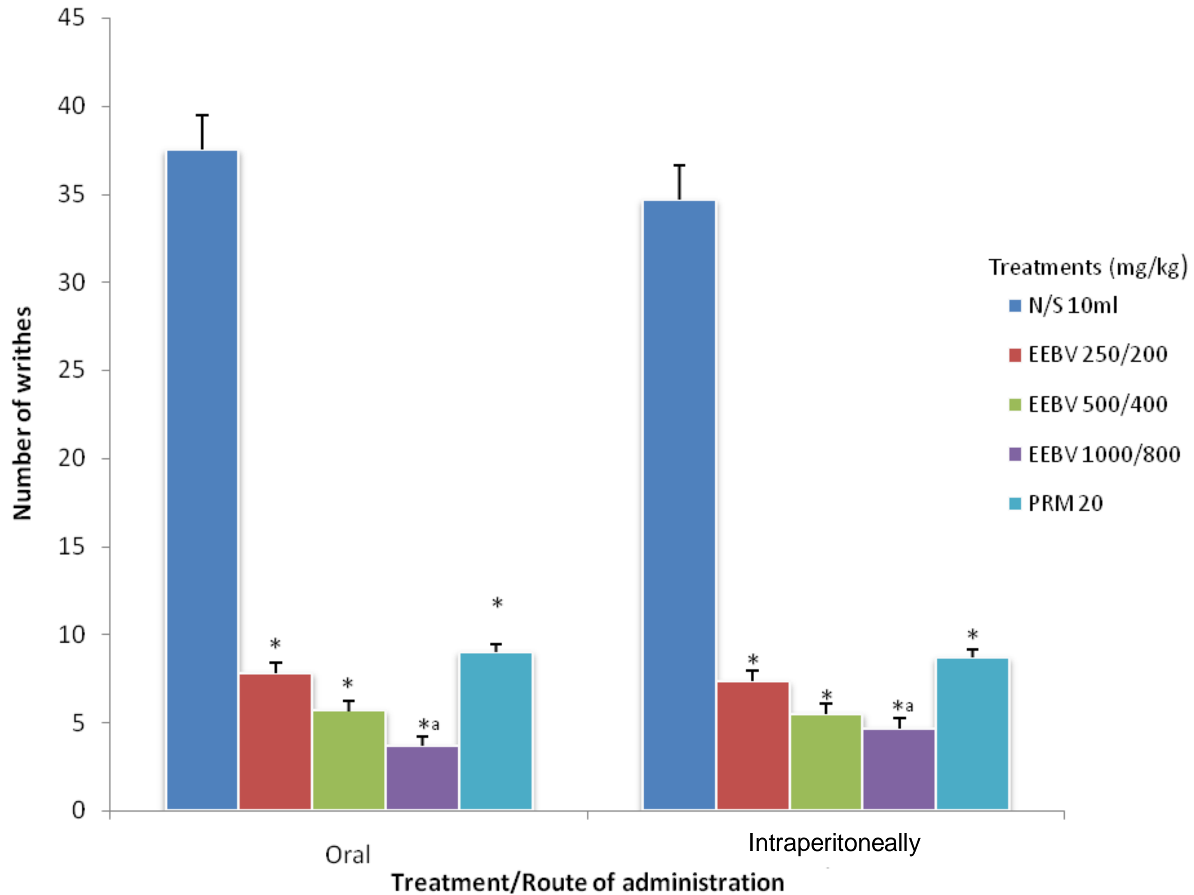

Figure 1. Effect of intraperitoneal and oral administration of EEBV on acetic acid induced abdominal writhes in mice. *p<0.001 compared with normal saline, [a]p<0.05 (n=6) significant compared with piroxicam, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata,* N/S = Normal Saline, and PRM = Piroxicam.

Analgesic studies

The effect of ethanol leaf extract of B. verticillata on acetic acid-induced writhes in mice

The extract significantly (p<0.001) inhibited the number of acetic acid-induced writhes in mice in a dose-dependent manner by both intraperitoneal and oral route of administration. The highest percentage of inhibition (90.21%) of writhes response was observed with ethanol leaf extract of *B verticillata* (EEBV) 1000 mg/kg (oral). Piroxicam, the standard drug, showed a maximum writhes inhibition of 76.00%. The lower doses of the extracts 200 (intraperitoneally) and 250 mg/kg (orally) showed higher percentage writhes inhibition of 78.85 (intraperitoneally) and 79.12 (orally) compared with piroxicam of 74.99 (intraperitoneally) and 76.0 (orally), respectively (Figure 1).

The effect of EEBV on thermally-induced pain stimulus in mice

EEBV significantly (p<0.001) elevated the reaction time to thermal stimulus in mice in a dose dependent manner. The highest protection was observed with morphine at 180 min in both oral (Figure 2) and intraperitoneal (Figure 3) routes were 126.94 and 128.57%, respectively. The reaction time at dose of 800 and 1000 mg/kg were found to be the highest at 180 min (Figures 2 and 3).

The effect of EEBV on formalin induced pain

The extract at doses of 500 and 1000 mg/kg significantly (p<0.05) reduced the severity pain in both phases I and II, while at 250 mg/kg the reduction in pain severity was not significant (Figure 4). Morphine 5 mg/kg, the standard drug used in the study inhibited both phases of nociception, while piroxicam inhibited only the second phase significantly (Figure 4).

The effect of EEBV on carrageenan-induced paw oedema in rats

The injection of 1% saline carrageenan solution into the

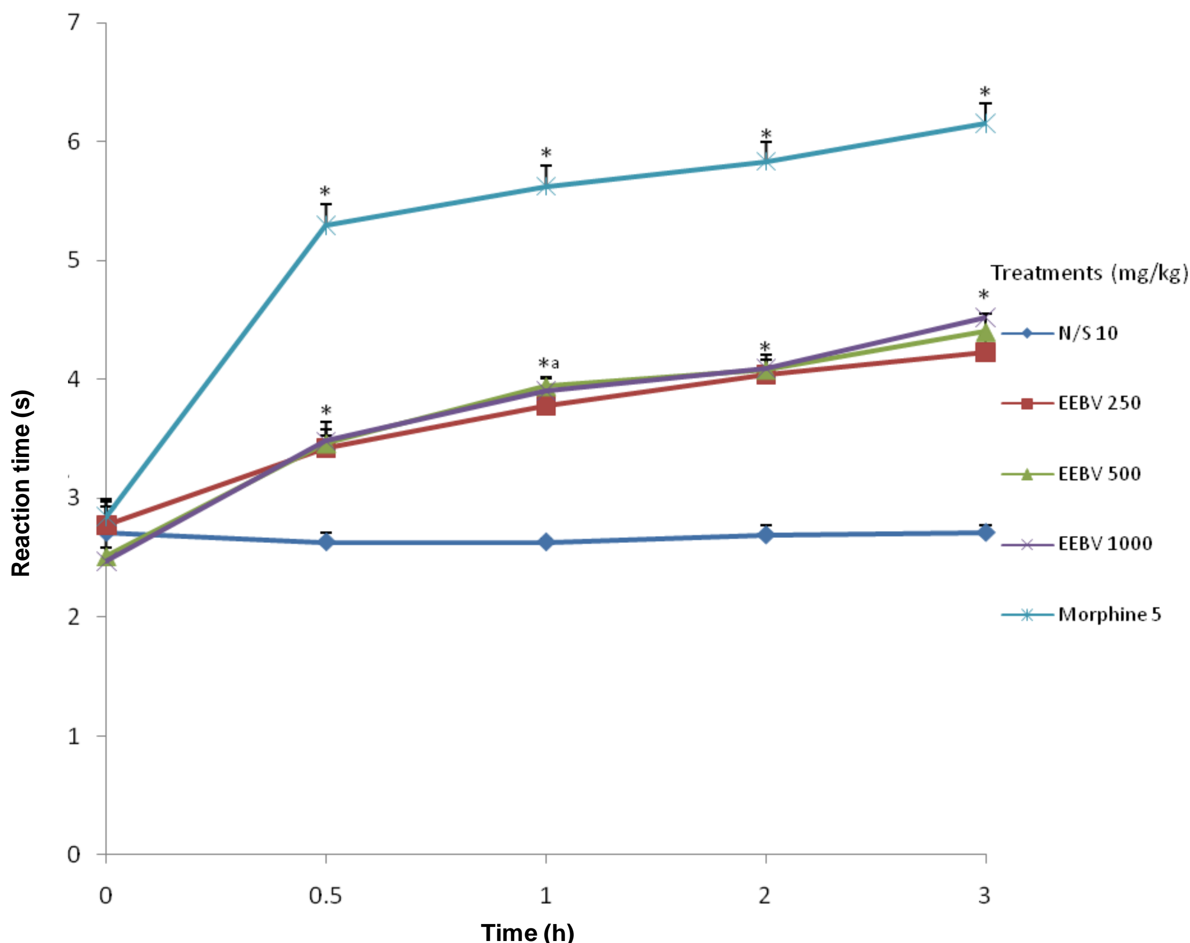

Figure 2. Effect of oral administration of EEBV on thermally induced pain in mice. *p<0.001 compared to normal saline, [a]p<0.001 (n=6) compared to morphine, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata* and N/S = Normal Saline

sub plantar region of the hind paw of the normal saline (negative control) treated group produced local oedema reaching its maximum at the fourth hour. EEBV, at all doses (250, 500 and 1000 mg/kg oral) significantly (p<0.001) inhibited the paw oedema. However, maximum oedema inhibition was observed at dose of 500 mg/kg (57.42%) in the fifth hour (Figure 5). The percentage anti inflammatory effect of piroxicam at the time of maximum oedema inhibition was 64.86% (Figure 5).

The effect of intraperitoneal administration of EEBV on carrageenan-induced paw oedema in rats

The injection of 1% saline carrageenan solution into the sub plantar region of the hind paw of the normal saline (negative control) treated group produced local oedema reaching its maximum at the third hour. EEBV, at all doses (200, 400 and 800 mg/kg) significantly (p<0.001) inhibited the paw oedema. However, maximum oedema inhibition was observed at dose of 800 mg/kg (55.77%) in the fourth hour (Figure 6). The percentage anti-inflammatory effect of piroxicam at the time of maximum oedema inhibition was 59.36% (Figure 6).

The effect of EEBV on formalin-induced paw oedema in rats

The injection of formalin into the sub planter region of the rats' hind paw produced a localized oedema, evident at the first hour and reached peak at the 24th hours in the control group. EEBV significantly (p<0.001) inhibited the paw oedema at doses (250, 500 and 1000 mg/kg oral). The maximum inhibition (63.86%) was observed with EEBV (1000 mg/kg) at the 24th hour when compared with the control group. Piroxicam, a standard anti-inflammatory drug produced 66.92% inhibitions at 24th hour (Figures 4 to 7). The inhibition of paw oedema produced by EEBV was comparable to that of piroxicam, the standard anti-inflammatory drug used in the study (Figure 7).

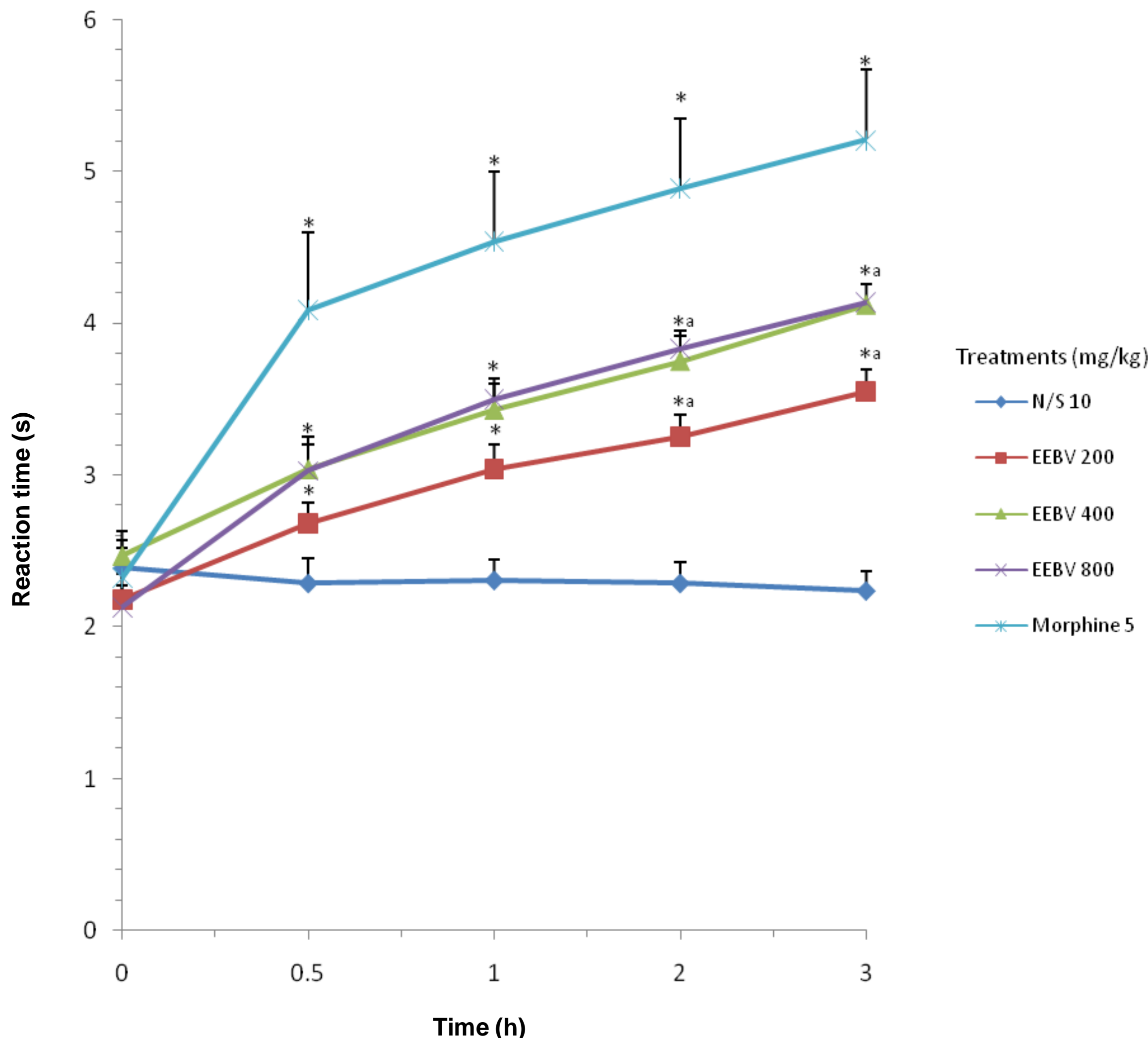

Figure 3. Effect of intraperitoneal administration of EEBV on thermally induced pain in mice. *p<0.001 compared to normal saline, [a]p<0.001 (n=6) compared to morphine, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata* and N/S = Normal Saline

DISCUSSION

The preliminary phytochemical screening of *B. verticillata* showed the presence of alkaloids, saponins, steroids, glycosides, flavonoids, tannins and phenols. This finding is consistent with those of Ushie and Adamu (2010) and Conserva and Ferreira (2012). These compounds have well known pharmacological activities including analgesic, anti-inflammatory and antioxidant effects (Perez, 2001; Park et al., 2001). A number of flavonoids as well as tannins isolated from plants have been discovered to have significant analgesic and/or anti-inflammatory effects (Duke, 1992; Ahmadiani et al., 1998, 2000). The inhibitory effect of flavonoids on eicosanoids synthesis has been documented (Damas et al., 1985; Raj et al., 2001; Danjuma et al., 2011). The ability of certain flavonoids to inhibit a wide array of enzymes such as protein kinase C, protein tyrosine kinases, phospholipase A_2, phosphodiesterases and others have been reported (Middleton, 1998). Alkaloids and saponins possess analgesic and antispasmodic effects, while the healing of wounds and inflamed mucus membranes are hastened by tannins (Okwu and Okwu, 2005). Thus, the presence of these constituents may be contributory to the analgesic and anti-inflammatory effects of the plant.

The oral median lethal dose (LD_{50}) of the extract in both mice and rats were greater than 5000 mg/kg, the intraperitoneal LD_{50} was greater than 5000 and 3807.88 mg/kg in rats and mice, respectively. This suggests that the extract is relatively non toxic orally and may be slightly toxic when administered intraperitoneally. This analysis is based on the toxicity classification proposed by Loomis and Hayes (1996) which states that substances with an LD_{50} values between 500 and 5000 mg/kg

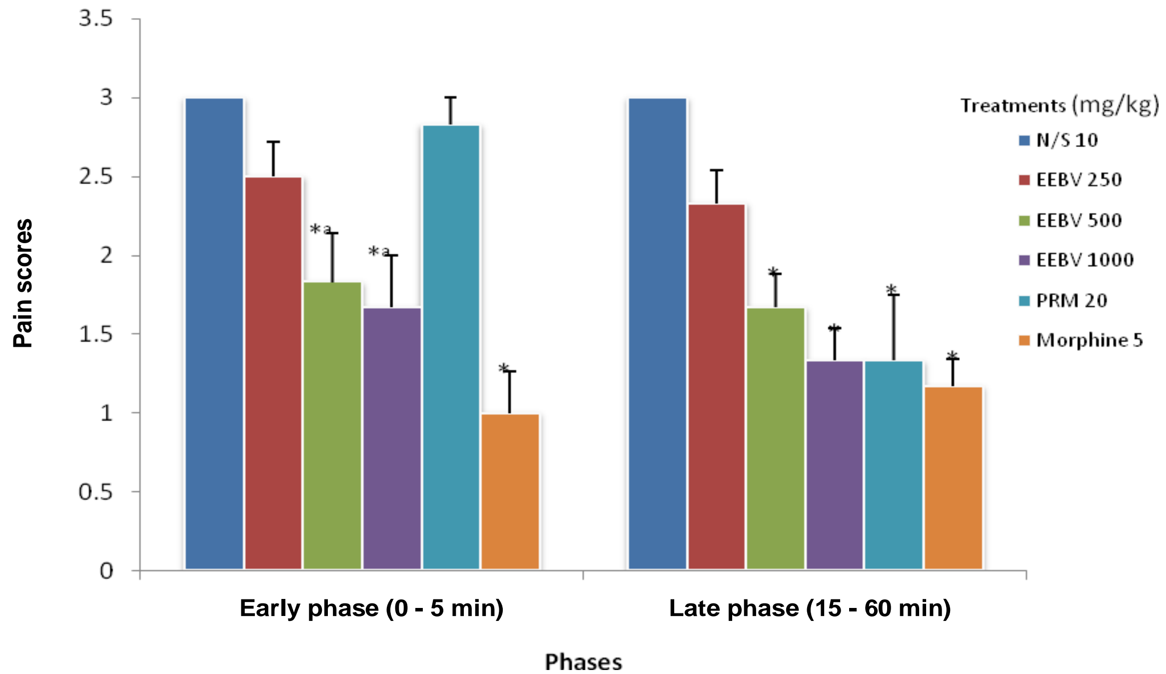

Figure 4. Effects of oral administration of EEBV on formalin-induced pain in rats. *p<0.05 compared to normal saline, ^{a}p<0.05 (n=6) compared to morphine, not significant compared to piroxicam, Kruskal wallis followed by Mann Whitney test. EEBV = Ethanol leaf extract of *B. verticillata,* N/S = Normal Saline and PRM = Piroxicam.

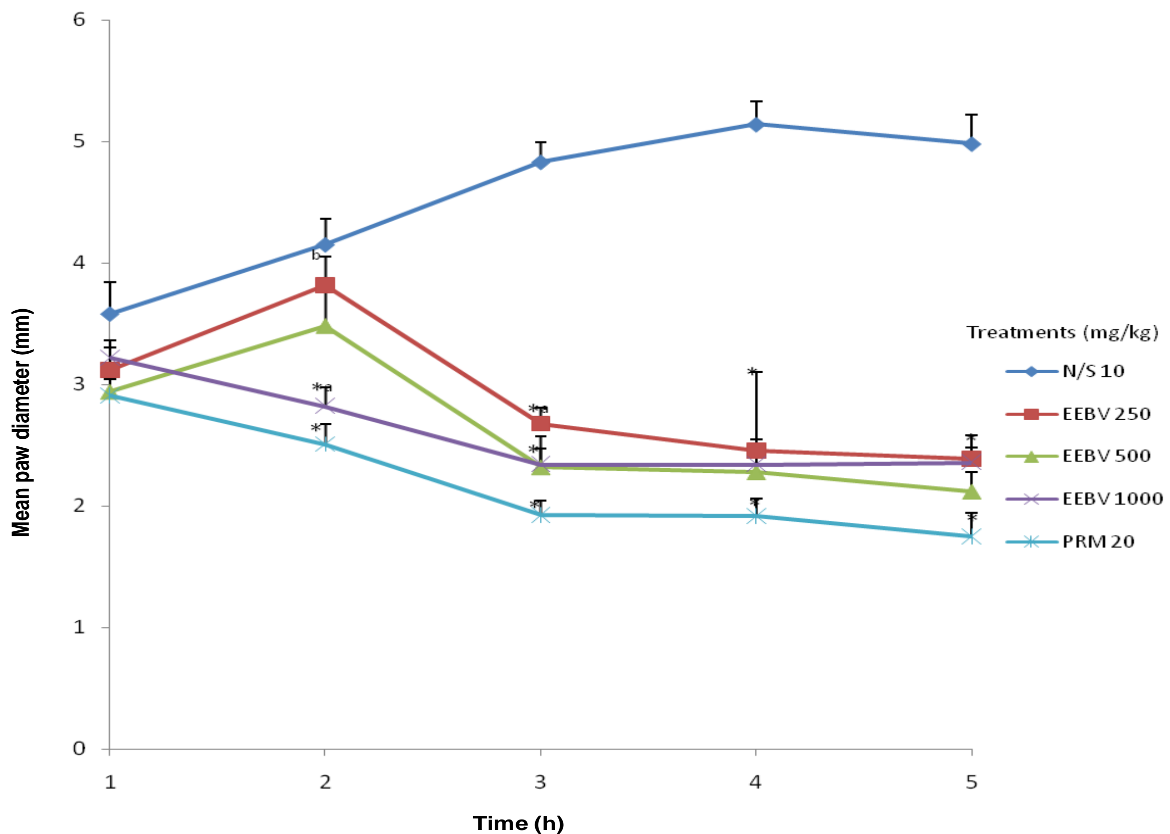

Figure 5. Effect of oral administration of EEBV on carrageenan induced paw oedema in rats. *p<0.001, ^{a}p<0.05 compared to normal saline, ^{b}p< 0.05 (n=6) significant compared to piroxicam, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata*, N/S = Normal Saline and PRM = Piroxicam.

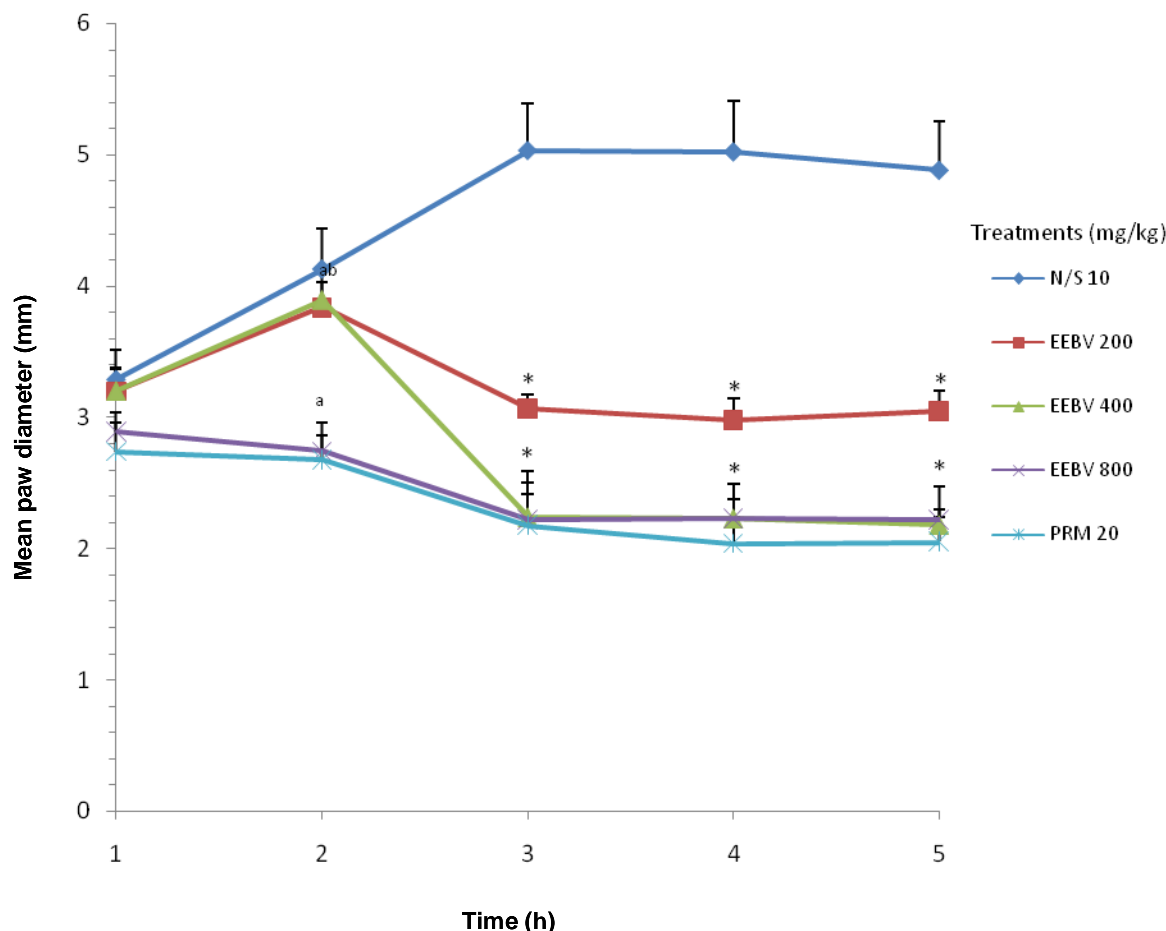

Figure 6. Effect of intraperitoneal administration of EEBV on carrageenan induced paw oedema in rats. *p<0.001, [a]p<0.05 compared to normal saline, [b]p<0.05 (n=6) significant compared to piroxicam, Tukey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata* N/S = Normal Saline and PRM= Piroxicam.

and 5000 mg to15,000 mg/kg body weight are regarded as slightly toxic and practically non toxic, respectively.

Four different animal models were employed to investigate the potential analgesic and anti-inflammatory activities of the plant extract. The models selected enabled the investigation of both central and peripherally-mediated effects.

The acetic acid-induced writhes response is a procedure to evaluate peripheral acting analgesics (Gene et al., 1998). The ethanol leaf extract of *B. verticillata* (EEBV), significantly (p<0.001) and dose-dependently inhibited acetic acid-induced writhes response in mice at doses investigated both by oral and intraperitoneal routes of administration. The acetic acid-induced writhes test is a sensitive test for the screening of peripheral analgesic activity of compounds (Gene et al., 1998). Increased level of prostanoids such as prostaglandins E_2 (PGE_2) and F_2 (PGF_2) in the peritoneal fluid has been implicated in the acetic acid writhes test (Deraedt et al., 1980). Some researchers have also postulated the involvement of lipoxygenase (LOX) and cyclo-oxygenase (COX) (Levine et al., 1984; Dhara et al., 2000). Local peritoneal nociceptive receptors are presumed to be partly involved in the abdominal constriction response (Bentley et al., 1983). The high sensitivity of the acetic acid-induced writhes test allows for detection of nociceptive activity of compounds at dose levels that might seem inactive in other methods like the tail flick test (Collier et al., 1968; Bentley et al., 1981).

The hot plate test described by Eddy and Lambach (1953) is suitable for the evaluation of centrally acting analgesics. Thermally induced nociception suggests narcotic involvement (Besra et al., 1996). The pain threshold in mice is generally elevated by centrally acting analgesics. The nociceptors appear to be sensitized by sensory nerves and endogenous peptides such as prostaglandins may have minimal involvement in this model (Mohan et al., 2009).

Another reliable method of evaluating analgesic activity of compounds is the formalin test. It is better correlated with clinical pain (Tjolsen et al., 1992; Ghannadi et al., 2005). The formalin induced pain elucidates both central

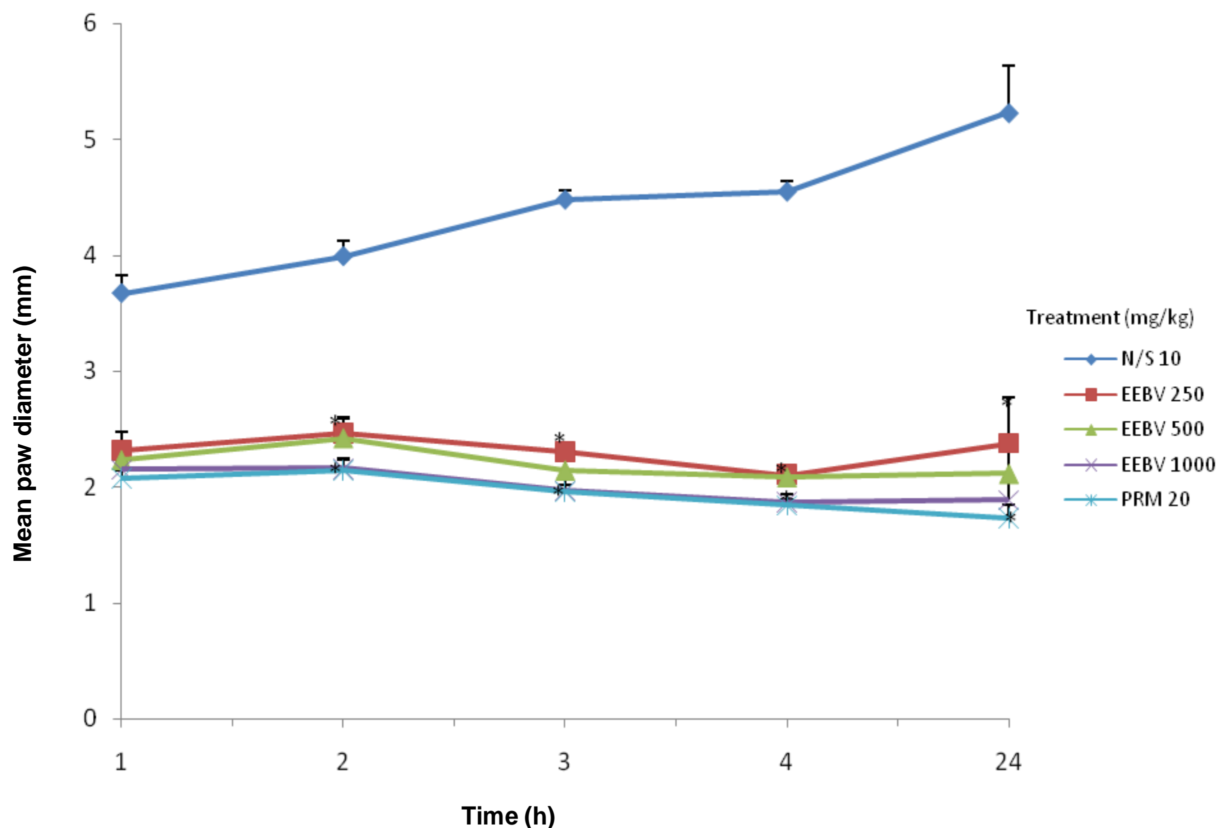

Figure 7. Effect oral administration of EEBV on formalin induced paw oedema in rats. *p<0.001, (n=6) compared to normal saline, not significant compared to piroxicam, Turkey's post hoc test. EEBV = Ethanol leaf extract of *B. verticillata*, N/S = Normal Saline and PRM = Piroxicam.

and peripheral mediated mechanism. The formalin test showed a unique biphasic responses termed early and late phases. The early phase response represents a direct chemical stimulation of pain due to the irritant effect of formalin on sensory C fibres (Hunskaar et al., 1985; Tjolsen et al., 1992) whereas the late phase response is due likely to the development of an inflammatory response and the release of allergic mediators such as serotonin, histamine and bradykinin (Hunskaar and Hole, 1987). Drugs that act centrally inhibit both phases, while those that act peripherally inhibit only the late phase (Chen et al., 1995). The significant ($p<0.05$) suppression of both phases of pain observed with EEBV (500 and 1000 mg/kg) indicates that the extract has both central and peripheral analgesic effect. This inference is further strengthened by the significant activity observed with both acetic acid writhes and the hot plate tests.

Oedema induced by phlogistic agents is a widely accepted model for the evaluation of the anti-inflammatory effect of drugs (Winter et al., 1962; El-Shenawy et al., 2002). To evaluate the anti-inflammatory activity, EEBV was assessed using two popular screening models widely used for NSAIDs, namely, carrageenan and formalin-induced rat paw oedema.

Carrageenan-induced hind paw oedema is the standard experimental model for acute inflammation. Carrageenan is the phlogistic agent of choice for testing anti-inflammatory drugs as it is not known to be antigenic and is devoid of apparent systemic effect (Chakraborty et al., 2004). The model is also known to show a high level of reproducibility (Winter et al., 1962). The probable mechanism of action of carrageenan is biphasic (Vinegar et al., 1969). The first phase is due to the release of histamine and serotonin (0 to 2 h), kinin like substances maintains the plateau phase (3 h) and the second phase is due to the release of prostaglandins (Perianayagam et al., 2006; Fotio et al., 2009). In the carrageenan-induced rat paw oedema test, EEBV at 500 mg/kg body weight showed slightly higher effect than 1000 mg/kg orally. This may be as a result of possible biological variation within test groups and possible interactions between constituents of the extract.

The formalin-induced rat paw oedema also showed a biphasic response and may originate mainly from neurogenic inflammation (up to 24 h) followed by the participation of kinins and leukocytes with their pro-inflammatory factors including prostaglandins (Wheeler- Aceto and Cowan, 1991; Popov et al., 2005). Inflammation induced

by formalin results in cell damage leading to the production and release of endogenous mediators like histamine and bradykinin. The response observed with formalin test correlates with that of carrageenan-induced rat paw oedema, thus re-affirming the anti-inflammatory effect of the plant extract.

The presence of both anti-inflammatory and analgesic effects seen with the extract (EEBV) is well defined for a variety of NSAIDs, especially the salicylates and their derivatives. It is therefore fascinating that the extract behaved like an NSAID in the study and this correlates with the traditional application of the plant in the management of painful and inflammatory conditions. The differences in activity of the extract at, 250 mg/kg body weight in the formalin-induced pain compared to those observed with the hot plate and the acetic acid induced writhes test could also be attributed to possible biological variation within the test groups. The visible lack of significant difference in the effects of different doses in some of the tests for example hot plate (orally and intraperitoneally) could be attributed to a ceiling effect at 250 mg/kg after which increase in dose may not result in any significant increase in observed effects (Vongtau et al., 2004).

Substances are administered to laboratory animals through several routes including oral and intraperitoneal routes. The oral route is economical, convenient and relatively safe; moreover, when substances are investigated for safety, the oral route mimics the common mode of use in humans. Substances administered by oral route may be poorly absorbed or inactivated by gastric secretions in the stomach and possibly first pass effect in the liver. Intraperitoneal route of administration provides faster absorption through the large vascular beds of the intestines. The intraperitoneal route is also accepted for use in laboratory animals due to difficulties of other extravascular routes and the inconsistencies of oral administration in laboratory animals (Turner et al., 2011). In this study, EEBV exhibited anti-nociceptive and anti-inflammatory effects in oral and intraperitoneal routes of administration and no difference in responses were observed, suggesting the possible similarity in oral and intraperitoneal pharmacokinetic profile of EEBV.

ACKNOWLEDGEMENT

The authors are grateful to the authorities of the Ahmadu Bello University, Zaria for providing the facilities for conducting this research.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Ahmadiani A, Fereidoni M, Kamalinejad M, Serami S (1998). Antinociceptive and Anti-inflammatory Effects of *Sambucus ebulus* Rhizome Extract in Rats. J. Ethnopharmacol. 61:229-235.

Ahmadiani A, Hosseiny J, Semnanian S, Javan M, Saeedi F, Kamalinejad M, Serami S (2000). Antinociceptive and Anti-Inflammatory Effects of *Elaeagnus angustifolia* Fruit Extract. J. Ethnopharmacol. 72:287-292.

Ajibesin KK, Benjamin EE, Danladi NB, Essien EE, Saburi AA (2008). Ethnobotanical Survey of Akwa Ibom State of Nigeria. J. Ethnopharmacol. 115:387-408.

Almeida RN, Navarro DS, Barbosa-Filho JM (2001). Plants with Central Analgesic Activity. Phytomedicine 8(4):310-322.

Ayenzu ES (1978). Medicinal plants of West Indies, Unpublished manuscript. Office of Biological Conservation. Smithsonian Institute, Washington D.C. 20560:110.

Benjamin TV (1979). Investigation of *Borreria verticillata*: An Anti Eczematic Plant of Nigeria. Q. J. Crude Drug Res. 17:137-137.

Bentley GA, Newton SH, Starr J (1981). Evidence for an Action of Morphine and the Enkephalins on Sensory Nerve Endings in the Mouse Peritoneum. Brit. J. Pharmacol. 73:325–332.

Bentley GA, Newton SH, Starr J (1983). Studies on the Antinociceptive Action of α-Agonist Drugs and Their Interaction with Opioid Mechanisms. Brit. J. Pharmacol. 79:125–134.

Besra SE, Sharma RM, Gomes A (1996). Anti-inflammatory Effect of Petroleum Ether Extract of Leaves of *Litchi chinensis* Gaertn (Sapinadaceae). J. Ethnopharmacol. 54:1-6.

Burkill HM (2000). The Useful Plants of West Tropical Africa, 2nd ed., Vol. 4. Royal Botanic Gardens, Kew, United Kingdom, p. 686.

Calixto JB (2000). Efficacy, safety, quality and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz. J. Med. Biol. Res. 33:179-189.

Calixto JB, Berritto A, Ferreira,J, Santos AR, Vaidin F, Yunes RA (2000a). Naturally Occurring Anti-nociceptive Substances from Plants. Phytother. Res. 14:401-418.

Chakraborty A, Devi RK, Rita S, Sharatchandra K, Singh TI (2004). Preliminary Studies on Anti-inflammatory and Analgesic Activities of *Spilanthes acmella* in Experimental Animal Models. Indian J. Pharmacol. 36:148-150.

Chen YF, Tsai HY, Wu TS (1995). Anti-inflammatory and Analgesic Activity from roots of *Angelica pubescens*. Planta Med. 61:2–8.

Collier HOJ, Dinneen LC, Johnson CA, Schneider C (1968). The Abdominal Constriction Response and its Suppression by Analgesic Drugs in the Mouse. Brit. J. Pharmacol. 32:295–310.

Conserva LM, Ferreira JC (2012). *Borreria* and *Spermacoce* species (Rubiaceae): A Review of Their Ethnomedicinal Properties, Chemical Constituents and Biological Activities. Pharmacogn. Rev. 6(11):46-55.

Damas J, Bourdon V, Remacle G, Lecomte J (1985). Pro-anti-inflammatory Flavonoids which are Inhibitors of Prostaglandin Biosynthesis. Prostaglandins Leukot. Med. 19:11-24.

Danjuma NM, Sani AA, Yaro AH, Ahmad A, Zezi AU, Hussaini IM (2011). Preliminary evaluation of methanol leaf extract of *Burkea africana* linn for analgesic, anti-inflammatory and antioxidant effects. J. Pharmacol. Trop. Therapeut. 1(2):7-11.

Deraedt R, Joughney S, Delevakee F, Falhour M (1980). Release of prostaglandins E and F in an algogenic reaction and its inhibition. Eur. J. Pharmacol. 51:17-24.

Dhara AK, Suba V, Sen T, Pal S, Nag CAK (2000). Preliminary studies on the anti-inflammatory and analgesic activity of the methanolic fraction of the root extract of *Tragia involucrate*. J. Ethnopharmacol. 72:265–268.

Dubuisson D, Dennis SG (1977). The formalin Test: a quantitative study of the analgesic effect of morphine, meperidine and brain stem stimulation in rats and cats. Pain 4:161-174.

Duke JA (1992). Handbook of Biologically Active Phytochemicals and Their Activities. CRC Press, Boca, Raton, FL.

Eddy NB, Lambach DJ (1953). Synthetic analgesics II-Dithienyl-butenyl and Dithienyl-butyl amines. J. Pharmacol. Exp. Ther. 107(3): 385-393.

El-Shenawy SM, Abdel-Salam OM, Baiuomy AR, El-Baeran S, Arbid MS (2002). Studies on the anti-inflammatory and antinociceptive effects of Melatonin in rat. Pharmacol. Res. 46:235-243.

Evans WC (2009). Trease and Evans Pharmacognosy, 16th edition.

Saunders Elsevier. P 196.
Fotio AL, Dimo T, Nguelefack TB, Dzeufiet PD, Lemba EN, Temdi RJ, Ngueguim F, Olleros ML, Vesin D, Dongo E, Kamtchouing P, Garcia I (2009). Acute and chronic anti-inflammatory properties of the stem bark aqueous and methanol extracts of *Sclerocarya birrea* (Anacardiaceae). Inflammopharmacology 17(4):229-237.
Gene RM, Segura L. Adzet T, Marin E, Inglesias J (1998). *Heterotheca inuloides*: Anti-inflammatory and Analgesic Effects. J. Ethnopharmacol. 60:157-162.
Ghannadi A, Hajhashemi V, Jafarabadi H (2005). An Investigation of the Analgesic and Anti-inflammatory Effects of *Nigella sativa* Seed Polyphenols. J. Med. Food 8:488-493.
Hess SM, Milong RC (1972). Inflammation Mechanism and Control. Lepow IH, Ward PA (eds.). Academic Press, New York. pp. 1-2.
Hunskaar S, Fasmer OB, Hole K (1985). Formalin test in mice, a useful technique for evaluating mild analgesic. J. Neurosci. Methods 14:69-76.
Hunskaar S, Hole K (1987). The formalin test in mice: dissociation between inflammatory and non inflammatory pain. Pain 30:103-114.
Koster R, Anderson N, De Beer JM (1959). Acetic acid used for analgesic screening. Fed. Proc. 18:412-416.
Levine JD, Lau W, Kwait G, Goetzl EJ (1984). Leukotriene B_4 produces hyperalgesia that is dependent on the polymorphonuclear leucocytes. Science 225:743–745.
Loomis TA, Hayes AW (1996). Loomis's Essentials of Toxicology, fourth edition. Academic Press, California, U.S.A. P 282.
Lorenzi H, Matos FJ (2002). Plants Medicinais do Brasil. Sao Paulo: Nova Odessa.
Lorke DA (1983). A new approach to practical acute toxicity testing. Arch. Toxicol. 54:275-287.
Middleton E (1998). Effect of plant flavonoids on immune and inflammatory cell function. Adv. Exp. Med. Biol. 439:175-186.
Mohan M, Gulecha VS, Balaraman R, Austin A, Thirugnanasampathan S (2009). Analgesic and anti-inflammatory activity of a Polyherbal formulation (PHF-AROGH). Oriental Pharm. Exp. Med. 9(3):232-237.
Mohanbabu AV, Shanbhag T, Kumari MK, Bairy, KL, Shendy S (2011). Evaluation of Anti-inflammatory and analgesic activities of Alcoholic extract of *Kaempferia galangal* in Rats. Indian J. Physiol. Pharmacol. 55(1):13-24.
Moreira VF, Oliveira RR, Mathias L, Braz-filho R, Viera IJ (2010). New Chemical Constituents from *Borreria verticillata* (Rubiaceae). Helvetica Chim. Acta 93(9):1751-1757.
Okwu DE, Okwu ME (2005). Chemical Composition of *Spondias Mombia* Linn. Plant Parts. J. Sustain Agric. Environ. 6:140-147.
Park EH, Kahng JH, Lee SH, Shin KH (2001). An anti-inflammatory principle from Cactus. Fitoterapia 72:288-290.
Perez GRM (2001). Anti-inflammatory activity of compounds isolated from plant. Sci. World 1:713-784.
Perianayagam JB, Sharma SK, Pillai KK (2006). Anti-inflammatory activity of *Trichodesma indicum* root extract in experimental animals. J. Ethnopharmacol. 104(3):410-414.
Popov SV, Popova GY, Ovodova RG, Ovodov YS (2005). Anti-Inflammatory Activity of The Pectic Polysaccharide From *Comarum Palustre*. Fitoterapia 76:281-287.
Raj NK, Reddy MS, Chluvadi MR, Krishna DR (2001). Bioflavanoids Classification, Pharmacological, Biochemical Effects and Therapeutic Potentials. Indian J. Pharmacol. 33:2-16.
Sofowora A (2008). Medicinal Plants and Traditional Medicines in Africa, Third edition. Spectrum Books Limited, Ibadan. pp. 197-204, 306-310.
Tjolsen A, Berge DG, Hunskaar S, Rosland JH, Hole K (1992). The formalin test: an evaluation of the method. Pain 5:5-17.
Turner PV, Brabb T, Pekow C, Vasbinder MA (2011). Administration of Substances to Laboratory animals: Routes of Administration and factors to Consider. J. Am. Assoc. Lab. Anim. Sci. 50(5):600-613.
Ushie OA, Adamu HM (2010). Phytochemical Screening of *Borreria verticillata* leaves. J. Agric. Biotechnol. Ecol. 3(1):108-117.
Vieira IJ, Mathias L, Braz-filho R, Schripsema J (1999). Iridinoids from *B. verticillata*. Organ. Lett. 1:1169-71.
Vinegar R, Schreiber W, Hugo RJ (1969). Biphasic Development of Carrageenan Oedema in rats. J. Pharmacol. Exp. Ther. 166:96-103.
Vogel HG (2002). Drug Discovery and Evaluation: Pharmacology Assay. Springer. P 670.
Vongtau HO, Abbaha J, Ngazal IE, Kunle OF, Chindo BA, Otsapa PB, Gamaniel KS (2004). Anti-nociceptive and Anti-inflammatory Activities of the Ethanolic Extract of *Parinari polyandra* Stem Bark in Rats and Mice. J. Ethnopharmacol. 90:115–121.
Wheeler-Aceto H, Cowan A (1991). Neurogenic and Tissue Mediated Components of Formalin Induced oedema. Agents Action 34:264-269.
World Health Organization (WHO). (2003). Fact Sheet No. 134: Traditional medicine. Geneva, Switzerland: WHO. Available at: http://www.who.int/mediacentre/factsheets/2003/fs134/en/
Winter CA, Risley EA, Nuss GW (1962). Carrgeenan induced oedema in the Hind paw of rats, an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111:544-547.
Winter CA, Risley EA, Nuss GW (1963). Anti inflammatory and antipyretic activities of indomethacin, 1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid. J. Pharmacol. Exp. Ther. 141:369-376.

academicJournals

Vol. 8(20), pp. 747-755, 25 May, 2014
DOI: 10.5897/JMPR2014.5324
Article number: 0289AED44842
ISSN 1996-0875
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Chemical and biological study of *Mentha suaveolens* Ehrh. cultivated in Egypt

El-Sayeda A. El-Kashoury[1], Hesham I. El-Askary[1], Zeinab A. Kandil[1]*, Shahira M. Ezzat[1], Mohamed A. Salem[1] and Amany A. Sleem[2]

[1]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Kasr El-Aini Street, Cairo 11562, Egypt.
[2]Department of Pharmacology, National Research Center, El-Behoose St. 31, Dokki, Giza, Egypt.

Received November 21, 2013; Accepted 18 May, 2014

Investigation of the different fractions of the ethanolic extract of the aerial parts of *M. suaveolens* growing in Egypt yielded nine compounds; two new triterpenes [3*β*-acetyl -22α-hydroxy ursa-12,20-diene (compound 1) and 2*α*, 3*β*-dihydroxy-olean-18-en-29-oic acid (compound 7)] and nine known compounds: a sterol and its glucoside; *β*-sitosterol (compound 2) and *β*-sitosterol-3-O- β-D-glucoside (compound 4), a triterpene; oleanolic acid (compound 3), two monoterepenes; dihydrolimonene (compound 5) and 7-hydroxy-*p*-cymene (compound 6), two flavonoids; isoquercitrin (compound 8) and rutin (compound 9) which were isolated from *M. suaveolens* Ehrh. for the first time. The structures of the isolated compounds were identified by spectral data (UV, MS, 1D and 2D-NMR) and comparison with authentic samples. Moreover, the ethanolic extract showed potent analgesic activity as compared to indomethacin. The ethyl acetate fraction was the most potent as anti-inflammatory (88%), followed by the ethanolic extract (82.9%) as compared with indomethacin. The ethanolic extract and its four subfractions showed a moderate inhibitory activity against the tested human pathogenic bacteria.

Key words: *Mentha suaveolens*, anti-inflammatory, analgesic, flavonoids, sterols, triterpenes.

INTRODUCTION

The genus *Mentha*, one of the important members of the Lamiaceae family, is represented by about 19 species and 13 natural hybrids. *Mentha suaveolens* Ehrh. is native of Africa, temperate Asia and Europe (Abbaszadeh et al., 2009).

M. suaveolens has been used in the traditional medicine of Mediterranean areas and has a wide range of effects: tonic, stimulating, stomachic, carminative, analgesic, choleretic, antispasmodic, sedative, hypotensive and insecticidal. It shows depressor activity, analgesic and anti-inflammatory activities (Moreno et al., 2002). On reviewing the current literature on *M. suaveolens,* flavonoids were the major constituents isolated from this species (Tomas-Barberan et al., 1998; Zaidi et al., 1998). Concerning the biological activities, it was found that *M. suaveolens* has antihypertensive (Bello et al., 2001), antioxidant and acetylcholinesterase inhibitory activities (Ferreira et al., 2006) and a monoamine oxidase inhibitory activity (López et al., 2010). Also, the essential oil of *M. suaveolens* was found to have a candidacidal activity

*Corresponding author. E-mail: zeinababdelazizmohamed@yahoo.com. Tel: +2 01006886793. Fax: +2 25320005.
Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

(Donatella et al., 2011). Moreover, in a previous publication, the authors proved that the essential oil of the aerial parts of *M. suaveolens* has analgesic, anti-inflammatory, antioxidant, cytotoxic, hepatoprotective, antioxidant and antifungal activities (El-Kashoury et al., 2012).

The aim of this study was to isolate and identify the major constituents of the aerial parts of *M. suaveolens* Ehrh. cultivated in Egypt and to investigate the possibility of introducing it as a new medicinal plant after screening for some of its biological activities such as analgesic, anti-inflammatory and antimicrobial activities.

MATERIALS AND METHODS

Chemicals

Sterols, triterpenes, phenolics and sugars used as references in co-chromatography (PC, TLC and HPLC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Authentic reference samples used in GLC analyses of the unsaponifiable matters (USM) and those of fatty acids methyl esters (FAME) were provided by the central laboratory at the Faculty of Agriculture, Cairo University. Carrageenan and ascorbic acid were purchased from Sigma, St. Louis, MO, USA. Indomethacin was purchased from EIPICO, Pharmaceutical Co., 6 October City, Egypt. Tetracycline was purchased from Sedico Pharmaceutical Co., 6 October City, Egypt. Diaion HP-20 AG for column chromatography was purchased from 75 to 150 µ, Mitsubishi Chemical Industries Co. Ltd. Silica gel H for vacuum liquid chromatography (VLC) was purchased from E-Merck (Darmstadt, Germany). Silica gel 60 and silica gel RP-18 (70-230 mesh) for column chromatography were obtained from Fluka. Sephadex LH-20 was purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} and silica gel RP-18 precoated plates (Fluka, Germany). The chromatograms were visualized under UV light (at 254 and 366 nm) before and after exposure to ammonia vapor, as well as spraying with *p*-anisaldehyde-sulfuric acid or natural products-polyethylene glycol (NP/PEG) spray reagents.

Apparatus and equipment

UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). Mass spectra were measured using Shimadzu QP-2010 Plus (Kyoto, Japan). NMR spectra were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury-300 instrument (Palo Alto, CA, USA). The NMR spectra were recorded in $CDCl_3$ or DMSO-d_6, and chemical shifts were given in δ (ppm) relative to trimethylsulphoxide (TMS) as internal standard. Electrothermal 9100 (Labequip, Markham, Ontario, Canada) was used for the determination of melting points (mp) (uncorrected). Hewlett-Packard HP 6890 N network GC system equipped with an MSD detector was used for analysis of unsaponifiable matters. Pye Unicam 304 series GC equipped with a dual flame ionization detector and a dual channel recorder was used for analysis of fatty acid methyl esters. The unsaponifiable matter of the *n*-hexane fraction was analyzed on TR-5-MS column (5% phenyl-polysil phenylene siloxane, 30 m × 0.25 mm D × 0.25 μm film thickness), injector temperature 270°C, the initial temperature was 70°C, kept isothermal for 5 min, increased to 280°C by the rate of 4°C min^{-1}, then kept isothermal for 10 min, using helium as a carrier gas at a flow rate 1 ml/min and MSD detector. The fatty acid methyl esters were analyzed on a coiled glass column (1.5 m × 4 mm D) packed with diatomite (100 to 120 mesh) and coated with 10% polyethylene glycol adipate (PEGA), the injector temperature was 250°C. Initial temperature, 70°C increased to 190°C by the rate of 8°C min^{-1}, then kept isothermal for 25 min, using nitrogen as a carrier gas at a flow rate 30 ml/min and FID detector.

Plant

The fresh aerial parts of *M. suaveolens* Ehrh. cultivated in Egypt were collected during the years 2009 to 2011 from plants cultivated in the Experimental Station of Medicinal and Aromatic Plants, Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was kindly authenticated by Dr. Gemma L. C. Bramley, curator of the Lamiaceae collections, Herbarium Department, Library, Art and Archives, Royal Botanic Gardens, Kew, Richmond, Surrey, United Kingdom. Voucher specimen (M-20/313) was kept at the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The plants were left to dry under normal air at ambient temperature (mean temperature, 28°C) in a dark, well-ventilated room for 4 to 5 days, reduced to powder and then subjected to extraction.

Preparation of extracts and fractions

The air-dried powdered aerial parts of *M. suaveolens* Ehrh. (1.5 kg) were exhaustively extracted with 90% ethanol by cold maceration. The total extract was evaporated under reduced pressure to yield a brownish green semi-solid residue (407 g). The residue (400 g) was suspended in water (800 ml) and successively subjected to liquid-liquid fractionation with *n*-hexane (8 × 400 ml), chloroform (10 × 400 ml), ethyl acetate (8 × 400 ml) and *n*-butanol saturated with water (8 × 400 ml). The solvents were evaporated under reduced pressure, yielding 14.0, 7.0, 11.4 and 54.0 g from the *n*-hexane, chloroform, ethyl acetate and *n*-butanol, respectively.

Isolation of the major constituents

Spots from the essential oil were neglected as it was studied separately. *n*-Hexane fraction: 10 g of the *n*-hexane fraction were chromatographed over a VLC column (Silica gel H, 150 g, 13 cm L × 7 cm D). Gradient elution was carried out using *n*-hexane, *n*-hexane/chloroform mixtures, chloroform, chloroform/ethyl acetate mixtures and ethyl acetate. Fractions (200 ml, each) were collected and monitored by TLC (precoated silica gel plates) to yield 3 main subfractions (A to C). Subfraction A (eluted with 100% *n*-hexane, 25% chloroform in *n*-hexane, 0.86 g) was rechromatographed over a silica gel 60 column using *n*-hexane as eluent to give compound 1 (60 mg). Subfraction B (eluted with 70-95% chloroform in *n*-hexane, 1.3 g) was rechromatographed over a silica gel 60 column using *n*-hexane/ethyl acetate mixture (95:5 v/v) as eluent to give compound 2 (17 mg) and compound 3 (85 mg). Subfraction C (eluted with 50 to 95% ethyl acetate in chloroform, 2 g) was rechromatographed over a silica gel 60 column using chloroform/methanol mixture (97:3 v/v) as eluent to give compound 4 (45 mg).

Chloroform fraction: 5 g were chromatographed over a VLC column (Silica gel H, 100 g, 9 cm L × 7 cm D) in the same manner as the *n*-hexane fraction. The collected fractions were monitored by TLC (precoated silica gel plates) to yield 2 main subfractions (D and E). Subfraction D (eluted with 100% *n*-hexane -45% chloroform in *n*-hexane, 2.2 g) was rechromatographed over a silica gel 60 column using *n*-hexane/ethyl acetate mixture (95:5 v/v) as eluent to give compound 5 (20 mg) and compound 6 (61 mg). Subfraction E(eluted with 15% ethyl acetate in chloroform -100% ethyl acetate, 1.5 g) was rechromatographed over a silica gel 60 column using n-

hexane/ethyl acetate mixture (80:20 v/v) as an eluent to give compound 7 (42 mg).

n-Butanol fraction: 15 g were fractionated over a VLC column (Silica gel H, 300 g, 12 cm L × 7 cm D). Gradient elution was carried out using chloroform, chloroform/ethyl acetate mixtures, ethyl acetate, ethyl acetate/methanol mixtures and methanol. Fractions (200 ml, each) were collected and monitored by TLC (precoated silica gel plates) to yield 2 main subfractions (F to G). Subfraction F (eluted with 100% ethyl acetate till 30% methanol in ethyl acetate, 0.5 g) was rechromatographed over a RP-18 column using water as eluent to give compound 8 (40 mg). Subfraction G (eluted with 40% methanol in ethyl acetate till 100% methanol, 5 g) was rechromatographed over a Diaion column using water/methanol mixtures as eluent. A semi-purified subfraction was obtained. Further purification on a Sephadex LH-20 column using methanol as eluent, it gave compound 9 (40 mg). Purification of the ethyl acetate fraction is under publication in another article (Figure 1).

Compound 1

Oily yellow liquid, R_f value (0.83, chloroform-methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, relative intensity, m/z): m/z at 482 (12.5%), 467 (27.5%), 422 (39.11%), 232 (5.32%), 250 (10.04%) 218 (100%), 207 (5.1%), 204 (15.08%), 203 (14%) and 189 (37.61%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.80 (3H, s, Me-28), 0.83 (3H, s, Me-25), 0.85 (3H, d, J= 6.9 Hz, Me-29), 0.87 (3H, s, Me-23), 0.98 (3H, s, Me-24), 1.01 (3H, s, Me-26), 1.07 (3H, s, Me-27), 1.68 (3H, s, Me-30), 1.99 (1H, m, H-18), 2.28 (3H, s, CH_3COO), 4.57 (1H, s, H-3), 4.59 (1H, s, H-22), 5.12 (1H, br. s, H-12), 5.33 (1H, m, H-21) ppm.^{13} C-NMR: δ (75 MHz, $CDCl_3$) 16.2 (C-24), 16.7 (C-25), 16.7 (C-26), 18.1 (C-6), 21.3 (C-32), 22.6 (C-11), 23.2 (C-29), 23.5 (C-30), 24.7 (C-27), 25.0 (C-15), 27.9 (C-2), 29.3 (C-23), 29.4 (C-28), 34.2 (C-7), 34.7 (C-17), 36.6 (C-16), 37.2 (C-10), 37.3 (C-4), 38.3 (C-1), 39.9 (C-19), 41.4 (C-8), 41.9 (C-14), 47.5 (C-9), 55.1 (C-5), 58.9 (C-18), 61.0 (C-22), 80.4 (C-3), 118.1 (C-21), 124.1 (C-12), 139.4 (C-13), 142.3 (C-20), 173.7 (C-31).

Compound 2

White needle crystals, m.p. 140 to 141°C, R_f value (0.62, Chloroform-Methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity), m/z at 414 $[M]^+$ (100%), 396 (51.2%), 329 (41.8%), 303 (43.7%), 273 (59.6%) and 255 (82.5%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.72 (3H, d, J=5.4, Me-21), 0.86 (3H, t, J=6.3, Me-29), 0.91 (3H, d, J=6.3, Me-26), 0.95 (3H,d, J=6.3, Me-27), 1.04 (3H, s, Me-18), 1.56 (3H, s, Me-19), 3.52 (1H, m, H-3), 5.38 (1H, br.s., H-6) ppm.

Compound 3

White amorphous powder, R_f value (0.56, chloroform-methanol 98:2 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, relative intensity, m/z): m/z at 456 $[M]^+$(1.59 %), 411 (0.33%), 438 (2.51%), 248 (100%). 208 (4.80%), 203 (53.14%) and 190 (11.37%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.67 (3H, s, Me-25), 0.71 (3H, s, Me-30), 0.85 (3H, s, Me-29), 0.87 (3H, s, Me-24), 0.89 (3H, s, Me-27), 1.09 (3H, s, Me-26), 1.23 (3H, s, Me-23), 3.00 (1H, m, H-18), 4.25 (1H, br.d, H-3) and 5.16 (1H, br.s, H-12) ppm. ^{13} C-NMR: δ (75 MHz, DMSO) 15.1 (C-25), 16.0 (C-24), 16.9 (C-26), 18.0 (C-6), 22.6 (C-30), 22.9 (C-11), 23.4 (C-16), 25.6 (C-27), 27.2 (C-2), 28.2 (C-15), 29.0 (C-23), 30.4 (C-20), 32.1 (C-21), 32.4 (C-22), 32.8 (C-29), 33.3 (C-7), 36.6 (C-10), 38.4 (C-1), 38.7 (C-4), 40.4 (C-8), 40.8 (C-14), 41.3 (C-18), 45.5 (C-19), 45.7 (C-17), 47.1 (C-9), 54.8 (C-5), 76.8 (C-3), 121.5 (C-12), 143.8 (C-13), and 178.5 (C-28).

Compound 4

White amorphous powder, R_f value (0.21, chloroform-methanol-formic acid 95:5:0.2 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity), m/z at 415 (8.71%), 414 (26.74%), 399 (9.83%), 396 (14.45%), 381 (8.36%), 329 (12.76%), 303 (11.04%), 275 (4.91%), 273 (11.97%), 255 (20.20%), 246 (3.77%), 231 (12.73%), 229 (7.43), 218 (12.48%), 213 (18.60%), 57 (100%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.66 (3H, d, J=5.5 Hz, Me-21), 0.78 (3H, t, J=6.3,Me-29), 0.83 (3H, d, J=6.2 Hz, Me-26), 0.90 (3H, d, J=6.3 Hz, Me-27), 0.92 (3H, s, Me-18), 0.96 (3H, s, Me-19), 3.03 (1H, m, H-3), 4.21 (1H, d, J=7.5, H-1\`), 5.33 (H, br.s, H-6) ppm.

Compound 5

Colorless oily liquid, R_f value (0.57, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). EI-MS (70 ev, relative intensity, m/z): 138 (3.62%), 125 (21.76%), 123 (5.94%), 111 (42.96%), 97 (69.49%), 95 (14.98%), 83 (64.42%), 80 (2.49%), 70 (33.74%), 68 (12.67%), 67 (20%), 57 (100%), 55 (80.01%) and 54 (10.46%). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.87 (3H, br.s, Me-10), 0.89 (3H, br.s, Me-9), 1.56 (1H, br.s, H-5a), 1.73 (H, br.s, H-5b), 2.02 (3H, s, Me-7), 2.04 (2H, br.s, H-6), 2.06 (1H, br.s, H-4), 2.08 (2H, br.s, H-3), 2.29 (1H, br.s, H-8), 5.81 (1H, m, H-2). ^{13} C-NMR: δ (75 MHz, $CDCl_3$) 22.7 (C -9,10), 28.9 (C-7), 29.7 (C-5), 30.0 (C-3), 30.1 (C-6), 31.9 (C -8), 33.8 (C-4), 114.0 (C -2), 139.2 (C-1).

Compound 6

Oily yellow liquid, R_f value (0.46, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (violet). ^{1}H-NMR: δ (300 MHz, $CDCl_3$) 0.89 (3H, d, J= 7.8, Me-10), 0.92 (3H, d, J= 7.8, Me-9), 1.32 (1H, m, H-8), 4.20 (2H, s, H-7), 7.53 (2H, d, J=8.4 Hz, H-2,6), 7.69 (2H, d, J=8.4 Hz, H-3,5) ppm. ^{13} C-NMR: δ (75 MHz, $CDCl_3$) 22.9 (C -10), 23.6 (C -9), 38.6 (C-8), 68.0 (C-7), 128.6 (C-3,5), 130.7 (C -2,6), 132.2 (C -1), 139.0 (C-4).

Compound 7

White powder, R_f value (0.23, chloroform-methanol 95:5 v/v); color in *p*-anisaldehyde/H_2SO_4 (purple). EI-MS (70 ev, rel. int., m/z): 472 (5.73%), 457 (73.4%), 395 (1.46%), 248 (100%), 218 (12.3%), 203 (49.3%), 189 (22.3%). ^{1}H-NMR: δ (300 MHz, DMSO) 0.76 (3H, s, Me-27), 0.80 (3H, s, Me-23), 0.82 (3H, s, Me-24), 0.875 (3H, s, Me-25), 0.90 (3H, s, Me-26), 1.04 (3H, s, Me-28), 1.24 (3H, s, Me-30), 3.00 (1H, m, H-2), 4.20 (1H, m, H-3), 5.14 (1H,br.s, H-19) ppm.^{13} C-NMR: δ (75 MHz, DMSO) 18.9 (C -26),19.8 (C -24), 21.9 (C-25), 27.4 (C -30), 26.3(C-28), 25.5 (C -27), 28.0 (C -23), 33.2 (C-22), 32.3 (C-21), 24.6 (C-11), 20.1 (C-6), 26.4 (C-12), 30.5 (C-15), 36.4 (C-16), 37.7 (C-17), 35.3 (C-10), 36.9 (C-7), 40.4 (C-8), 39.0 (C-4), 56.4 (C-20), 49.7 (C-13), 51.0 (C-14), 38.4 (C-1), 54.2 (C-9), 56.2 (C-5), 64.2 (C-2), 86.2 (C-3), 134.0 (C-19), 147.6 (C-18), 187.7 (C -29).

Compound 8

Yellow powder, R_f value (0.58, in ethyl acetate- formic acid -glacial acetic acid- water 100:11:11:10 v/v), Color of the spot in UV (yellow) and in NP-PEG/ UV (orange-yellow). ^{1}H-NMR: δ (300 MHz, DMSO) 4.12 (1H,d, J= 6.9 Hz, H-2\`\`), 5.43 (1H,d, J= 7.5 Hz, H-1\`\`),6.19 (1H, d, J=1.8, H-6), 6.39 (1H, d, J=2.1, H-8), 6.82 (1H, d, J=9 Hz, H-5\`), 7.13 (1H, br.s., H-2\`), 7.56 (1H, dd, J=2.7,8.1 Hz, H-6\`).UV (nm): MeOH 257, 273sh, 365 (3-OH substituted flavonol) NaOMe 279, 329, 410 (free OH on ring A and B) $AlCl_3$ 279, 303sh,

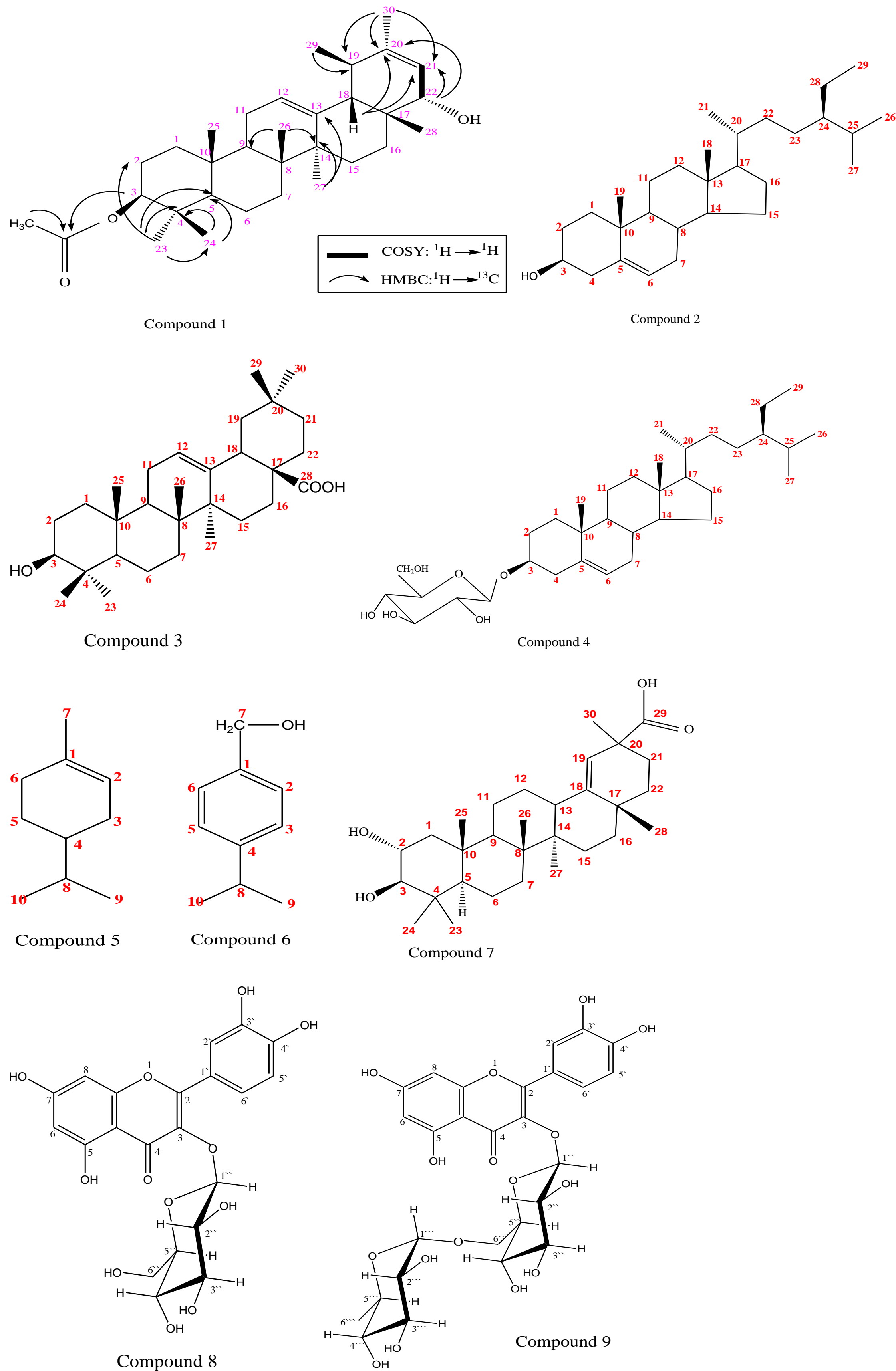

Figure 1. Structures of the isolated compound.

Table 1. Components identified by GLC analysis of the USM of the *n*-hexane fraction of *M. suaveolens* Ehrh.

Peak	RR_t* (min)	Identified components	Number of carbons	Relative percentage
1	0.810	*n*-Dodecane	C_{12}	9.70
2	0823	*n*-Tridecane	C_{13}	7.49
3	0.826	*n*-Tetradecane	C_{14}	2.61
4	0.839	*n*-Pentadecane	C_{15}	4.66
5	0.867	*n*-Hexadecane	C_{16}	4.80
6	0.873	*n*-Heptadecane	C_{17}	3.16
7	0.889	*n*-Octadecane	C_{18}	1.08
8	0.922	*n*-Nonadecane	C_{19}	2.86
9	0.936	*n*-Eicosane	C_{20}	2.65
10	0.991	*n*-Heneicosane	C_{21}	3.50
11	1	*n*-Docosane	C_{22}	13.76
12	1.015	*n*-Tricosane	C_{23}	7.87
13	1.028	*n*-Tetracosane	C_{24}	3.22
14	1.282	*n*-Pentacosane	C_{25}	3.64
15	1.286	*n*-Hexacosane	C_{26}	2.27
16	1.291	*n*-Heptacosane	C_{27}	3.98
17	1.294	*n*-Octacosane	C_{28}	1.21
18	1.295	*n*-Nonacosane	C_{29}	2.24
19	1.299	*n*-Triacontane	C_{30}	1.03
20	1.472	*n*-Hentriacontane	C_{31}	0.73
21	1.623	*n*-Dotriacontane	C_{32}	0.99
22	1.680	Stigmasterol	C_{27}	1.90
23	1.705	*β*-Sitosterol	C_{27}	5.61
% Total identified components				90.96
% Identified hydrocarbons				83.45
% Identified sterols				7.51

*Retention time relative to *n*-Docosane (R_t = 38.21 min).

435 (free OH on ring A and B) $AlCl_3$/HCl 276, 365sh, 408 (free OH at C5) CH_3COONa 278, 328sh, 398 (free OH at C7 and ring B) CH_3COONa/H_3BO_3 267, 291sh, 390 (ortho OH at ring B).

Compound 9

Pale buff powder, R_f value (0.4, ethyl acetate- formic acid -glacial acetic acid- water 100:11:11:10 v/v), Color of the spot in UV (brown) and in NP-PEG/ UV (orange). ^{1}H-NMR: δ (300 MHz, DMSO) Aglycone: 6.43 (1H, br.s, H-6), 6.69 (1H, br.s, H-8), 6.83 (1H, d, J=8.4 Hz, H-5'), 7.38 (1H, br.s, H-2'), 7.42 (1H, dd, J=1.2, 8.4, H-6'). Sugar: 1.02 (3H, d, J =6.3Hz, Me-6```), 4.54 (1H, br.s, H-1'''), 5.04 (1H, d, J=7.8Hz, H-1''). UV (nm): in CH_3OH 258, 300sh, 358 (flavonol) CH_3ONa 268, 328sh, 410(free OH on ring A & B) $AlCl_3$ 270, 306sh, 426 (free OH on ring A & B) AlCl3/HCl 268,298sh, 366, 400 (free OH at 5 and ortho OH at ring B) CH_3COONa 264, 300sh, 382 (free OH at 7 & ortho OH at ring B) CH_3COONa/H_3BO_3 262, 308sh, 378 (ortho OH at ring B).

GLC analysis of the lipoidal matter

The saponifiable and unsaponifiable fractions were obtained from the *n*-hexane fraction (1 g) and the separated fatty acids were methylated by adopting the method described by Vogel (1975). Each of the unsaponifiable matter and fatty acid methyl esters was subjected to GLC analysis. Components were identified by GLC analysis and shown in Table 1 and 2.

Biological evaluation

Animals

Adult male rats of Sprague-Dawley strain [130 to 150 g body weight] and Swiss male Albino mice (20 to 25 g) were obtained from the animal house of the National Research Centre, Giza, Egypt. They were kept under the same hygienic conditions and were fed by the basal diet recommended by the American Institute of Nutrition (AIN, 1977). All experimental procedures were performed in accordance with internationally accepted principles for laboratory animal use and were approved by the Ethics Committee of the National Research Centre (No. 9-031).

Analgesic activity

Analgesic activity of the oral administration of the ethanolic extract and its subfractions (*n*-hexane, choloform, ethyl acetate and *n*-butanol) were evaluated using acetic acid induced writhing test in mice (Koster et al., 1959) using indomethacin as a standard drug.

Table 2. Components identified by GLC analysis of the FAME of the *n*-hexane fraction of *M. suaveolens* Ehrh.

Peak	R_t* (min)	Fatty acids corresponding to identified FAME	Relative percentage
1	0.72	Lauric acid (C_{12})	0.84
2	0.896	Myristic acid (C_{14})	2.43
3	1	Palmitic acid (C_{16})	45.27
4	1.039	Palmitoleic acid ($C_{16:1}$)	11.21
5	1.246	Stearic acid (C_{18})	5.67
6	1.284	Oleic acid ($C_{18:1}$)	5.30
7	1.361	Linoleic acid ($C_{18:2}$)	7.86
8	1.459	Linolenic acid ($C_{18:3}$)	19.26
% Total identified components			97.84
% Saturated fatty acids			54.21
% Unsaturated fatty acids			43.63

*Retention time relative to palmitic acid (R_t = 12.833 min).

Table 3. Analgesic activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using acetic acid induced writhing test.

Animal group	Body weight (mg/kg)	Number of writhes (Mean ± SE)	Inhibition as compared to the control (%)	Potency as compared to the standard (%)
Control	1 ml saline	46.8±1.2	00.0	00.0
Ethanolic extract	100	24.9±0.7*	46.8	78.5
n-Hexane fraction	100	36.5±1.4	22.0	36.9
Choloform fraction	100	39.7±1.3	15.0	25.2
Ethyl acetate	100	28.3±0.6*	39.5	66.3
n-Butanol fraction	100	31.2±1.1*	32.7	54.7
Indomethacin	20	18.9±0.3*	59.6	100.0

*Statistically significant difference from zero time at P<0.01, n=6.

Table 4. Acute anti-inflammatory activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using carrageenan-induced rat paw oedema test

Animal group (Dose)	Body weight (mg/kg)	Percentage of oedema (Mean ± SE)	Inhibition as compared to the control (%)	Potency as compared to the standard (%)
Control	1 ml saline	59.7± 1.8	0	0
Ethanolic extract	100	28.7± 0.6*	51.9	82.9
n-Hexane fraction	100	48.6± 1.7	18.6	29.7
Choloform fraction	100	47.5± 1.2	20.4	32.6
Ethyl acetate	100	26.8± 0.6*	55.1	88.0
n-Butanol fraction	100	36.3± 1.4*	39.2	62.6
Indomethacin	20	22.3± 0.4*	62.6	100.0

*Statistically significant difference from zero time at P<0.01, n=6.

The number of writhes was counted over a period of 30 min after acetic acid injection. Results are as shown in Table 3.

Acute anti-inflammatory activity

Acute anti-inflammatory activity of the oral administration of the ethanolic extract and its subfractions were evaluated and compared with that of indomethacin as a standard drug using carrageenan-induced rat paw oedema test (Winter et al., 1962). The paw oedema was measured 3 h after injection of carrageenan. Results are recorded as shown in Table 4.

Agar disc diffusion method

The antimicrobial screening of the ethanolic extract and its sub-fractions were performed by the agar disc diffusion method (Bauer

Table 5. Antimicrobial activity of *M. suaveolens* Ehrh. ethanolic extract and its fractions using agar disc diffusion method.

Tested microorganism	Diameter of inhibition zone in mm (percentage of efficiency)					
	Ethanolic extract	*n*-Hexane fraction	Chloroform fraction	Ethyl acetate fraction	*n*-Butanol fraction	Tetracycline
Gram-positive bacteria *Staphylococcus aureus* (ATCC12600)	12 (43)	12 (43)	12 (43)	11 (39)	10 (36)	28 (100)
Streptococcus faecalis (ATCC19433)	12 (39)	13 (42)	15 (48)	11 (35)	11 (35)	31 (100)
Bacillus subtilis (ATCC6051)	10 (31)	13 (41)	13 (41)	13 (41)	10 (31)	32 (100)
Gram-negative bacteria *Escherichia coli* (ATCC11775)	12 (40)	13 (43)	12 (40)	12 (40)	10 (33)	30 (100)
Neisseria gonorrhoeae (ATCC19424)	12 (35)	10 (29)	12 (35)	12 (35)	10 (29)	34 (100)
Pseudomonas aeruginosa (ATCC10145)	12 (39)	11 (35)	13 (42)	12 (39)	10 (32)	31 (100)

et al., 1966). Standard discs of tetracycline (5 µg/disc) served as a postive control. The test samples were dissolved in DMSO at a concentration of 20 mg/ml. Aliquots of 50 µl (equivalent to 1 mg of the tested extracts) were aseptically added to the cups of the inoculated plates. Results are shown in Table 5.

Statistical analysis

The data obtained were presented as mean ± standard error and the significance of difference between test and control groups was statistically analyzed using student's t-test. P values of 0.05 or less was considered as criteria for significance.

RESULTS AND DISCUSSION

The EI-MS spectrum of compound 1 showed a molecular ion peak at m/z 482 calculated for the molecular formula $C_{32}H_{50}O_3$. In addition to the peak at m/z 467 $[M-CH_3]^+$. A prominent peak appeared at m/z 422 which is the characteristic for $[M-CH_3COOH]^+$. The EI-MS spectrum of this compound was similar to pentacyclic triterpenes of the ursane series in which ring C is unsaturated at C_{12} (Goad and Akihisa, 1997). The retro-Diels-Alder fragmentation generated two fragments, a (at m/z 232) and b (at m/z 250). The fragment at m/z 232 gives the base peak at m/z of 218 (by the loss of methylene group). In addition to peaks at m/z of 204 and 189 which are characteristic to pentacyclic triterpenes of the ursane nucleus (Shiojima et al., 1992). The ^{1}H-NMR spectrum showed signals for eight methyl groups, seven of which were positioned at quaternary carbons corres-ponding to the singlets at δ 1.68 ppm (CH_3-30), 1.07 ppm (CH_3-27), 1.01 ppm (CH_3-26), 0.98 ppm (CH_3-24), 0.87 ppm (CH_3-23), 0.83 ppm (CH_3-25) and 0.80 ppm (CH_3-28). The CH_3-29 methyl appeared as three-proton doublet at δ 0.85 ppm (J=6.9 Hz). So, the ^{1}H-NMR spectrum suggested that it is urasne type triterpene with the olefinic proton at C-12 and extra double bond at C-21 (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). The assignments of protons were supported by the correlations in ^{1}H-^{1}H COSY. The ^{1}H-NMR spectrum also showed, two downfield protons at δ 5.33 and 5.12 ppm which were assigned to H-21 and H-12, respectively. The two protons at δ 4.59 and 4.57 ppm were assigned to H-22 and H-3, respectively. In addition to the signal at δ 2.28 ppm methyl were assigned to acetate moiety. The proton at δ 1.99 ppm was ascribed to 18-*β* proton (Goad and Akihisa, 1997). On the other hand, the ^{13}C-NMR of compound 1 showed 30 carbon signals corresponding to those of the urs-12-ene type framework of the molecule with extra double bond at C-21 (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). The signals of vicinyl carbons appeared at δ 124.1 (C-12), 139.4 (C-13), 142.3 (C-20) and 118.1 ppm (C-21). These assignments were deduced from HSQC correlations. The first double bond was established at C-12/C-13 from the HMBC correlations of CH_3-27 with C-13. The second double bond was established at C-20/C-21 from the HMBC correlations of H_1-22, H_1-18 and CH_3-30 with C-20 and C-21. The signal at δ 173.7 ppm was assigned for the carbonyl of acetate group. The acetyl group was established to C-3 position based on the HMBC correlations of protons of the acetate group with C-3. The signals at δ 80.4 and 61.0 ppm were assigned for the oxymethine groups of C-3 and C-22, respectively. From the aforementioned data, this compound was identified as 3β-acetyl -22α-hydroxy ursa-12,20-diene which is a new natural product.

Compounds 2 to 4 were identified as *β*-sitosterol (compound 2), oleanolic acid (compound 3) and *β*-sitosterol -3-O- β-D-glucoside (compound 4) from their mass spectra, ^{1}H and ^{13}C-NMR, direct

comparison of melting points and co-chromatography with authentic samples as well as the available literature (Goad and Akihisa, 1997; Hu et al., 1995; Shiojima et al., 1992).

The EI-MS of compound 5 represents the presence of a molecular ion peak at m/z 138, calculated for the molecular formula $C_{10}H_{18}$. In addition to the peak at m/z 123 $(M-15)^+$. The two peaks at m/z 70 and 68 are characteristic of a retro-Diels-Alder fragmentation of Δ^1-*P*-menthene. Moreover, the peak at m/z 67 (loss of proton from fragment at m/z 68) and the peak at m/z 95 represents the loss of isopropyl group $(M-43)^+$. The characteristic peaks at m/z 80 represents $(95-CH_3)^+$, the peak at m/z 55 represents a retro-Diels-Alder fragmentation of fragment at m/z 80 and the peak at m/z 54 represents the loss of proton from fragment at m/z 55 (Crews et al., 1998; Fang et al., 2010; Silverstein and Webster, 1996). ^{13}C-NMR spectrum of compound 5 showed 10 carbons indicating that it is a monoterpene. This suggestion was confirmed from ^{1}H-NMR spectrum by the presence of two signals at δ 0.87 and 0.89, each integrated as three protons and assigned to the two methyls of the isopropyl group. The presence of a signal at δ 5.81 ppm (1H, m, H-2) indicates the presence of a double bond, which is most probably at C-1, that was confirmed from the downfield shift of Me-7 (δ 2.02 ppm). The ^{13}C-NMR spectrum of the compound confirmed the presence of a double bond by the two olefinic carbons at δ 114.0 and 139.2 ppm assigned to C-2 and C-1, respectively (Fang et al., 2010). From the aforementioned data, compound 5 was identified as dihydrolimonene.

^{13}C-NMR spectrum of compound 6 showed 10 carbons indicating that it is a monoterpene (Crews et al., 1998; Silverstein and Webster, 1996). This suggestion was confirmed by the presence of two signals at δ 0.89 and 0.92 ppm, each integrated as three protons and assigned to the two methyls of the isopropyl group. The presence of two doublets at δ 7.53 and 7.69 ppm in its ^{1}H-NMR spectrum with a coupling constant (J= 8.4 Hz) indicating an ortho coupling, each integrated as two protons assigned to H-2,6 and H-3,5 suggests the presence of a *p*-disubstituted benzene ring. The presence of two signals at δ 128.6 and 130.7 ppm assigned to C-3,5 and C-2,6, in addition to two signals at δ 132.2 and 139.1 ppm assigned to C-1 and C-4, respectively confirmed the presence of a *p*-disubstituted aromatic system. The presence of a singlet at δ 4.20 ppm also integrated as two protons suggest the presence of a benzylic moiety at H-7. The downfield shift of C-7 (δ 68.0 ppm) indicated that it is a hydroxylated carbon (Crews et al., 1998; Silverstein and Webster, 1996). From the aforementioned data, compound 6 was identified as 7-hydroxy-*p*-cymene.

The EI-MS spectrum of compound 7 showed a molecular ion peak at m/z of 472, a base peak at m/z 248 in addition to peaks at m/z 203 and 189. In addition to other characteristic peaks of olean-18-ene skeleton (at m/z 395, 218, 203 and 189) (Shiojima et al., 1992). The ^{1}H-NMR spectrum of compound 7 showed the seven singlets assigned to the seven methyl groups of a pentacyclic triterpene and the ^{13}C-NMR spectrum also showed in addition a signal at δ 187.7 ppm indicating that it is a pentacyclic triterpene acid. Data of this compound showed signals characteristic of an olean-18-en skeleton, especially the two signals at δ 134.0 and 147.6 ppm assigned to C-19 and C-18, respectively (Goad and Akihisa, 1997; Mahatao and Kundu, 1994). In addition, the downfield shift of C-3 (δ 86.2 ppm with a downfield shift of about 6 ppm) indicated the presence of α-hydroxy group at C-2 in the ^{13}C-NMR spectrum (Mahatao and Kundu, 1994). This was confirmed from the downfield shift of C-2 (δ 64.2 ppm) also the downfield shift of C=O of the carboxylic acid group (δ 187.7 ppm) suggests its presence at C-29 (Mahatao and Kundu, 1994; Nakano et al., 1997). ^{1}H and ^{13}C-NMR spectra indicated that this compound was a Δ^{18} oleanane-type triterpene by the chemical shifts of the two olefinic carbons at δ 147.6 (C18) and 134 (C19). The position of the two hydroxy methine carbons at C-2 and C-3 and their configurations were confirmed as *α*- and *β*-oriented, respectively, by comparing their values with previously reported data (Mahatao and Kundu, 1994). From the aforementioned data, it can be concluded that compound 7 was identified as 2*α*, 3*β*-dihydroxy-olean-18-en-29-oic acid which is a new natural product.

Compounds 8 and 9, isolated from the *n*-butanol fractions, were identified as isoquercitrin and rutin, respectively, from their UV, ^{1}H and ^{13}C-NMR and co-chromatography with authentic samples as well as comparing with the available literature (Grace et al., 1998; Markham, 1982).

To the best of our knowledge, this is the first report on the isolation of these compounds from *M. suaveolens* Ehrh. In addition, compounds 1 and 7 are new compounds.

Concerning the biological screening, it was clear that the ethanolic extract showed the most potent analgesic activity as it caused the least number of writhes (24.9) (78.5% potency) as compared to indomethacin. It was followed by the ethyl acetate and n-butanol fractions whose potency percentages were 66.3 and 54.7%, respectively. While, the ethyl acetate fraction was the most potent anti-inflammatory (88% potency) as compared to indomethacin, followed by the ethanolic extract (82.9% potency) followed by *n*-butanol fraction which exhibited 62.6% potency. From the results, it is obvious that both the interesting analgesic and anti-inflammatory activities of *M. suaveolens* were exerted by the ethanol extract, the ethyl acetate and n-butanol fractions. So, it could be concluded that these activities may be attributed to their phenolic contents.

Concerning the antimicrobial activity, it was found that all the tested samples showed a moderate inhibitory activity against human pathogenic bacteria.

Results of GLC analysis of the unsaponifiable matter

(Table 1) revealed that the total identified hydrocarbon and sterol components represented 90.96% of the total unsaponifiable matter. Hydrocarbons constituted 83.45% of the total composition; among which, the major constituents were *n*-docosane (13.76%) and *n*-dodecane (9.70%). Sterols were represented only by β-sitosterol and stigmasterol (5.61 and 1.90%, respectively). GLC analysis of the fatty acid methyl esters (Table 2) revealed that the total identified fatty acids represented 97.84% of the total saponifiable matter. The saturated fatty acid constituted 54.21% of the total fatty acid composition; among which palmitic acid (45.27%) was the major constituent. The unsaturated fatty acids amounted to 43.63% of the total fatty acid composition. Linolenic acid was the major component (19.26%) followed by palmitoleic (11.21%), linoleic (7.86%) and oleic (5.30%) acids. The potent analgesic and anti-inflammatory activities of the ethanolic extract may be due to its content of sterols, triterpenes, phenolic acids and flavonoids which have been proved to exert anti-inflammatory activity. *β*-Sitosterol and its glucoside have profound anti-inflammatory activity (Bouic et al., 1996). In addition, flavonoids have been discovered to have anti-inflammatory activity (Ziaullah et al., 2013). More specifically, rutin is widely used in treating ailments through its anti-inflammatory activities (Yang et al., 2008). Oleanolic acid has also been long-recognized to have anti-inflammatory properties in laboratory animals (Liu, 1995). *β*-Sitosterol has been reported to reduce carcinogen-induced cancer in rats (Bouic et al., 1996). During the last two decades, pharmacological studies of oleanolic acid, the major triterpenoid compound isolated from the *n*-hexane fraction, indicated that this triterpenoid have many beneficial effects, notably hepatoprotection, antiinflammation and antitumor-promotion (Liu, 1995).

Conclusion

The present work showed that *M. suaveolens* cultivated in Egypt is a rich source of the important isolated compounds, described here for the first time from the plant. In addition, the plant revealed remarkable biological activities which need further clinical investigation.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Abbaszadeh B, Valadabadi S, Farahani H, Darvishi H (2009). Studying of essential oil variations in leaves of *Mentha* species. Afr. J. Plant Sci. 3:217–221.

AIN (1977). Report of the American institute of nutrition ad hoc. Committee on standards for nutritional studies. J. Nutr. 107:1340-1648.

Bauer AW, Kirby WM, Sherris C, Turck M (1966). Antibiotic susceptibilty testing by a standardized single method. Am. J. Clin. Pathol. 45:493-496.

Bello R, Calatayud S, Beltran B, Primo-Yufera E, Esplugues J (2001). Cardiovascular effects of the methanol and dichloromethane extracts from *Mentha suaveolens* Ehrh. Phytother. Res. 15:447-448.

Bouic PJD, Etsebeth S, Liebenberg RW, Albrecht CF, Pegel K, Van Jaarsveld PP (1996). Human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination. Int. J. Immunopharmacol. 18:693-700.

Crews P, Rodriguez J, Jaspars M (1998). Organic Structure Analysis, Oxford University Press, New York.

El-Kashoury EA, El-Askary HI, Kandil ZA, Salem MA, Sleem AA (2012). Chemical composition and biological activities of the essential oil of *Mentha suaveolens* Ehrh. Z. Naturforsch. 67c:571-579.

Fang R, Jiang CH, Wang XY, Zhang HM, Liu ZL, Zhou L, Du SS, Deng ZW (2010). Insecticidal activity of essential oil of *Carum carvi* fruits from China and its main components against two grain storage insects. Molecules 15:9391-9402.

Ferreira A, Proenca C, Serralheiro MLM, Araujo ME (2006). The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J. Ethnopharmacol. 108:31-37.

Goad J, Akihisa T (1997). Analysis of Sterols. 1st edition, Blackie Academic and Professional Press, Chapman and Hall, London.

Grace MH, Mohamed TK, Khattab AM (1998). Flavonoids of *Carduncellus eriocephalus*, Egypt. J. Pharm. Sci. 39:409-416.

Hu M, Ogawa K, Sashida Y, Xiao PG (1995). Triterpenoid glucuronide saponins from root bark of *Aralia armata*. Phytochemistry 39:179-184.

Koster R, Anderson M, De Beer E (1959). Acetic acid for analgesic screening. J. Fed. Proc. 18:412-415.

Liu J (1995). Pharmacology of oleanolic acid and ursolic acid: review article. J. Ethnopharmacol. 49:57-68.

López V, Martín, S. Gómez-Serranillos M, Carretero M, Jäger A, Calvo M (2010). Neuroprotective and neurochemical properties of mint extracts. Phytother. Res. 24(6):869-874.

Mahatao SB, Kundu AP (1994). ^{13}C NMR Spectra of pentacyclic triterpenoids-a compilation and some salient features: Review Article. Phytochemistry 37:1517-75.

Markham KR (1982). Techniques of Flavonoid Identification; Academic Press, London.

Moreno L, Bello R, Primo-Yufera E, Esplugues J (2002). Pharmacological properties of the methanol extract from *Mentha suaveolens* Ehrh. Phytother. Res. 16:10-13.

Nakano K, Oose Y, Takaishi Y(1997). A novel epoxy-triterpene and nortriterpene from callus cultures of *Tripterygium wilfordii.* Phytochemistry 46:1179-1182.

Shiojima K, Arai Y, Masuda K, Takase Y, Ageta T, Ageta H (1992). Mass spectra of pentacyclic triterpenoids. Chem. Pharm. Bull. 40:1683-1690

Silverstein RM, Webster FX (1996). Spectrometric identification of organic compounds, 6th Ed., John Wiley & sons, INC., New York.

Tomas-Barberan FA, Husain S, Gil MI (1998). The distribution of methylated flavones in the *Lamiaceae*. Biochem. Syst. Ecol. 16:43-49.

Vogel AJ (1975). A Textbook of Practical Organic Chemistry; 3rd ed., English Language Book Society and Longmans Group Ltd., London, pp. 969-971.

Winter GA, Risley EA, Nuss GW (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiinflammatory drugs. P. Soc. Exp. Biol. Med. 111:544-547.

Yang J, Guo J, Yuan J (2008). *In vitro* antioxidant properties of rutin. Leben.Wissen. Technol. 41:1060–1066.

Zaidi F, Voirin B, Jay M, Viricelt M (1998). Free flavonoid aglycones from leaves of *Mentha pulegium* and *Mentha suaveolens* (Labiatae). Phytochemistry 48:991-994.

Ziaullah KS, Bhullar SN, Warnakulasuriya HP, Rupasinghe V (2013). Biocatalytic synthesis, structural elucidation, antioxidant capacity and tyrosinase inhibition activity of long chain fatty acid acylated derivatives of phloridzin and isoquercitrin. Bioorg. Med. Chem. 21:684–692.

academicJournals

Vol. 8(20), pp. 756-762, 25 May, 2014
DOI: 10.5897/JMPR2013.5351
Article No: F7703BC44844
ISSN 1996-0875
Copyright © 2014
Author(s) retain the copyright of this article
http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Rapid and easy molecular authentication of medicinal plant *Zingiber officinale* Roscoe by loop-mediated isothermal amplification (LAMP)-based marker

Anis Ahmad Chaudhary[1]*, Mohsin Khan[2], Waleed M. Al-Shaqha[1], Mohammad Alharbi[1] and Osama A. Al-Khamees[1]

[1]College of Medicine, Al-Imam Muhammad Ibn Saud Islamic University (IMSIU), Riyadh-11432, Kingdom of Saudi Arabia.
[2]Department of Biosciences, Jamia Millia Islamia (A Central University), New Delhi-110025, India.

Received 31 December, 2013; Accepted 6 May, 2014

The *Zingiber* genus, which includes the herbs known as gingers with maximal therapeutic properties, is well known for its medicinal importance as a purificant of body. Some morphological similar members of the genus are available but differ in their pharmacological and therapeutic properties. So there is an existing demand in herbal drug industry for an authentication system for gingers in order to facilitate their commercial use as genuine phytoceuticals. To this end, the objective of the present study was to develop a novel loop mediated isothermal amplification (LAMP)-based marker for authentication of the commercially important *Zingiber officinale* Roscoe from the closely related species. The twelve rhizome samples of these plants were collected from different geographical locations in India and analyzed with randomly amplified polymorphic DNA (RAPD). A prominent DNA fragment in RAPD that is common to all accessions was eluted, cloned and sequenced. Based on the DNA sequences four specific LAMP primers (two inner and outer primers) were in house designed for LAMP based marker. LAMP reaction was performed by using designed specific LAMP primer and total DNA extracted from *Z. officinale* as template. The developed LAMP-based markers were tested in several non-*Zingiber* species. The LAMP was observed for approximately 30 min at DNA concentrations of 10 to 15 ng. The resulting amplicon was visualized by adding SYBR Green-I to the reaction tube without using further technique as gel electrophoresis, to shorten reaction time considerably, since the assay method is simple, sensitive and rapid, for identifying and authentication of *Z. officinale* Roscoe.

Key words: *Zingiber officinale,* randomly amplified polymorphic DNA (RAPD), loop-mediated isothermal amplification, polymerase chain reaction, cloning.

INTRODUCTION

Common or culinary ginger, rhizome of the plant *Zingiber officinale* Roscoe has been used as a spice and for traditional medicine purposes to treat headache, nausea, colds, arthritis, rheumatic disorders and muscular discomfort (Dedov et al., 2002) in Asian herbal traditions since ancient times. Preclinical and clinical studies have

*Corresponding author. E-mail: anis.chaudhary@gmail.com.
Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

established anti-inflammatory (Wei et al., 2005; Young et al., 2005), antiemetic (Surh et al., 1999; Dorai et al., 2004) and cholagogic (bile-flow-inducing) activities in crude, semi-processed and processed forms of ginger. Ginger is a household remedy for dyspepsia, flatulence, colic and diarrhoea (Govindarajan, 1982). In addition to the medicinal uses, ginger is valued globally as an important cooking spice. Authentication of raw materials is essential for botanical drug quality, safety and efficacy. Ginger is identified by its macroscopic and organoleptic characteristics, including its characteristic form, color, pungent taste and volatile oil content and by micro-chemical tests (World Health Organization (WHO), 1999). In the case of dried, chipped or ground samples, the problem becomes even more complex, as closely related species are morphologically very similar, making it difficult to differentiate them. The aim of the present study was to develop simple and easy DNA-based molecular markers for *Z. officinale* Roscoe to facilitate its proper identification, avoiding the unintentional adulteration that affects the quality and efficacy of its botanical preparations. The *Zingiber* species were selected on account of their similar traditional medicinal uses and morphology, which could promote their use as adulterants or contaminants of *Z. officinale* Roscoe (Tao et al., 2009).

Genetic tools that use hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Zhang et al., 2007; Shcher et al., 2008). In PCR, RAPD and AFLP analysis has been applied in herbal medicine to discriminate between species in various genera (Williams et al., 1990; Hosokawa et al., 2000; Belaj et al., 2001), simple sequence repeat (Rallo et al., 2000; Sefc et al., 2000; Carriero et al., 2002; Cipriani et al., 2002) and SCAR marker (Paran and Michelmore 1993) have been widely applied. These markers were detected by PCR using specific oligonucleotide primers designed based on the sequence data and following PCR amplification under more stringent conditions. SCAR markers, a more accurate and reliable technique also need the analysis of gels, time-consuming and sophisticated polymerase chain reaction (PCR) machine.

To overcome these problems associated with the discussed technique, isothermal amplification of DNA is useful method. Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method developed by Notomi et al. (2000) and relies on an autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (Notomi et al., 2000; Nagamine et al., 2001, 2002). LAMP requires two specially designed inner and two outer primers to perform the amplification of the target gene, as such, LAMP amplifies DNA with high specificity, efficiency and rapidity. The amplification uses a single temperature step at 60 to 66°C for about 30 to 60 min. Simple incubitors, such as a water bath or block heater are sufficient for the DNA amplification, and the amplification products have many types of structures in large amounts. Thus, LAMP is faster and easier to perform than PCR, as well as being more specific. Furthermore, gel electrophoresis is not needed for LAMP products because the LAMP products can be detected indirectly by the turbidity that arises due to a large amount of by-product, pyrophosphate ion, being produced, yielding an insoluble white precipitate of magnesium pyrophosphate in the reaction mixture (Mori et al., 2001).

MATERIALS AND METHODS

Plant

Fresh plants rhizomes of twelve accessions of *Z. officinale* were used in the present study for comparison and they were collected from Patna, Kolkata, Pune, Kerla, Nasik, Bangalore, Chandigarh, Gujarat, Assam, Hyderabad, Chennai and Lukhnow which cover four ecological zones like south, north, west and east India (Table 1). Collected rhizome sample were frozen in liquid nitrogen and stored at -20°C until used for DNA isolation.

DNA Isolation

DNA was isolated from frozen rhizome sample using a modified cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). Briefly, rhizome samples (0.2 to 0.5 g) were ground to fine powder in liquid nitrogen and transferred to a microcentrifuge tube containing freshly prepared equal volume of extraction buffer (100 mmol/L Tris buffer, pH 8.0, 20 mmol/L Na_2EDTA, 1.4 mol/L NaCl, 2% CTAB, 1% polyvinyl pyrrolidone). The suspension was gently mixed and incubated at 60°C for 60 min with occasional mixing. The suspension was then cooled to room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was centrifuged at 13,000 × g for 10 min. The clear upper aqueous phase was then transferred to a new tube containing 0.5 ml ice-cooled isopropanol and incubated at -20°C for 30 min. The nucleic acid was collected by centrifuging at 13,000 × g for 10 min. The resulting pellet was washed twice with 70% ethanol containing 10 mmol/L ammonium acetate. The pellet was air-dried under a sterile laminar hood and the nucleic acid was dissolved in TE (10 mmol/L Tris buffer, pH 8.0, 1 mmol/L Na_2EDTA) at 4°C. The contaminating RNA was eliminated by treating the sample with RNase A (20 µg/µl) for 30 min at 37°C. DNA concentration and purity were determined by measuring the absorbance of diluted DNA solution at 260/280 optical density (OD) value. The quality of the DNA was determined by using 0.8% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide and photographed using gel documentation system (Image Master VDS, Pharmacia, USA). All DNA samples taken for RAPD study showed a 260/280 OD value of 1.8 to 1.9.

RAPD amplification

The RAPD amplification was performed according to the method developed by McClelland et al. (1995). PCR reactions were carried out in 25 µl reaction tubes using 25 random decanucleotide primers, OPAA-1, OPAA-2, OPAA-3, OPAA-4, OPAA-5, OPAA-6, OPAA-7, OPAA-8, OPAA-9, OPAA-10, OPAA-11, OPAA-12, OPAA-13, OPAA-14, OPAA-15, OPAA-16, OPAA-17, OPAA-18, OPAA-19, OPAA-20 (Operon Technologies Inc., USA), Bg26, Bg27, Bg28,

Table 1. Location of accessions of *Z. officinale*

S/No.	Code	Location
1	G1	Kolkata
2	G2	Pune
3	G3	Kerla
4	G4	Nasik
5	G5	Bangalore
6	G6	Chandigarh
7	G7	Gujarat
8	G8	Assam
9	G9	Hyderabad
10	G10	Chennai
11	G11	Lucknow
12	G12	Patna

Bg29 and Bg30 (BangloreGenei, India). Each reaction tube contained 50 ng template DNA, 1.5 mmol/L $MgCl_2$, 300 μmol/L of dNTPs, 1×*Taq* DNA polymerase buffer, 25 pmol decanucleotide primer and 2 units of *Taq* DNA polymerase (Promega, USA). Amplification was performed in a thermal cycler (Ependroff, USA), using the following conditions: 95°C for 3 min; 40 cycles at 94°C for 30 s, 50°C for 1 min and 72°C for 1 min; final extension at 72°C for 5 min. PCR products were resolved on 1.2% agarose gel in 1×TAE buffer, agarose gel containing 0.5 μg/ml ethidium bromide visualised under UV light and photographed using gel documentation system (Image Master VDS, Pharmacia, USA). RAPD markers suffer from a lack of reproducibility. Consequently, to confirm the electrophoretic patterns and the obtained polymorphic bands, every PCR was repeated twice under the same conditions of composition of reaction volume and amplification profile.

Cloning and sequencing of specific RAPD fragment

A band of 780 bp, which is common in all twelve accessions, was excised from gels and eluted using a Gel extraction Kit (QIAGEN, Germany). The eluted DNA was cloned into pGEM®-T easy vector (Promega, USA) following the manufacturer's instruction. The ligated plasmid was introduced into *Escherichia coli* strain $DH_{5\alpha}$, following the protocols for preparing competent cells and transformation using the calcium chloride method (Sambrook and Russell, 2001). White colonies were picked from LB-X-gal plates and grown overnight in LB medium containing ampicilin. The plasmid DNA was isolated from the bacterial culture using plasmid isolation kit (QIAGEN, Germany). The inserted fragment was sequenced at The Center for Genomic Application, New Delhi, India with T7 primer. Nucleotide sequence of 780 bp was identical for all twelve accessions. This sequence was used for designing primers for LAMP reaction.

Primers design for LAMP

Four oligonucleotide primers, forward inner primer (FIP), back inner primer (BIP) and two outer primers (F3 and B3), were designed by using the sequence of DNA from 780 bp RAPD amplicon. All primer sequences were designed with the software program Primer Explorer V3 (http://primerexplorer.jp/elamp3.0.0/index.html). The primers were selected based on the criteria described by Notomi et al. (2000). Briefly, the design of the two outer primers, F3 and B3, is the same as that of regular PCR primers, while the design of the two inner primers, FIP and BIP, is different from that of PCR (Notomi et al., 2000). FIP consists of the sense sequence of F2 at the 3` end and the F1c region at the 5` end that is complementary to the F1 region. BIP consists of a B2 region at the 3` end that is complementary to the B2c region and the same sequence as the B1c region at the 5` end (Figure 2B).

LAMP assay

LAMP reaction (25 μl) contained the one of outer primers (0.2 μM) and one pair of inner primers (1.6 μM), 2.5 μl of 10× *Bst* DNA polymerase reaction buffer [1 μl containing 20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 (pH 8.8)], 400 μM each dNTP, 1 μl of an 8 U/μl concentration of *Bst* DNA polymerase (New England Biolabs, Inc., MA), 2 mM $MgSO_4$ (2 μl), 5 μl of betaine (Sigma-Aldrich, St. Louis, MO) and 5 μl of double-stranded target DNA. The LAMP reaction was performed in a heating block (Genei, India) at 65°C for 30 min. For comparison, the reaction was also performed by using a conventional thermal cycler (Bio-Rad, USA) at constant temperature 65°C for 30 mins.

Visualization of LAMP product

The inspection for amplification was performed through observation of a color change following addition of 1 μl (1:1000) of SYBR Green I dye to the tube. This color was visualized by naked without any UV source and produced florescence in under UV transillumenator (Image Master VDS, Pharmacia, USA).

RESULTS

Twenty five RAPD primers were used in RAPD analysis for search DNA polymorphisms, which used for generating informative LAMP-based molecular markers and defining the individuals. Only the fragments confirmed by repeated amplifications were considered for generating LAMP-based markers. Out of 25 random decanucleotide primers (OPAA-1 to OPAA-20 and Bg26 to Bg30) showed that 24 primers were able to amplify genomic DNA of *Z. officinale* and 1 primer showed no amplification and resulted in amplified PCR products of a variable number of DNA bands (48 to 86 bands per primer) (Table 2). A total of 2,779 DNA bands were obtained. Amplification of *Z. officinale* species with BG-29 produced good quality (Figure 1), reproducible fingerprint patterns and showed a high level of consistency of fingerprints among samples of the same species collected from different localities. Several specific RAPD fragments of high intensity and reproducibility were eluted, cloned and sequenced. Nucleotide sequence of 780 bp amplicons (Figure 2A), identical in all the twelve accessions of *Z. officinale,* were used for designing primers for LAMP reaction (Figure 2B). Only the fragments confirmed by repeated amplifications were identical for all the twelve accessions of *Z. officinale,* was used for designing primers for LAMP reaction. The LAMP reaction relies mainly on autocycling strand displacement DNA synthesis that is similar to the cascade rolling-circle amplification reported by Notomi (Hafner et al., 2001).

Table 2. Number of amplified products generated by 25 arbitrary primers in all twelve accession of *Z. officinale*.

Primer	Genotype											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
OPAA -01	7	10	4	1	8	8	6	10	10	8	8	8
OPAA -02	6	5	3	5	3	4	4	7	1	5	6	9
OPAA -03	6	4	3	4	4	7	6	6	5	3	1	6
OPAA -04	5	6	6	6	5	4	5	7	7	9	7	8
OPAA -05	12	13	11	8	13	9	10	11	10	10	0	11
OPAA -06	7	2	12	5	2	10	11	11	11	9	7	6
OPAA -07	9	8	8	11	11	10	7	12	10	10	11	7
OPAA -08	6	9	9	10	11	10	0	9	8	10	9	9
OPAA -9	9	11	9	9	10	7	9	9	10	7	4	2
OPAA -10	6	7	4	5	6	8	4	5	4	4	5	4
OPAA -11	9	8	8	9	8	8	6	6	6	10	7	9
OPAA -12	4	5	4	5	3	4	2	5	5	4	3	4
OPAA -13	7	8	9	10	8	8	9	7	10	10	12	13
OPAA -14	7	8	7	9	8	7	6	9	8	6	7	8
OPAA -15	12	13	12	11	13	13	13	10	14	14	15	7
OPAA -16	16	19	20	17	15	13	14	15	15	14	14	12
OPAA -17	15	13	14	14	14	15	13	13	9	10	11	11
OPAA -18	10	12	9	10	11	10	10	11	11	10	9	11
OPAA -19	10	12	10	11	11	12	8	10	11	13	10	11
OPAA-20	0	0	0	0	0	0	0	0	0	0	0	0
BG-26	8	6	6	7	6	8	8	8	9	7	6	6
BG-27	11	11	12	11	13	12	10	11	12	10	11	12
BG-28	5	4	3	7	7	4	6	7	5	5	4	3
BG-29	12	12	14	15	16	15	14	14	14	13	14	15
BG-30	15	18	18	19	15	17	20	18	14	12	9	11
Total	214	224	215	221	225	223	300	233	219	310	190	205

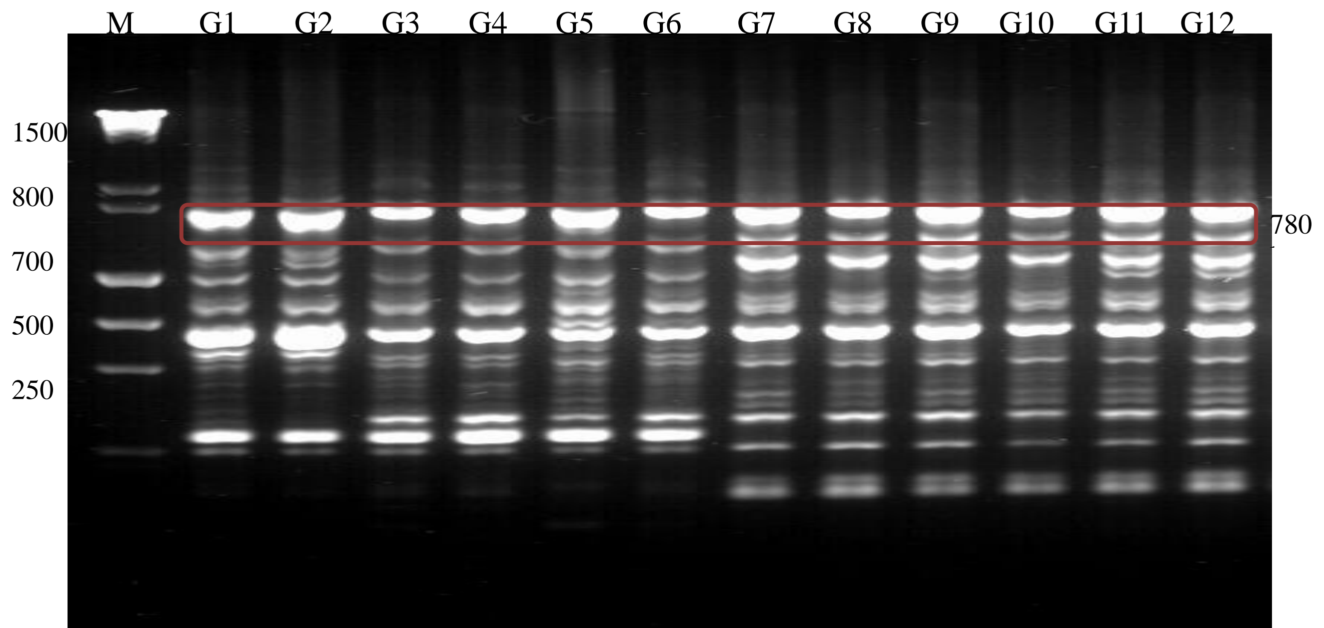

Figure 1. RAPD electrophoresis profile of *Z. officinale* amplified with **BG-26.** Lanes 1–12 correspond to the twelve accessions. Lane M, molecular marker 250-1500 bp (Bio Basic Inc, Canada).

```
0   GATTTTTCAA ATGGAGCAAA AGAGAGGCAG CAAATGGGAG TTTTAAATTC
51  TGGATCTTGG TGTATTTTAA ACGGGTGATT TATTCTTTCT TATACTTTCA
101 GGTGTTTCCA ATGTGGACAC TGAAGAGACA GTTTCCTATC CTTTTTAACA
151 TGATCCTAAT TTCTGAACTC CTTGGGGCTA GATGGTTTCC TAAAACTCTG
201 CCCTGTGATG TCACTCTGGA TGCTCCAAAT GCCCATGTGA TTGTGGACTG
251 CACAGACAAA CATTTGACAG AAATTCCTGG AGGTATTCCT GCCAATGCCA
301 CCAACCTCAC CCTCACCATT AACCACATAG CAGGCATCTC TCCAGCCTCC
351 TTCCACCGGC TGGACCATCT GGTGGAGATC GATTTCAGGT GCAACTGCAT
401 ACCTGTTCGA CTGGGGCCAA AAGACAACGT GTGCACCAAA AGGCTACAGA
451 TTAAACCCAA CAGCTTTAGC AAACTCACGT ATTTAAAATC TCTTTACCTG
501 GATGGAAACC AGCTTCTAGA AATACCTCAG GATCTTCCTC CCAGCTTACA
551 GCTGCTGAGC CTGGAGGCCA ACAACATCTC TTGATCATGA AGGAGAATCT
601 AACAGAACTG GCCAACCTAG AAATACTCTA CCTGGGCCAA AACTGTTACT
651 ATCGTAACCC TTGTAATGTT TCATTTACTA TCGAAAAAGA TGCTTTCCTA
701 AATATGAGAA ATTTAAAATT GCTCTCCCTA AAAGATAACA ATATCTCAGC
751 TGTCCCCACT GTTTTGCCAT CTAGTTTGA
```

(A)

Primers	Outer forward and backward Primers	Bases
GNF3	5'-tgcaccaaaaggctacag-3'	18
GNB3	5'-taggttggccagttctgt-3'	18
	Inner forward and backward Primers	
GMFIP (F1c+F2)	5'-ctggtttccatccaggtaaagagattaaacccaacagctttagc-3'	35
GNBIP (B1+B2c)	5'-ctcaggatcttcctcccagccttcatgatcaagagatgttgtt-3'	38

(B)

Figure 2. (A) Nucleotide sequence of RAPD amplicon (780 bp) of *Z. officinale* and underlined indicate the designed LAMP primers, (B) LAMP Primer for *Z. officinale.*

The minimum LAMP reaction unit consists of two outer (F3 and B3), two inner primers (FIP and BIP) and target DNA. Each inner primer contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA and form stem-loop structures at both ends of the minimum LAMP reaction unit. These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction. The LAMP products were visualized by naked eye without using any UV source (Figure 3) on a UV transilluminator at 302 nm (Figure 4).

DISCUSSION

The rhizomes of other three species of the *Zingiber* genus (*Z. officinale* Roscoe, *Z. montanum* (J. Koeing) Link ex A. Dietr and *Z. zerumbet* (L.) Roscoe ex Sm) used in the present study are morphologically almost similar and can be used as its adulterants or contaminants. It is even almost impossible for classical taxonomists to differentiate between these two species in the non-flowering stage (Ghosh et al., 2011). Various methods, including macroscopy, microscopy and chemoprofiling have been reported for the quality control of crude ginger and its products. These methods are reported to have limitations in distinguishing *Z. officinale* from closely related species: molecular based identification is a great promise in resolving issues of controversial identity and quality control, when botanical identification, based on morphology becomes difficult, such as in the case of incomplete or damaged samples and in dried herbal products, but has not yet been capitalized by the traditional medicine sector (Chaudhary et al., 2012). DNA fingerprinting patterns provide the ultimate in individualization due to the stability of DNA in

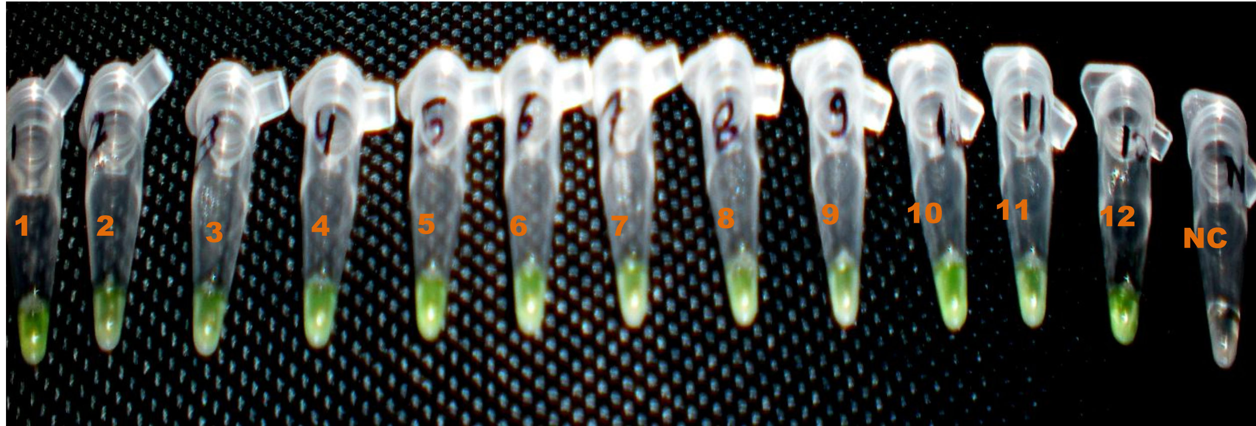

Figure 3. Analysis of LAMP result under naked eye without using any UV source.
1-12 showed accessions of *Z. officinal* NC = Negative control.

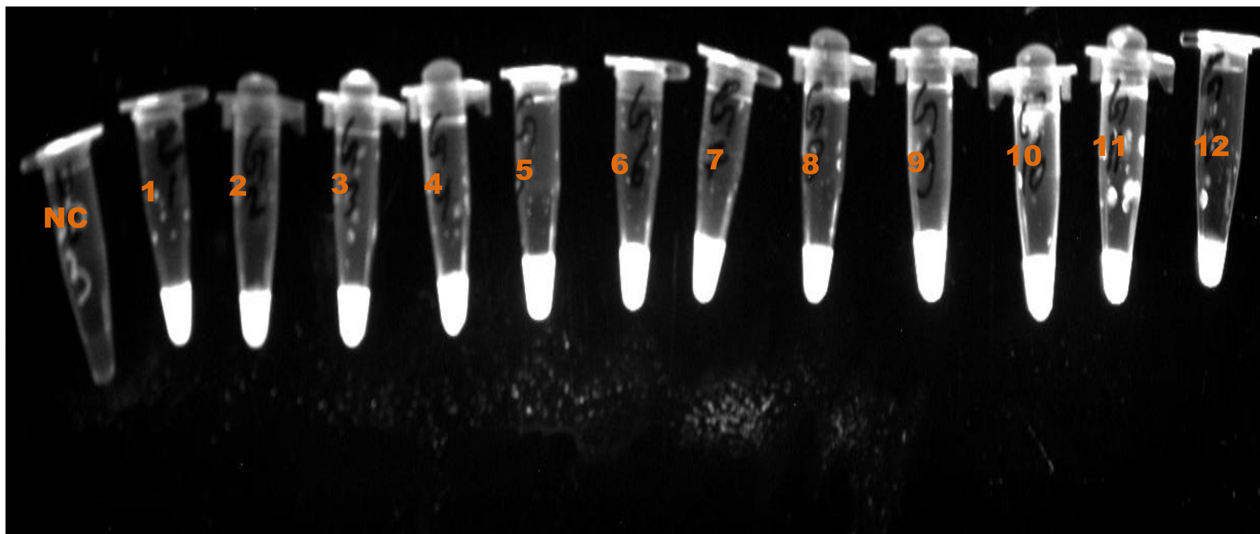

Figure 4. Analysis of LAMP result under UV-transilluminator.
1-12 showed accessions of *Z. officinale,* NC = Negative control.

any plant part and also through variation in environment and also variation in phase of life cycle. So we propose the LAMP-based markers developed in the present study as a complementary tool for identification of *Z. officinale* and demonstrate their applicability in the detection of *Z. officinale* from fresh rhizomes, dried rhizome powders and multicomponent Ayurveda-based formulation.

On the basis of sequence information, longer species-specific LAMP primers were developed to amplify specific LAMP based markers to distinguish *Z. officinale* from the other selected Z*ingiber* species, as commonly used in ginger-containing formulations.

The LAMP operation is quite simple; it starts with the mixing of buffer, primers, DNA lysates and DNA polymerase in a tube and then the mixture is incubated at 65°C for a certain period. For the visualization of product, SYBR Green I was added. The tubes can also be inspected for white turbidity with the naked eye after a pulse spin to deposit the precipitate in the bottom of the tube (Mori et al., 2001). However, detecting a small amount of the white precipitate by the naked eye is not always easy; therefore, the detection limit is apparently inferior to that of electrophoresis. To increase the rate of recognition by the naked eye, addition of SYBR Green I to the reaction solution is convenient (Hill et al., 2008). LAMP amplification is rapid (results can be obtained in less than 1 h), easy to perform and low in cost (chaudhary et al., 2011). Because of its easy operation without any sophisticated equipment, it will be simple enough for use in small-scale industries, hospitals and

testing laboratories in developing countries. This is the first report of species-specific LAMP marker development in ginger. Hence, newer complementary method for correct identification of ginger is very useful.

Conflict of Interest

Authors declare no conflict of interest.

REFERENCES

Belaj A, Trujillo I, Rosa R, Rallo L (2001). Polymorphism and discrimination capacity of randomly amplified polymorphic markers in an Olive germplasm bank. J. Am. Soc. Hortic. Sci. 126:64–71.

Carriero F, Fontanazza G, Cellini F, Giorio G (2002). Identification of simple sequence repeats (SSRs) in olive (*Olea europea*). Theor. Appl. Genet. 104:301–307.

Chaudhary AA, Yadav D, Hemant, Jamil SS, Asif M (2012). Real Time Sequence Characterized Amplified Region (RT-SCAR) marker - Development and its application for authentication and quantification of *Catharanthus roseus* L. Don. J. Med. Plants Res. 6(32):4748-4754.

Cipriani G, Marrazzo MT, Marconi R, Cimato A, Testolin R (2002). Microsatellite markers isolated in olive (Olea europea) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theor. Appl. Genet. 104:223–228.

Dedov VN, Tran VH, Duke CC (2002). Gingerols: a novel class of vanilloid receptor (VR1) agonists. Brit. J. Pharm. 137:793–798.

Dorai T, Aggarwal BB (2004). Antitumor promoting activities of selected pungent phenolic substances present in ginger. Cancer Lett. 215:129-140.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Ghosh S, Majumder PB, Mandi SS (2011). Species-specific AFLP markers for identification of *Zingiber officinale*, *Z. montanum* and *Z. zerumbet* (Zingiberaceae). Genet. Mol. Res. 10(1):218-229.

Govindarajan VS (1982). Ginger-chemistry, technology and quality evaluation: part 1. CRC. Crit. Rev. Sci. Nutr. 17:1-96.

Hafner GJ, Yang IC, Wolter LC, Stafford MR, Giffard PM (2001). Isothermal amplification and multimerization of DNA by *Bst* DNA polymerase. Biotechniques 30:852–860.

Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T, Wadowsky MR, Singh V, Goyal A, Jahnukainen T, Johnson JR, Tarr IP, Vats A (2008). Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. J. Clin. Microbiol. 46:2800–2804.

Hosokawa K, Minami M, Kawahara K, Nakamura I, Shibata T (2000). Discrimination among three species of medicinal *Scutellaria* plants using RAPD markers. Planta Med. 66:270–272.

McClelland M, Mathieu-Daude F, Welsh J (1995). RNA fingerprinting and differential display using arbitrarily primed PCR. Trends Genet. 11:242–246.

Mori Y, Nagamine K, Tomita N, Notomi T (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289:150–154.

Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16:223–229.

Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T (2001). Loopmediated isothermal amplification reaction using a nondenatured template. Clin. Chem. 47:1742-1743.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanebe K, Amino N, Hase T (2000). Loop- mediated isothermal amplification of DNA. Nucleic Acids Res. 28:E63.

Paran I, Michelmore RW (1993). Development of reliable PCR based markers linked to downy mildew resistance genes in lettuce. Theor. Appl. Genet. 85:985–993.

Rallo P, Dorado G, Martin A (2000). Development of simple sequence repeats (SSRs) in olive tree (Olea europea). Theor. Appl. Genet. 101:984–989.

Sambrook J, Russell DW (2001). Molecular Cloning: a Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press. New York.

Sefc KM, Lopes MS, Mendonca D, Rodrigues Dos Santos M, Laimer Da Camara Machado M, Da Camara MA (2000). Identification of microsatellite loci in olive (Olea europea) and their characterization in Italian and Iberian olive trees. Mol. Ecol. 9:1171–1193.

Shcher NJ, Carles MC (2008). Genome-Based Approaches to the Authentication of Medicinal Plants. Planta Med. 74:603–623.

Surh YJ, Park KK, Chun KS, Lee L, Lee E, Lee S (1999). Antitumor promoting activities of selected pungent phenolic substances present in ginger. J. Environ. Pathol. Toxicol. Oncol. 18:131-139.

Tao Y, Li W, Liang W, Van-Breeman RB (2009). Identification and quantification of 6-gingerol and related compounds in ginger dietary supplements using high performance liquid chromatography-tandem mass spectrometry. J. Agric. Food Chem. 57:10014-10022.

Wei QY, Ma JP, Cai YJ, Yang L, Liu ZL (2005). Cytotoxicnd apoptotic activities of diarylheptanoids and gingerol related compounds from the rhizome of Chinese ginger. J. Ethnopharmacol. 102:177-184.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531–6535.

World Health Organization (1999). WHO Monographs on Selected Medicinal Plants, vol. 1, World Health Organization, Geneva.

Young HY, Luo YL, Cheng HY, Hsieh WC (2005). Analgesic and anti-inflammatory activities of 6-gingerol. J. Ethnopharmacol. 96:207-210.

Zhang YB, Shaw PC, Sze CW, Wang ZT, Tong YJ (2007). Molecular authentication of Chinese herbal materials. Food Drug Anal. 15:1–9.

Related Journals Published by Academic Journals

- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals