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Original Research

Wound healing properties of ethyl acetate fraction of *Moringa oleifera* in normal human dermal fibroblasts

Sivapragasam Gothai[1#], Palanisamy Arulselvan[1#], Woan Sean Tan[1], Sharida Fakurazi[1,2]

[1]Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia, [2]Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia
[#]These authors are contributed equally

Address for correspondence:
Palanisamy Arulselvan, Research Fellow
Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia.
E-mail: arulbio@gmail.com

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ABSTRACT

Background/Aim: Wounds are the outcome of injuries to the skin that interrupt the soft tissue. Healing of a wound is a complex and long-drawn-out process of tissue repair and remodeling in response to injury. A large number of plants are used by folklore traditions for the treatment of cuts, wounds and burns. *Moringa oleifera* (MO) is an herb used as a traditional folk medicine for the treatment of various skin wounds and associated diseases. The underlying mechanisms of wound healing activity of ethyl acetate fraction of MO leaves extract are completely unknown. **Materials and Methods:** In the current study, ethyl acetate fraction of MO leaves was investigated for its efficacy on cell viability, proliferation and migration (wound closure rate) in human normal dermal fibroblast cells. **Results:** Results revealed that lower concentration (12.5 μg/ml, 25 μg/ml, and 50 μg/ml) of ethyl acetate fraction of MO leaves showed remarkable proliferative and migratory effect on normal human dermal fibroblasts. **Conclusion:** This study suggested that ethyl acetate fraction of MO leaves might be a potential therapeutic agent for skin wound healing by promoting fibroblast proliferation and migration through increasing the wound closure rate corroborating its traditional use.

KEY WORDS: Active fractions, cell proliferation, migration, natural products, scratch assay

INTRODUCTION

Skin plays a crucial role in the sustenance of life by acting as a barrier to external noxious agents. When this barrier is disrupted, skin may not be able to adequately perform its crucial function; therefore, it is vital to restore its integrity forthwith. A normal wound healing necessitates a series of dynamic and overlapping process. A traumatized skin, expose underlying tissue to outside environment, provide an open access to infection and often results in the development of unpleasant exudates and toxins, which is associated with concomitant killing of regenerating cells [1,2]. Thus, interplay between cellular and extracellular components is required to restore tissue integrity. Modulation of diverse growth factors in tissue repair influence the cellular proliferation, migration and other cellular metabolic activity of the skin. The culmination of these biological processes results in the replacement of normal skin structures [3]. Though the healing process takes place naturally, wounds may enter a state of pathologic inflammation due to a postponed, incomplete, or uncoordinated healing process that exhibits impaired or delayed acute wounds and chronic wounds. Besides health status, age factors, body built, nutritional status, and physiological stress are speculated to be the result of impaired wound healing [4-6].

Wound healing is categorized into four classic stages and that are hemostasis, inflammation, fibroplasia, and maturation [7]. Hemostasis takes place immediately after injury and its

form of protection to vascular system and bridges invading cells required for the following healing phases. The platelet aggregation and clot formation in hemostasis event recruit growth factors and cytokines such as transforming growth factors-β (TGF-β), platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF). These molecules act as promoters to following inflammatory phase in wound healing cascade. An influx of inflammatory cells including neutrophils, monocytes, and macrophages in the fibrin scaffold [8] enhances further tissue debridement and recruitment of additional growth factors vital for wound healing. Epithelialization, angiogenesis, granulation tissue formation, and collagen deposition, characterize the proliferative phase. The sequential events of epithelialization encompass cell detachment, proliferation migration, and differentiation, which are facilitated by TGF-β, VEGF, and multiple cytokines. Angiogenic response is critical as newly developed cell and tissue requires formation of new blood vessels to provide nutrients needed for homeostasis. The hypoxic condition allows proliferation of endothelial cells under influence of angiogenic factors. Fibroblasts multiply and increase collagen production to produce greater tensile strength about 80% of the original or uninjured tissue. Concurrently, cell and capillary density decrease in the final phase of wound healing which involves maturation or remodeling [9].

Achievement in developing the new-fangled information of unique biological markers linked with normal and pathological wound healing responses aids in discovery of new natural therapeutic agent to replacing those existing costly drugs such as silver sulfadiazine, vasolex and santyl accompanied by like skin irritation, rashes blood dyscrasias and life-threatening cutaneous reactions [10]. Many medicinal plants have a very significant role in the course of wound healing [11]. Plant-based therapy not only accelerate healing process but also maintains the aesthetics in a natural way [12].

At present, there has been growing interest on *Moringa oleifera* (MO) from biomedical researcher due to high potential and nutritive value. MO tree is native to the sub-Himalayan regions of Northwest India and cultivated throughout tropical and sub-tropical areas of the world including India, Pakistan, Malaysia, and Sri Lanka [13]. The pharmacological attributes of MO leaves mainly consist of glycosides, β-sitosterol, α-tocopherol, pyridoxine, ascorbic acids, lysine methionine and proteins [14]. This compound has been shown to be very rare in nature and exhibited anti-hyperthyroidism, antitumor, antispasmodic, antioxidant, hepatoprotective and antimicrobial activities [15-18]. The current work was aimed to investigate the *in vitro* wound healing potential of ethyl acetate fraction of MO leaves on normal human dermal fibroblast (HDF-N) cells and these activities were compared with positive control drug, Allantoin. Allantoin holds numerous therapeutic activities including wound healing, remover of necrotic tissue and promoter of epithelial stimulation and it has been used in pharmaceutical preparations for more than 70 years [19]. Besides neutrophils, endothelial cells, and keratinocytes, fibroblasts holds an important role in cutaneous wound repair and remodeling. They proliferate to expand, migrate into the wound bed and synthesize new extracellular matrix [20]. Understanding the mechanisms that regulate the cell proliferation and migration of fibroblasts cells by bioactive compound could be favorable in developing novel therapies to improve the wound healing process.

MATERIALS AND METHODS

Plant Collection, Extract Preparation and Isolation of Ethyl Acetate Fraction

The MO leaves were collected from Garden No.2 at Universiti Putra Malaysia (UPM), Malaysia with the voucher specimen (SK 1561/08) and deposited in the IBS Herbarium unit. The powdered leaves were taken and extracted with 90% ethanol using maceration technique in room temperature. The filtrate were collected and allowed for drying through rotary evaporator at 25°C (Virtis Bench Top K, United States. Further, by using solvent-solvent partition technique, ethyl acetate fraction (EtOAc) was isolated from leaves crude extract, and this was stored at −20°C until further use.

In-vitro Wound Healing Study

Cell culture maintenance

HDF-N cells were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA, CRL-2301) and thawed as well as maintained according to the ATCC protocol. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) premixed with 5% fetal bovine serum, growth supplements, and antibiotics consisting of L-glutamine 15 mmol/L, streptomycin 100 μg/mL, and penicillin 100 U/Ml and incubated in 5% CO_2 and 37°C. Cell passages between 12 and 15 at 70-80% confluence were used for seeding and treatment throughout the experiment.

3-(4, 5-DImethylthiazol-2-yl)-2, 5-diphenyl Tetrazolium Bromide [MTT]) Assays

HDF-N cells passage numbers between 12 to 15, were seeded into 96-well plate at a density of 1×10^5 (in 100 μL DMEM medium) per well and grown for 24 h. The medium was replaced with serial dilutions of EtOAc MO leaves concentration of 15.62, 31.25, 62.5, 125, 250 and 500 μg/ml and plates were incubated for 24 h. 10 μL of 5 mg/mL MTT reagent was then added to each of the wells and incubated for another 4 h. The purple formazan formed was solubilized by adding 100 μL dimethyl sulfoxide to all the wells including control (without any treatment), then swirled gently to mix well and this was then kept in the dark place at room temperature for about 30 min. Microplate reader was used to read absorbance at 570 nm with reference of 630 nm. Graph of absorbance against number of cells was plotted to determine the HDF-N cells viability as per the standard methods [21]. Experiments were performed in triplicate and the data were recorded and analyzed statistically using SPSS.

Cell Proliferation Assay

HDF-N cells were seeded on 96 well plates at a density of 1 × 10^5 (in 100 μL medium) per well and incubated at 37°C until confluent, and medium was replaced with serial dilutions of EtOAc MO leaves (15.62, 31.25, 62.5, 125, 250 and 500 μg/ml) and incubated for 24 h. 10 μL of cell counting kit-8 (CCK-8) was then added to each of the wells and incubated for another 4 h according to the manufacturer's instructions (Dojindo Lab, Japan). Microplate reader was used to read absorbance at 450 nm with reference of 630 nm. Graph of absorbance against number of cells was plotted to determine the HDF cells proliferation according to the kit manufactures instructions. Experiments were performed in triplicate and the data were recorded and analyzed statistically using SPSS.

Wound Scratch Assay

The migration of HDF-N was examined using the scratch assay method [18]. HDF-N (2 × 10^5 cells) were seeded into each well of a 24-well plate and incubated with complete medium at 37°C and 5% CO_2. After 24 h of incubation, the cells were treated with EtOAc fraction of MO leaves with varying concentration (12.5 μg/mL, 25 μg/mL and 50 μg/mL). Confluent cells were scrapped horizontally with a P200 pipette tips. The medium was replaced with fresh medium and wound closure were monitored and photographed by phase contrast microscopy using ×4 magnification at 0 h. After 24 h of incubation, the second set of images was photographed. To determine the migration rate, the images were analyzed using image-j software and percentage of the closed area was measured and compared with the value obtained before treatment. An increase of the percentage of closed area indicated the migration of cells. Experiments were performed in triplicate and the data were recorded and analyzed statistically using SPSS.

Statistical Analysis

Data are given as the mean ± standard deviation (SD) and statistical analyses were performed using one-way ANOVA (ANOVA) SPSS version 21.0 software (SPSS, USA). Results were obtained at the end of the experiment and it was compared with control and treated groups using Student's *t*-test. Differences were considered as statistically significant at $^{*}P < 0.05$, $^{**}P < 0.01$, $^{###}P < 0.001$ versus control group.

RESULTS

Effect of MO on Cell Viability

The cytotoxic effect of MO EtOAc fraction was determined by MTT assay in HDF-N cells treated with different gradient concentration (15.62, 31.25, 62.5, 125, 250, and 500 μg/mL concentration). Cell viability scrutiny showed that MO ethyl acetate fraction had no toxic effect on HDF-N cells even at higher concentration [Figure 1]. By increasing the concentration of fraction, cell viabilities were not significantly different in percentage therefore, we have selected a lower concentration (12.5, 25 and 50 μg/mL) of ethyl acetate fraction for further wound healing studies.

Effect of *M. oleifera* on Cell Proliferation

The cell proliferation as represented in Figure 2 demonstrate significant increase in the rate of cell proliferation on HDF-N cells on treatment with MO EtOAc fraction using concentrations up to 62.5 μg/ml. However, concentration from 125 μg/ml and higher of MO EtOAc fraction reduced the HDF-N cells proliferative rate. Accordingly, the proliferative investigations, the concentration at 12.5 μg/mL, 25 μg/mL and 50 μg/mL were chosen for further wound healing experiments.

Effect of MO on Wound Healing Activity

Based on cytotoxicity and proliferation experiment, an optimized concentration (non-toxic) of MO EtOAc fraction

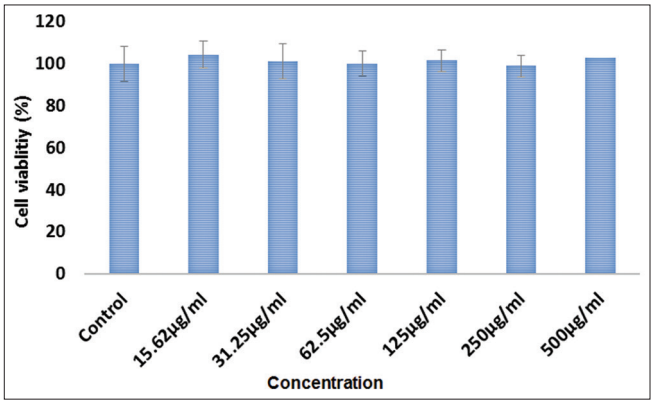

Figure 1: Cyto-toxicity effects of *Moringa oleifera* (MO) EtOAc fraction treatment in human dermal fibroblast - normal cells. At 24 h of treatment, effects of MO ethyl acetate fraction against the viability of treated cells were evaluated through mitochondrial activity using the MTT assay and calculated by comparing the values from the MO ethyl acetate fraction treatment group with the control group. Values are presented as the mean percentage ± standard deviation (n = 3) from three individual experiments.

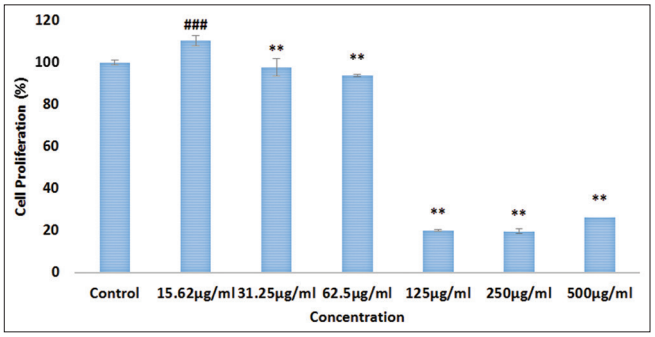

Figure 2: The effect of *Moringa oleifera* (MO) EtOAc fraction treatment on the proliferation rate of human dermal fibroblast-normal cells. The proliferation effect was estimated by cell counting kit-8 assay and calculated by comparing the values from the *M. oleifera* ethyl acetate fraction treatment group with the control group. Data are expressed as mean ± standard deviation from three individual experiments. $^{**}P < 0.01$, $^{###}P < 0.001$ versus control group

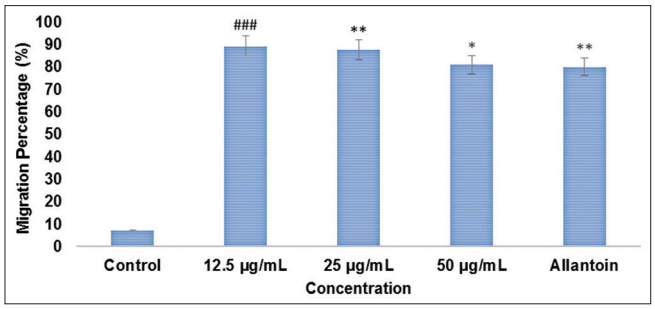

Figure 3: The migration rate in percentage for normal human dermal fibroblast after treatment with *Moringa oleifera* (MO) EtOAc fraction for 24 h. Quantitative analysis of the migration rate was analyzed with the use of Image-J software in MO ethyl acetate fraction-treated normal human dermal fibroblast. Data are expressed as mean ± standard deviation from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{###}P < 0.001$ versus control group

was assessed in scratch assay [Figures 3 and 4] to determine its effects on spreading and migration activities of HDF-N cells. Though, there is slight migration of cells in control, MO EtOAc treated HDF-N cells were found to migrate faster following a 24 h incubation period. The results revealed that MO EtOAc fraction administration created ± 9% difference in wound closure rate between treated and the positive control. It was also noticed that lower concentration of fraction (12.5 μg/ml) proliferate and migrate more rapidly than higher concentration (50 μg/ml). Despite the increased migration rate of HDF-N cells at 50 μg/ml, the morphology in terms of size and shape (fusiform) were altered, showing evidence of toxicity.

DISCUSSION

Impaired wounds healing may occur in any individual but are more frequent in the elderly and chronically ill people. With an ageing population and a dramatically increasing prevalence of chronic diseases like cancer, diabetes, wound care will certainly become an even more noteworthy issue for health systems [22,23]. Delayed wound healing is signified with alteration in physical properties of collagen, flattening of the dermo-epidermal junctions, nutritional depletion and cellular immunity leading to abnormal changes in pro-inflammatory and anti-inflammatory cytokines [24]. A number of evidence has been collected to show immense potential of medicinal plants used in various traditional systems. Certain plants pose a serious risk of toxicity from a human health standpoint such as cycads in contempt of its rich source of alkaloids [25]. This encourage the healthcare team to shorten the time required for healing and to minimize the undesired consequences such as skin inflammation, allergy as plants are more potent healers because they promote the repair mechanisms without adverse effects. These efforts ensure the patient is given holistic wound care and offer wounds the best chance to heal [26].

Traditional healers claim that the leaves of the MO are used as antiviral, anti-inflammatory, and analgesic. Collateral to that, the discovery of scientific evidence of phytochemical compounds from MO leaves which consist of various clinically important constituents such as 4-[(4'-O-acetyl-alpha-L-rhamnosloxy)benzyl] isothiocyanate,4-[(3'-O-acetyl-alpha-i-rhamnosyloxy)benzyl]isothiocyanate and S-methyl-N-{4-[(alpha-I-rhamnosyloxy)benzyl]}thiocarbamate, which is an anti-inflammatory phenolic glycosides [27]. With this background, the present study objective was undertaken to emphasize the effect of ethyl acetate fraction of MO leaves on *in-vitro* wound healing properties. The study clearly submits

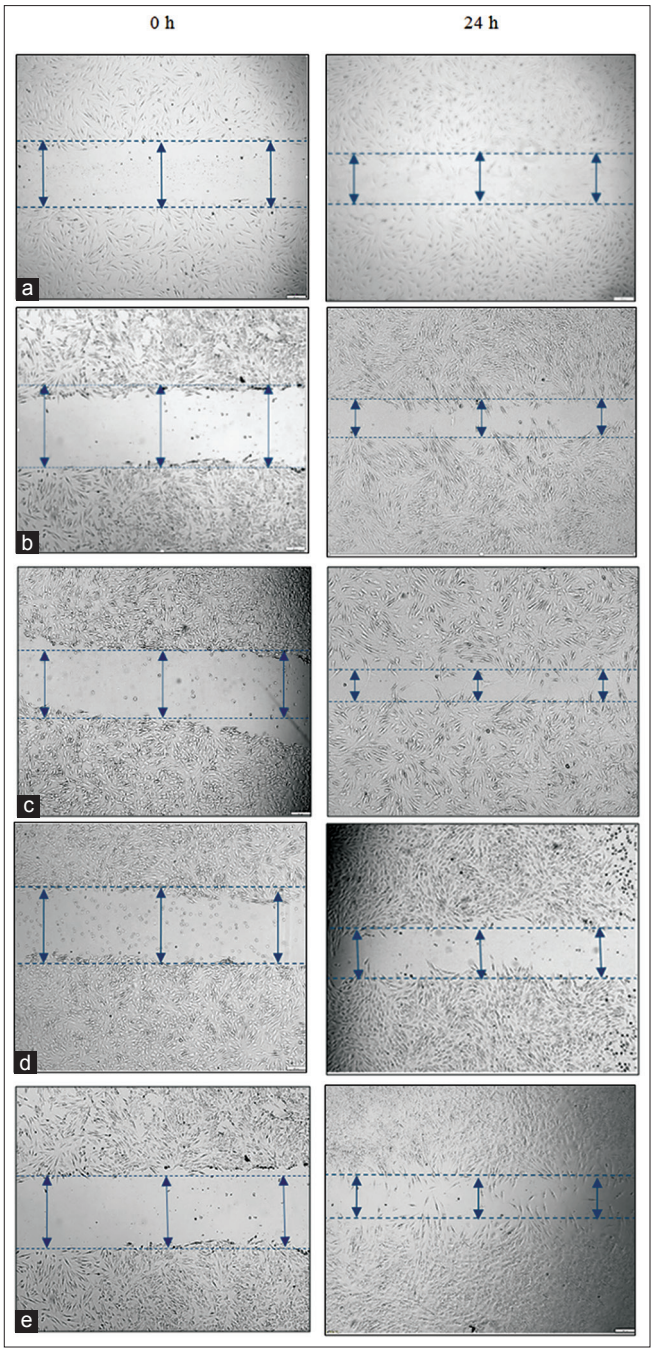

Figure 4: Effect of *Moringa oleifera* (MO) EtOAc fraction on migration rate (wound scratch assay) in normal human dermal fibroblast. The migration rate was quantified by the Image-J software and data's are expressed as mean ± standard deviation from three individual experiments for migration assay (wound scratch assay) (a) Control, (b) MO EtOAc fraction 12.5 µg/mL, (c) MO EtOAc fraction 25 µg/mL, (d) MO EtOAc fraction 50 µg/mL, (e) Allantoin

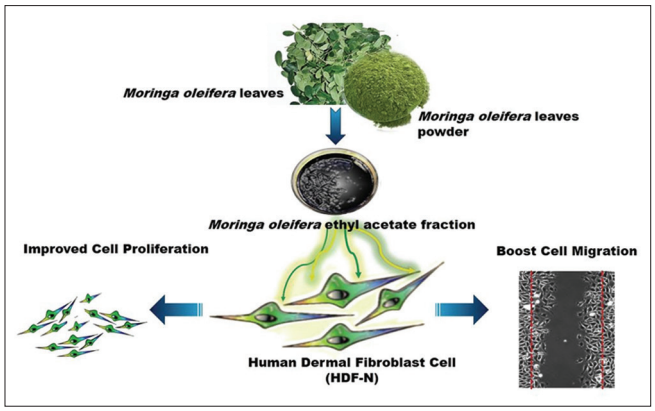

Figure 5: The potential role of ethyl acetate fraction of *Moringa oleifera* leaves in *in-vitro* wound healing model

that MO EtOAc fraction at the concentration of 12.5 and 25 μg/mL enhances the cell proliferation and migration of HDF. Cell proliferation and cell migration are two important events necessary for wound healing and an essential event during re-epithelialization, so proliferating fibroblasts at the wound site ensure an adequate supply of cells to migrate and cover the wound surface [28,29]. Both criteria were witnessed in *in-vitro* studies of cell proliferation and scratch assay [Figures 2-4]. In addition, increased response with concentration in the migration of fibroblast cell in scratch assay indicates EtOAc fraction of MO leaves was shown to be potent in promoting angiogenesis [Figure 3]. However at higher concentration, EtOAc fraction of MO leaves confer a strong anti-proliferative activity, possibly due to the strong accumulation of phenolic compounds which may be linked to activation of caspases and inducing apoptosis [30].

The results discussed above established the scientific basis of a traditional claim for the use of MO leaves as a wound healing agent. In the literature, the clinical importance of MO leaves including wound healing effect has not been fully studied with systematic manner up to now. This study confirms that ethyl acetate fraction of MO leaves might have wound healing effects based on the data of *in-vitro* assays on normal human fibroblast cells (Figure 5). Future studies will be centered on the identification and purification of the active component(s) of EtOAc fraction of MO leaves responding to the underlying wound healing mechanisms for novel cost-effective drug lead. In addition, *in-vivo* wound healing studies are still lacking to show the pharmacological activity of EtOAc fraction on the mammalian system to support the specific mechanism tangled in regulating the anti-inflammatory activity in wound healing.

CONCLUSION

This study demonstrated that EtOAc fraction of MO leaves was effective in promoting and accelerating wound closure process in HDF-N cells. This natural agent is rich target for development as alternative therapeutic wound healing agent, though, there is a need for scientific validation and safety evaluation before this plant could be commercialized for alternative wound treatment.

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Original Research

ScopeMed

Central additive effect of *Ginkgo biloba* and *Rhodiola rosea* on psychomotor vigilance task and short-term working memory accuracy

Hayder M. Al-Kuraishy

Department of Pharmacology, Toxicology and Medicine College of Medicine Al-Mustansiriya University, P.O. Box 14132, Iraq, Baghdad

Address for correspondence: Hayder M. Al-Kuraishy, Department of Pharmacology, Toxicology and Medicine College of Medicine Al-Mustansiriya University, P.O. Box 14132 Iraq, Baghdad. E-mail: hayderm36@yahoo.com

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ABSTRACT

Aim: The present study investigates the effect of combined treatment with *Ginkgo biloba* and/or *Rhodiola rosea* on psychomotor vigilance task (PVT) and short-term working memory accuracy. **Subjects and Methods:** A total number of 112 volunteers were enrolled to study the effect of *G. biloba* and *R. rosea* on PVT and short-term working memory accuracy as compared to placebo effects, the central cognitive effect was assessed by critical flicker-fusion frequency, PVT, and computerized N-back test. **Results:** Placebo produced no significant effects on all neurocognitive tests measure $P > 0.05$ in normal healthy volunteers, *G. biloba* or *R. rosea* improve PVT and low to moderate working memory accuracy, The combined effect of *R. rosea* and *G. biloba* leading to more significant effect on PVT, all levels of short-term working memory accuracy and critical fusion versus flicker $P < 0.01$, more than of *G. biloba* or *R. rosea* when they used alone. **Conclusion:** The combined effect of *R. rosea* and *G. biloba* leading to a more significant effect on cognitive function than either *G. biloba* or *R. rosea* when they used alone.

KEY WORDS: *Ginkgo biloba, Rhodiola rosea*, neurocognitive tests

INTRODUCTION

Most of the registered psychotropic drugs that were licensed are synthetic and natural agents prior to 2007, although the contemporary trend includes an array of natural agents that produced a significant neuroprotection against different neurological diseases and age-induced cognitive dysfunctions [1]. Since, some of the synthetic agents are toxic with low efficacy profile; therefore, psycho neuroactive herbal extracts and their phytochemical are tried for their potential effects on memory, cognitive, and vigilance functions [2]; therefore, *Ginkgo biloba*, *Rhodiola rosea*, and other herbal medicines are marketed widely with expectation of preventing and delaying cognitive impairment with augmenting of normal brain functions [3].

G. biloba is confidential in its own partition as class *Ginkgoopsida*, order *Ginkgoales*, family *Ginkgoaceae* and genus *Ginkgo*, it has widely used in traditional medicine, *G. biloba* extracts contain various constituents which include, ginkgolides, bilobalides, flavonoid glycosides, phenolic acid, and quercetin [4]. In addition, *G. biloba* biflavones and polyphenols have potential neuroprotective effect in Alzheimer dementia via inhibition of amyloid fibrils accumulation [5]. Moreover, *G. biloba* delayed age-induced cognitive decline and may have more delicate and subtle therapeutic effects on the speed of cognitive alterations [6].

R. rosea is a traditional medicinal herb have about 140 active constituents such as flavonoid, alkaloids, rosin, rosarin, and phenols, its belong to *Crassulaceae* family, grow mainly at high altitude in Asia and Europe, the medicinal and pharmacological properties of *R. rosea* are species dependent and most of the human and animal studies conducted on *R. Rosea*. Since, it passed sophisticated and extensive pharmacological studies and had been certified safe for both humans and animals [7].

R. rosea contain an active constituent called salidroside which has various and multiple pharmacological effects include, anti-oxidant, anti-ischemic, anti-hypoxic, and neuroprotection [8].

Moreover, long-term therapy with *R. rosea* leads to central nervous system stimulation; improve psychomotor performance and improvement of cognitive function [9].

Therefore, the purpose of the present study was assessing the effect of *G. biloba* and/or *R. rosea* on psychomotor vigilance

task (PVT) and short-term working memory accuracy in normal healthy volunteers.

SUBJECTS AND METHODS

This study was done in Department of Clinical Pharmacology, College of Medicine, Al-Mustansiriya University, in Baghdad-Iraq from January to April 2015. Enrolled volunteers were meeting and consulates on their healthy status. Exclusion criteria for the volunteers were psychiatric, metabolic, neurological, and other medical disorders. All volunteers were recommending not drinking caffeine, stimulant drugs, and alcohol containing beverage for at least 5 days before starting the study. The volunteers enrolled in this study signed well-read consent to their involvement in this study, according to the Declaration of Helsinki. The research protocol was endorsed and approved by a scientific board in Department of Clinical Pharmacology and College Medical Committee.

In this double-blind, randomized, the placebo-controlled study, a total number of 112 volunteers (60 males and 52 females) with mean age of 22 years were engaged from College of Medicine, Al-Mustansiriya University. They are diving into the following groups:

Group A: 30 volunteers (15 males, + 15 females) treated with starch 500 mg/day.

Group B: 27 volunteers (15 males + 12 females) treated with *G. biloba* capsule 60 mg/day (standardized to contain 24% *Ginkgo* flavone glycosides) SANTASYA Ltd.

Group C: 25 volunteers (15 males + 10 females) treated with *R. rosea* capsule 500 mg/day (standardized to contain 5% receiving) Rhodiola India UPC Code 790011140702.

Group D: 30 volunteers (15 males + 15 females) treated with both *G. biloba* capsule 60 mg/day and *R. rosea* capsule 500 mg/day. The duration of therapy was 10 days, subsequently, each volunteer in the groups performed neurocognitive tests that measured by a special device called a Leeds psychomotor battery tester (Zac-Gmbh.D-8346-Simbach/Inn), which measure the followings:

Critical Flicker-Fusion Frequency (CFFF)

A training period of the test was allowable and supported, this test made in a dim room. The device calculates records and lists the results. The Leeds psychomotor battery tester encloses four red emitting diodes situated in the corner of 1 cm^2 in surplus of a black panel, each volunteer be supposed to sit in front of device to guarantee 75-100 cm of distance between the device and eyes, which allows binocular vision for flicker-fusion awareness and discrimination, the flicker happen in frequency that ranged from 1 Hz to 60 Hz. On an elevating trail, the volunteer watches the four red lights flickering and should press the key as soon as possible when as they appear fused, this called ascending critical or fusion frequency (ACFF), whereas awareness of fusion light until to be flickering named as descending critical or flicker frequency (DCFF). The standard average of five fusions and flickers representing the cortical arousal activity, deterioration in either ACFF or DCFF indicating arousal disorders; furthermore, when ACFF value more than 30 Hz (near 60 Hz) and DCFF values <30 Hz (near 1 Hz) indicating a good arousal activity, and from the exceeding values a CFFF can be estimated where CFFF=DCFF+ACFF/2 [10].

PVT

The Leeds psychomotor performance tester advice was useful for estimations of total reaction time in ms (TRT), which observed as a marker for the assessment of sensorimotor reaction to the critical stimuli. The volunteer asked to place the index finger on the central button and teaches to press urgent red light appearance sites as soon as possible, the mean of five successive readings is recorded and listed on digital screen as TRT in ms and recognition reaction time in ms (RRT). TRT represents the time for the onsets of a stimulus to the end of the reaction in ms, while RRT represents the time for the onsets of a stimulus to the beginning of motor action consequently, TRT minus RRT equal to movement reaction time which represent the time from the end of stimulus recognition to the end of motor actions [11].

Short Term Working Memory Accuracy Test (Computerized N-Back Test)

This test was performed on the laptop screen; the eight squares at different sites were reachable consecutively on laptop monitor at a rate of three seconds, an answer was requested each time, and then single site reverses in sequence. In one-back test, the volunteer detect and seek squares site in relation to the preceding square, in two-back test, the volunteer detect and seek square site in relation into two reverse trails of the preceding square, while in a three-back the volunteer detects and seek square site in relation into three reverse trails of the preceding square. The laptop monitor consecutively measuring and counting short-term working memory accuracy (number of corrected responses) through pressing the letter A on laptop keyboards, one back (I-BACK) representing low-level, two back (II-BACK) representing a moderate level, whereas, three back (III-BACK) represent a high level of accuracy% [12].

All neurocognitive tests were measured before taking drugs as the first measure while the second measure performed on the 10^{th} day of the experiment after 4 h of the last dose of the drug, since repeated measurements may lead to adaptation.

Data Analysis

Data obtained were presented as mean ± standard error; different groups were compared using paired Student's *t*-test. The significance of differences was considered when $P < 0.05$ regarded as the lower border of significance.

RESULTS

Consort flow diagram demonstrated the number of participants in this randomized and placebo-controlled study a total number of 120 participants were enrolled, only eight participants were withdrawn from this study due to non-compliances, not met inclusions criteria and other reasons, while 112 participants were continued the neurocognitive studies [Figure 1].

Placebo produced no significant effects on all neurocognitive tests measure $P > 0.05$ in normal healthy volunteers after 10 days of treatment [Table 1].

G. biloba 60 mg/day for 10 days therapy produced significant effects on PVT $P < 0.01$, insignificant effects on CFFF parameters $P > 0.05$ with mild significant effect on ACFF $P < 0.05$ and significant effects on short term working memory accuracy only for I-BACK WMA and II-BACK WMA $P < 0.01$ but not for III-BACK WMA $P > 0.05$ [Table 2].

Therefore, *G. biloba* improves PVT and low to moderate working memory accuracy.

R. rosea 500 mg/day for 10 days therapy produced significant effects on PVT $P < 0.01$, insignificant effects on CFFF parameters $P > 0.05$ with mild significant effect on ACFF $P < 0.05$ and significant effects on short term working memory accuracy only for I-BACK WMA and II-BACK WMA $P < 0.01$ but not for III-BACK WMA $P > 0.05$ [Table 3].

Therefore, *R. rosea* improves PVT, ACFF, and low to moderate working memory accuracy.

Therefore, *R. rosea* and *G. biloba* have similar central effects, but combined *G. biloba* 60 mg/day plus *R. rosea* 500 mg/day

Table 1: The placebo effects on the neurocognitive variables on normal healthy volunteers

Neurocognitive variables	Before n=30	After n=30	P
TRT (ms)	675.78±46.44	678.56±33.38	0.06
RRT (ms)	456.87±33.72	458.77±31.65	0.11
MRT (ms)	218.91±12.72	219.77±1.73	0.86
ACFF (Hz)	31.23±1.64	31.22±1.55	0.29
DCFF (Hz)	29.38±2.38	29.33±2.33	0.95
CFFF (Hz)	30.31±1.005	30.27±1.94	0.96
I-BACK WMA (%)	85.79±6.75	87.8±4.67	0.10
II-BACK WMA (%)	80.49±5.67	82.73±4.22	0.08
III-BACK WMA (%)	76.59±3.83	77.21±4.11	0.47

Data expressed as mean±SE, TRT: Total reaction time, RRT: Recognition reaction time, MRT: Movement reaction time, ACFF: Ascending critical fusion frequency, DCFF: Descending critical flicker frequency, CFFF: Critical flicker-fusion frequency, WMA: Working memory accuracy, SE: Standard error

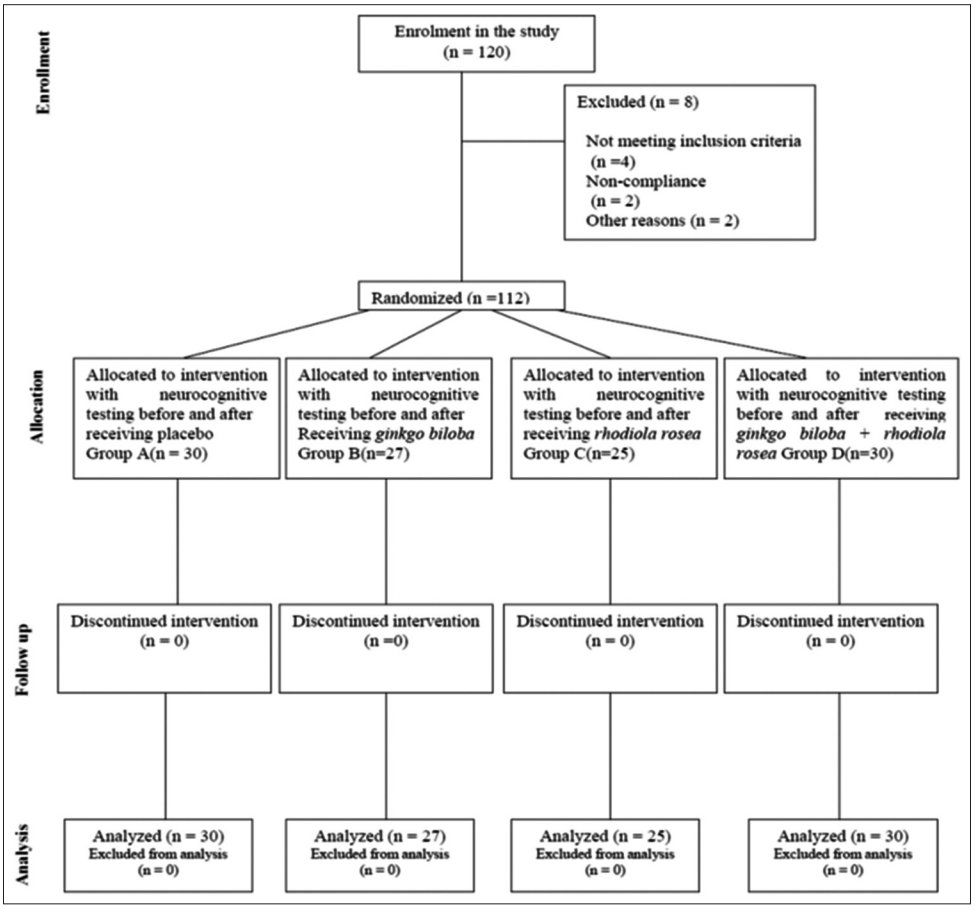

Figure 1: Flow diagram of study design

for 10 days therapy in normal healthy volunteers' demonstrated central additive effects more than either *R. rosea* or *G. biloba* when they used alone.

The combined effect of *R. rosea* and *G. biloba* leading to more significant effect on PVT $P < 0.01$, improve both ACFF and DCFF significantly $P < 0.05$ without amelioration of CFFF $P > 0.05$ also, they produced significant effects on short-term working memory accuracy at all levels, i.e. they significantly improve I-BACK WMA, II-BACK WMA, and III-BACK WMA $P < 0.01$ [Table 4].

60 males and 52 females enrolled in this study revealed insignificant differences in the response for the neurocognitive tests, which indicating a gender in significant differences to the responses of neurocognitive stimuli after the combined effects of *R. rosea* and *G. biloba* $P > 0.05$ [Figure 2].

Differential effects of *G. biloba* and/or *R. rosea* on working memory accuracy compared with placebo effect [Figure 3].

Table 2: *G. biloba* effects on the neurocognitive variables on normal healthy volunteers

Neurocognitive variables	Before n=27	After n=27	P
TRT (ms)	669.84±34.7	601.22±22.68	0.0001*
RRT (ms)	405.46±21.39	388.43±11.73	0.0017*
MRT (ms)	264.38±13.31	212.79±10.95	0.0001*
ACFF (Hz)	30.44±1.34	33.54±2.49	0.04**
DCFF (Hz)	29.79±1.58	27.77±1.64	0.10
CFFF (Hz)	30.11±1.46	30.66±2.06	0.51
I-BACK WMA (%)	80.33±8.99	87.99±11.32	0.004*
II-BACK WMA (%)	77.24±6.83	84.44±7.83	0.009*
III-BACK WMA (%)	72.93±4.63	75.64±6.39	0.06

Data expressed as mean±SE, *P<0.01, **P<0.05, TRT: Total reaction time, , RRT: Recognition reaction time, MRT: Movement reaction time, ACFF: ascending critical fusion frequency, DCFF: Descending critical flicker frequency, CFFF: Critical flicker-fusion frequency, WMA: Working memory accuracy, *G. biloba: Ginkgo biloba*

Table 3: *Rhodiolarosea* effects on the neurocognitive variables on normal healthy volunteers

Neurocognitive variables	Before n=25	After n=25	P
TRT (ms)	677.65±33.47	611.22±32.68	0.0001*
RRT (ms)	408.43±32.39	368.43±10.63	0.0003*
MRT (ms)	269.22±1.08	242.79±0.18	0.0007*
ACFF (Hz)	31.44±1.34	34.74±2.49	0.042**
DCFF (Hz)	28.19±1.58	26.67±1.44	0.16
CFFF (Hz)	29.86±1.46	28.67±1.75	0.23
I-BACK WMA (%)	72.13±7.29	89.49±11.32	0.001*
II-BACK WMA (%)	74.24±5.83	86.54±7.83	0.003*
III-BACK WMA (%)	73.93±3.63	74.64±6.39	0.42

Data expressed as mean±SE, *P<0.01, **P<0.05, TRT: Total reaction time, RRT: Recognition reaction time, MRT: Movement reaction time, ACFF: Ascending critical fusion frequency, DCFF: Descending critical flicker frequency, CFFF: Critical flicker-fusion frequency, WMA: Working memory accuracy, SE: Standard error

Table 4: Combined effect of *Rhodiolarosea* and *G. biloba* on the neurocognitive variables on normal healthy volunteers

Neurocognitive variables	Before n=30	After n=30	P
TRT (ms)	665.35±23.27	511.12±32.28	$5.2E^{-05}$*
RRT (ms)	408.43±32.39	318.13±7.93	$6.3E^{-05}$*
MRT (ms)	269.22±1.08	242.79±0.18	0.0007*
ACFF (Hz)	31.34±1.14	36.14±1.29	0.021**
DCFF (Hz)	29.28±1.18	25.17±0.14	0.028**
CFFF (Hz)	29.86±1.46	28.67±1.75	0.23
I-BACK WMA (%)	62.13±7.29	88.22±12.35	0.0007*
II-BACK WMA (%)	64.14±3.63	82.56±7.83	0.0014*
III-BACK WMA (%)	62.13±1.22	74.64±6.39	0.003*

Data expressed as mean±SE, *P<0.01, **P<0.05, TRT: Total reaction time, RRT: Recognition reaction time, MRT: Movement reaction time, ACFF: Ascending critical fusion frequency, DCFF: Descending critical flicker frequency, CFFF: Critical flicker-fusion frequency, WMA: Working memory accuracy, SE: Standard error, *G. biloba: Ginkgo biloba*

DISCUSSION

A neurocognitive test which includes PVT, critical fusion versus flicker frequency, and short-term working memory accuracy are a reliable and simple test for estimation and evaluation of central arousal state [13] that used in the present study to investigate different herbal agents, which mainly acts as cognitive enhancers. These tests are affected by many factors which include, gender, age, race, and healthy factors thus in the present study a younger age group of both males and females Iraqi medical students were included to exclude gender, race, and age difference in the neurocognitive response from the present study. Indeed, many studies shown significant deterioration of cognitive performances associated with elderly and diseased stating also; there are a significant controversy about race factor effects on cognitive functions of psychomotor performance testing [14].

The present study demonstrated that placebo may produce changes before and after 10 days duration of therapy on the

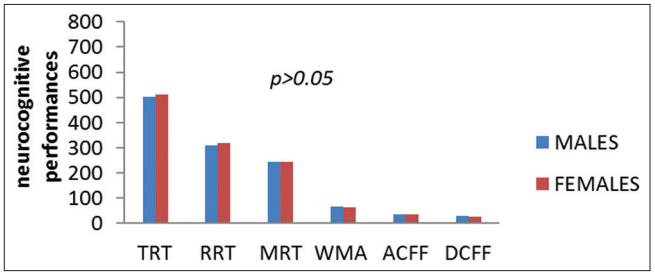

Figure 2: Gender differences in response to the neurocognitive stimuli

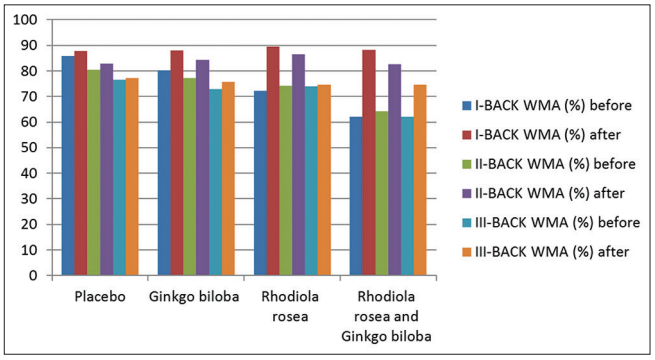

Figure 3: Improvements in working memory accuracy regarding *Ginkgo biloba* and/or *Rhodiola rosea* effects

neurocognitive variables mainly on total reaction time but, not reached to the level of significant $P > 0.05$. Draganich and Erdal, 2014 study revealed that placebo therapy may affect the cognitive function of both negative and positive directions, suggesting a means of controlling vigilance, memory, and cognitive functions [15].

The present study also demonstrated that *Ginkgo biloba* was significantly improved the neurocognitive variables of the cognitive function, chiefly on the PVT, upgrade low and moderate levels of short-term working memory accuracy and mild improved in ACFF without significant effects on CFFF and DCFF. Kennedy and Wighman, 2011 research revealed *G. biloba* extract significantly accelerating psychomotor performances, memory, and vigilance function throughout different mechanisms, *G. biloba* contains a numeral biologically active constituents (ginkgolides, bilobalide, and terepenes) which lead to potential central effects on induction of brain constitutive nitric oxide synthase, neuromodulation effect through inhibition of neural platelet activating factor, inhibition of monoamine oxidase enzyme that lead to significant neurotransmitters augmentation and neuroprotection through inhibition of oxidative stress and down-regulation of free radical generations [16], which may explain the positive effects of *G. biloba* on memory and psychomotor performances in the current study.

Moreover, a randomized human control trials have revealed cognitive improvement and enhancement in both older and younger age groups subsequent a single doses of *G. biloba* for 7 days [17], a study corresponding with the result of present study to the enhancement outcome of *G. biloba* on neurocognitive variables within 10 days duration of treatment, whereas animal models cognitive study done by Yoshitake *et al.*, 2010 exhibited that chronic but not acute therapy with *G. biloba* extracts improve cognitive function through increased dopaminergic neurotransmission in the prefrontal cortex [18].

Furthermore, Gavrilova *et al.*, 2014 study demonstrate that acute and chronic *G. biloba* treatment resulted in improvements in vigilance, attention, cognitive performance, decision-making function, and low to moderate but not high working memory accuracy [19], as demonstrated in the present study.

In additional, Al-Kuraishy *et al.*, 2014 psychometric study reported that dopaminergic advancing agents like sertraline and bupropion will accelerate working memory, and psychomotor performances in the similar manner of *G. biloba* effects due to augmentation of synaptic dopamine, also *G. biloba* improve muscarnic receptor that involved in modulation of memory and cognitive functions [20].

Indeed, central *G. biloba* effects may be through blocking over-activated NMDA receptors leading to neuroprotection from excitotoxicity during direct or indirect CNS stimulations [21].

Other possible explanations for the enhancement effects of *G. biloba* may be related to the up-regulation of genes transcription in cerebral cortex and hippocampus which were linked with learning, memory and cognition functions. Dietary *G. biloba* extract up-regulated 16 folds for genes responsible for a synthesis of transthyritin (protein transport retinol binding protein and thyroxine in CSF), transthyritin improve the neural integrity and cognitive performances [22], and unfortunately gene encoding assay is unavailable in Iraq.

Furthermore, the present study confirmed *R. rosea* was significantly improving the neurocognitive variables of cognitive function primarily on PVT; advance low and moderate levels of short-term working memory accuracy and mild progress in ACFF without significant effects on CFFF and DCFF. The present results are in corresponding with a numerous studies that showed *R. rosea* administration leads to CNS stimulation, enhance working memory, and improve cognitive function in addition to the antioxidant effect on free radicals scavenging effect and neuroprotection [23,24].

Moreover, the phytochemical analysis revealed that *R. rosea* contain strong constituents called salidroside which has time and dose dependent anti-oxidant and neuroprotective effects [25]. In addition, salidroside augments hippocampus serotonin levels and reduced inflammatory changes [26], which may explain the positive effects of *R. rosea* on working memory in the current study.

Animal experimental study of salidroside on cognitive function pointed out that *R. rosea* modulate cerebral neurotransmission and hypothalamic-pituitary axis since most of antidepressant and anti-inflammatory agents improve cognitive function [27].

Regarding the combined effect of both *R. rosea* and *G. biloba* on cognitive functions, they significantly improve the neurocognitive variables of cognitive function principally on PVT; advance low, moderate and high levels of short-term working memory accuracy and mild progress in ACFF and DCFF without significant effects on CFFF.

Our findings match Zang *et al.*, 2009 research that showed the combined effect of *G. biloba* and *R. rosea* accelerate and improve cognitive performance through increasing oxygen consumption and protecting against central and physical fatigues [28].

Therefore, this combination leads to central additive effect on cognitive function, PVT, and short-term working memory, since combines *G. biloba*, one of most broadly used herbs for a brain, with the *R. rosea* lead to maintain mental performance, mood balance, provides antioxidant protection for the brain, improve glucose metabolism, elevation of serotonin levels, and cognitive function [29,30].

Finally, there are insignificant gender differences in cognitive enhancement effect of this combination in response to the neurocognitive stimuli and reactions; this finding was supported

by Kim *et al*., 2015 study which showed a non-significant differences in the cognitive reaction in younger but not in older with or without Alzheimer dementia [31].

CONCLUSION

The combined effect of *R. rosea* and *G. biloba* leading to more significant effect on cognitive function than either *G. biloba* or *R. rosea* when they used alone.

Suggestions for Future Research

1. Study different doses of *R. rosea* and *G. biloba* on psychomotor vigilance task and short-term working memory accuracy
2. Estimation of orexin plasma levels to evaluate central effect of *R. rosea* and *G. biloba* on psychomotor vigilance task and short-term working memory accuracy
3. Study the central improvement effect of *R. rosea* and *G. biloba* on psychomotor vigilance task and short-term working memory accuracy on elderly patients with dementia
4. Gender differences in central effect of *R. rosea* and/or *G. biloba*.

Limitations of the Study

1. I study the central effect of *R. rosea* and/or *G. biloba* in normal healthy volunteers
2. Limitations of the availability of modern highly sensitive devices
3. Similarly, the educational level of the undergraduate medical student groups was known to affect vigilance and short term working memory but this indicator was not included in the present study or compared with other educational levels.

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Original Research

ScopeMed

Pharmacological evaluation of *Mallotus philippinensis* (Lam.) Muell.-Arg. fruit hair extract for anti-inflammatory, analgesic and hypnotic activity

Mayank Gangwar[1], Manish Kumar Gautam[1], Shivani Ghildiyal[2], Gopal Nath[3], Raj Kumar Goel[1]

[1]Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India, [2]Department of Dravyaguna, Government College & Hospital, S.S.V.V., Varanasi, India, [3]Department of Microbiology, Faculty of Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Address for correspondence:
Raj Kumar Goel, Department of Pharmacology, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India.
E-mail: rkgoelbhu50@gmail.com

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ABSTRACT

Objective: Recently, we observed wound healing activity of 50% ethanol extract of *Mallotus philippinensis* Muell. Arg (MP) fruit hairs extract (MPE). In several intestinal infections, localized inflammation is of common occurrence and hence we evaluated the anti-inflammatory, analgesic and hypnotic activity of MPE in different rat experimental models. **Materials and Methods:** Anti-inflammatory activity was evaluated by carrageenan (acute) and turpentine oil induced formalin (subacute) induced paw edema and while granuloma pouch (subacute) in rats. Analgesic and hypnotic activity of MPE was undertaken by tail-flick, hot-plate, and acetic acid-induced writhing tests while pentobarbitone-induced hypnotic potentiation in rats. **Results:** MPE at a dose of 200 mg/kg at 3 h after their administration showed inhibition of formalin-induced paw edema by 41.60% ($P < 0.001$) and carrageenan-induced paw edema by 55.30% ($P < 0.001$). After 7 days of treatments, MPE showed 38.0% ($P < 0.001$) inhibition against formalin-induced paw edema and reduced weight of turpentine-induced granuloma pouch by 29.6% ($P < 0.01$) and volume of exudates by 26.1% ($P < 0.01$), respectively. MPE (200 mg/kg) showed dose-dependent elevation in pain threshold and peak analgesic effect at 120 min as evidenced by increased latency period in tail flick method and increased reaction time in the hot-plate test while the reduction in the number of acetic acid-induced writhes by 45.7% ($P < 0.001$). The pentobarbitone-induced hypnosis model showed potentiation, as defined by increased duration of sleep in treated group rats as compared to control. **Conclusion:** Thus, the study revealed MPE is effective in reducing acute and subacute inflammation and showed effective and similar analgesic activity. This seemed to be safe in the treatment of pain and inflammation.

KEY WORDS: Carrageenan, Eddy's hot plate, formalin, granuloma pouch, *Mallotus philippinensis*, tail-flick method

INTRODUCTION

Inflammation is the symptom in various infective diseases such as inflammatory bowel disease bronchitis, cardiovascular diseases, asthma, inflammatory and autoimmune disorders, neurodegenerative conditions, and cancer [1]. Anti-inflammatory activity refers to the property of a substance or treatment that reduces inflammation. Anti-inflammatory drugs, steroids, mainly glucocorticoids, mainly decreases inflammation by combining with the cortisol receptors. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by counteracting the cyclooxygenase (COX) enzyme [2]. The inflammatory response are mediated *via.* immune system components at infectious region and will manifested by increased blood supply and vascular permeability which results in immigration of peptides, mononuclear cells, and neutrophils. Literature suggests various standard anti-inflammatory animal models *viz.* air pouch granuloma, paw edema, sponge implantation, and pleurisy using different chemical agents such as carrageenan [3], turpentine [4], and formalin [5] to screen new anti-inflammatory molecules [6]. It has been reported that rat paw edema model using different mediators are suitable *in vivo* animal model to predict the efficacy

of anti-inflammatory agents, which mainly act by inhibiting the mediators of acute inflammation [7].

According to the International Association for the Study of Pain, pain is defined as an unpleasant feeling, which may involve tissue damage, and could have physical and emotional components [8]. However, pain sensation will protect our body from external stimuli by causing us to perform certain actions. Pain may be defined as a predictor, protector, or simply a hassle [9]. The pain can be classified as acute or chronic. Acute pain is defined as short-term but extreme pain that comes on quickly but last only for a brief period. But in usual conditions, such pain can be resolved by the body's physiologic response from the endogenous pain modulating system [10]. The pain which persist for a longer duration compared to normal time course called as chronic pain, and mostly associated with a some sort of injury or disease condition. Most common nonmalignant chronic pain syndromes are divided into categories of neuropathic and non-neuropathic ("functional"). It can be made much worse by environmental and psychological factors [11]. The major classes of analgesics include NSAIDs, COX_2 inhibitors, opiates, and morphinomimetics. The main demerit about the present synthetic drug therapy against inflammation is their toxicity and reappearance of symptoms after discontinuation. Therefore, screening and development of natural drugs for analgesic and anti-inflammatory activity are still in progress, and there is much hope for finding these drugs from indigenous medicinal plants [12].

Insomnia is a sleep disorder which mainly perception or complaint of inadequate or poor-quality sleep due to various psychological and physical disorder. Sedatives are the drugs which calm the central nervous system (CNS) and have a relaxing effect. Sedatives at higher doses usually cause sleep; they can be defines as hypnotics. The basic difference between sedatives and hypnotics is the amount of the dose; calming effect achieved at low dose while sleep at a higher dose. Recent studies have shown that herbal drugs exert good sedative and hypnotic effect on the CNS [13,14].

Our experimental laboratory and research group have been engaged in the identification of various herbal plants for the anti-inflammatory, analgesic and hypnotic activity using anti-inflammatory, analgesic and hypnotic experimental animal models. This study evaluates the anti-inflammatory, analgesic and hypnotic potentiation of MPE against various animal experimental models. These activities are important for any ulcer healing property including wounds as anti-inflammatory activity would help in decreasing wound inflammation, while analgesic and hypnotic activity would reduce the reaction to pain and cause euphoria.

MATERIALS AND METHODS

Chemicals and Instruments

The drugs used in this study include carrageenan (Sigma-Aldrich, USA), turpentine oil (Loba Chemie, India), formaldehyde (Merck Limited, India), and diclofenac sodium (Jagsonpal Pharmaceuticals, India). However, standard drugs such as pentazocine (Ranbaxy Pharmaceuticals, India), acetic acid (Merck, India), and pentobarbitone (Loba Chemie, Mumbai, India) were used for the different pharmacological studies.

The instruments used in the study include tail flick apparatus (Socrel model, DS2, Milan, Italy), plethysomometer (Ugo basile, Mila, Italy), and hot plate (Harvards Apparatus Ltd, UK).

Preparation of Plant Extract

Mallotus philippinensis fruits were procured from Botanical Garden, Department of Dravyaguna, Institute of Medical Sciences, Banaras Hindu University, India. Fruiting season of the plant is in the month of March to April, whole fruits were collected initially to prepare the fruit glandular hair extract. Identification and authentication were done by Professor Asthana Department of Botany, Banaras Hindu University, India with a reference voucher number RKA/BOT/Sept. 10-12. Further, the plant sample was preserved in the Department of Botany, BHU, Varanasi, India. The glandular hairs of fruit (red color powder) adhering at the surface of shade dried fruits were collected. Approximately, 500g of powder was collected and added to 1000 mL of 50% ethanol in a bottom flask and was kept at room temperature for 3 days in shade. The organic fraction was collected and concentrated *in vacuum* in a rotary evaporator, and the residue was dried in desiccators over calcium chloride powder.

Experimental Animals

Healthy Charles-Foster albino rats (180-200 g) of either sex were selected and collected from the Central Animal House facility (Reg.no.542/02/ab/CPCSEA), IMS, BHU, Varanasi, India. All the animals were kept in Plexiglas cages in groups of six, with free access to water and food in the animal house of pharmacology departmental with standard temperature condition as 26 ± 2°C, 44-56% of relative humidity, light and dark cycles of 10 and 14 h, respectively, during the experiments. The diet for animals was purchased as a standard rodent pellet diet (Pashu Aahar Vihar, Ramnagar, Varanasi) and water ad libitum for all the animals during the experiment. Laboratory animal care principles and guidelines (NIH publication no. 82-23, revised 1985) will be followed after approval of Institutional Animal Ethical Committee for experimental work (Notification no. Dean/13-14/CAEC/331 dated 20.11.2013).

Anti-inflammatory Activity

Dose selection and treatment protocol

Considering the significant dose of MPE, and the extract yield (≈11.6%), the fruit extract dose in the rat was calculated according to the surface area in relation to the human. Thus in this experiment, we have planned initially graded anti-inflammatory doses of MPE (100, 200, and 400 mg/kg) against

formalin-induced pedal edema. 0.5% carboxymethylcellulose (CMC) was given to all animals in the control group as a vehicle, while test extracts/standard drug, diclofenac sodium were given orally in a volume of 1 mL/100 g body weight, once daily using the orogastric tube.

Indigenously prepared graduated plethysmograph was used for the study. The mercury displacement due to the dipping of the paw was directly read from the scale attached to the mercury column. A mark was made on both the hind paw (right and left) just beyond tibiotarsal joint, so that every time the paw is dipped in the mercury column up to the fixed mark to ensure constant paw volume. Mercury displacement measured the initial paw volume (both right and left) of each rat in formalin and carrageenan-induced paw edema animal models.

Formalin-induced pedal edema

All animals were divided into five groups ($n = 6$ in each group). 0.1 ml of 2% v/v formalin was injected in the subplanter region on the first and third day of the experiment of each rat in hind paw region.

Control, 1st group received 0.5% CMC while the treated groups from the 2nd to 4th groups received MPE (100, 200, and 400 mg/kg, p.o.) and the 5th group received diclofenac sodium (DFC, 10 mg/kg, p.o.), respectively. The initial dose of the extract was given 60 min before the injection of formalin and was continued until 7 days. The paw thickness was measured using a plethysmograph on 0 and 3 h and the 7th day according to the standard method mentioned by Singh *et al*. [15]. Percent inhibition in paw volume between treated and control groups was calculated as follows:

$$\text{Percent inhibition} = (1\text{-}V_T/V_C) \times 100$$

Where, V_T and V_C were mean paw volume of treated and control groups, respectively, at 3 h or 7th day.

Carrageenan-induced pedal edema

Carrageenan-induced inflammation in paw was performed according to the method described in detailed by Winter *et al*. [16]. Total of three groups of rats having six animal in each were used for the study. The animals in control group were administered with 0.5% CMC, while the treated group received MPE (200 mg/kg) and DFC (10 mg/kg) orally 60 min before carrageenan administration in 18 h fasted rats. For inducing inflammation, carrageenan suspension (1.0%) in 0.9% sodium chloride solution (sterile normal saline) were administered. Paw volume in each control and treated group were measured by means of volume displacement technique using plethysmometer (Ugo Basile no. 7140) immediately after injection of carrageenan and also after 1, 2, 3, and 24 h. duration. The percent inhibition in paw volume between treated and control groups were calculated as per equation mentioned below:

$$\text{Percent inhibition} = (1-V_T/V_C) \times 100$$

Where, V_T and V_C were mean paw volume of treated and control groups, respectively, at 1, 2, 3, or 24 h.

Turpentine oil-induced granuloma pouch

Subcutaneous dorsal granuloma pouch was made by injecting 25 ml of air in anesthetized rats, followed by 0.5 ml of turpentine oil injection into it 17. Extract, the standard drug, and CMC were administered orally 60 min prior to turpentine oil injection and will further continue until seven consecutive days. After last day of the experiment (on day 7), the pouch was removed and the content of exudates was collected after 60 min of the last dose of drugs. Further, the amount of exudate (in volume) and weight of pouch were calculated and presented as g and ml per 100 body weight of the animal. The percent inhibition of exudates volume and granuloma pouch weight between control and treated groups were calculated as follows [17]:

$$\text{Percent inhibition} = (1\text{-}V_T/V_C) \times 100$$

Where, V_T and V_C were mean weight and volume of treated and control groups, respectively, at 7th day.

Analgesic Activity

Dose selection and treatment protocol

Considering the significant dose of MPE in above experiment, authors have planned graded doses of MPE (100, 200, and 400 mg/kg) were tested to find an optimal effective analgesic dose in tail flick test. 0.5% CMC was given as vehicle to control group, while test extracts/standard drug, pentazocine (PTZ, positive control) in 0.5% CMC were administered (p.o.) using an orogastric tube of volume of 1 mL/100 g body weight of animal.

Tail flick test

The tail flick test with radiant heat is an extremely simplified and standard method used for analgesic activity assay. The mechanism behind thermal radiation model focuses on the time of tail withdrawn by a brief, vigorous movement [18]. It is the reaction time of this movement that is recorded (often referred to as "tail-flick latency"). A variation in the lengthening of the reaction time treated with extract, standard, and control vehicle are interpreted as an analgesic action.

CF rats of either sex were divided into five groups with six animals in each group. Analgesiometer was used to study the tail flick latencies study (i.e. reaction time) of the animals were recorded [19]. The basal reaction time of radiant heat was calculated by placing the last 2 cm of the tip of the tail on the source of radiant heat as per the standard manual instruction of instrument. Animal flicking response (tail withdrawn) was considered as the end point of the experiment. The rats were initially selected showing cut-off latency period was <5 s to prevent any damage to the animal tail. 0.5% CMC was administered to the control group, while treated groups (2-4th groups) received 100, 200, and 400 mg/kg graded doses

of MPE. However, last or the 5th group received the standard drug, PTZ (10 mg/kg). All the drugs (extracts/standard) were given *via* oral route just before the experiment. The tail flick latencies time as describes above was tabulated in sec at pre-drug (basal, 0 min reading) and at 30, 60, 120, 180, and 300 min after administration of CMC/MPE/PTZ. The percent increase in latency period was calculated following the formula:

$$\text{Percent increase} = (T_T/T_C\text{-}1) \times 100,$$

Where, T_C and T_T were defined as the mean basal (0 min) analgesic time and post-treatment time of 30, 60, 120, 180, and 300 min, respectively.

Hot plate test

Four groups of rats having six animals in each were used selected for the study. The hot plate assay was performed using Eddy's hot plate apparatus. All the steps were followed as per manufacturer's instructions. Paw licking and jumping responses of experimental animals were evaluated in terms of their reaction times and for this constant temperature were maintained during the experiment [20]. Both are considered to be supraspinally integrated responses. During the experiment, animals were placed on the top of the hot plate, which was kept at approximately 55 ± 0.5°C. Animals were placed for a maximum time of 10 s to avoid any thermal injury/damage in the paws. As soon as animal licked their fore- and hind-paws, and jumped from the top of hot plate, reaction time was recorded at before (basal/0 min) and after 30, 60, 120, 180, and 300 min following administration of CMC (control), MPE (Test drugs, 200 mg/kg), and PTZ (positive control, 10 mg/kg). Based on the data of reaction time, the percent increase in reaction time was calculated using the following formula:

$$\text{Percent increase} = (T_T/T_C\text{-}1) \times 100,$$

Where, T_C and T_T were defined as the mean analgesic time at basal (0 min) and post-treatment time of 30, 60, 120, 180, and 300 min, respectively.

Acetic acid-induced writhing response

For an acetic acid-induced writhing response, a total of four groups were selected having six animals in each group. The control group received CMC, and treated groups were received diclofenac (10 mg/kg) or MPE (200 mg/kg). After, thirty minutes 0.7% of acetic acid (i.e., 10 mL/kg) solution was injected intraperitoneal to all the animals in the different experimental groups. The number of writhes (abdominal constrictions) produced by each animal was observed individually under a glass jar for a period occurring between 5 and 20 min after acetic acid injection, and the same was counted. The antinociceptic response was considered as if the number of writhes was significantly reduced in the treated group compared to the control group [21]. The % protection of analgesic activity was calculated by using the formula:

$$\text{Percentage inhibition} = (1\text{-}W_T/W_C) \times 100,$$

Where, W_T and W_C were denoted as the number of writhing in the treated group and in the control group, respectively.

Hypnotic Activity

The sedative and hypnotic activity of the glandular hairs of *M. philippinensis* fruit extract is mainly depends on the nature of CNS depressant drugs, as they potentiate a subhypnotic dose of the standard drug, pentobarbitone. It mainly concludes the mild sedatives action, along with gives results with other depressants such as drugs which are analgesic, anticonvulsants, and anxiolytics. Sodium pentobarbitone was basically used for sleep induction. For the assessment, righting reflex was the useful measure to study whether or not animals are asleep.

The sleeping time in rats was studied by the method of Dandiya and Columbine [22]. Three groups having six male rats in each group were selected for the study. CMC was administered to the experimental control group, while MPE (200 mg/kg) was given to the treated groups. Pentobarbitone (20 mg/kg, intraperitoneal) was administered as subhypnotic dose before thirty minutes, to each animal of all the defined groups. Pentobarbitone-induced hypnosis and its potentiation was observed in each animal. The time between the administration of pentobarbitone to start losing the righting reflex is denoted as onset of sleep, while the time between the onset of sleep to start regaining of righting reflex is denoted as the duration of sleep, which was recorded in each animal and then compared with respect to the control group.

Statistical Studies

All the data of control and treated animals were analyzed using ANOVA, further the significance difference was calculated between mean values. The values expressed after the analysis were average ± standard error of means (SEM). The data were also subjected to correlation coefficient using Sigmastat version 3.1 statistical analysis software for further correlation between the samples. The correlation of the data was calculated using Pearson's test. *P* values <0.01, 0.05, and 0.001 were considered as statistically significant.

RESULTS

Anti-inflammatory Activity

Formalin-induced paw edema

Formalin-induced paw edema model was studied using dose-dependent inhibition at 3 h and 7th day was observed after the oral administration of MPE (100, 200, and 400 mg/kg). The percent inhibition ranged from 23.4% to 45.2% at 3 h and 15.5% to 41.5% at 7th day with MPE at different doses. The MPE at all doses showed a significant inhibition of paw edema at the third hour as compared to reference drug. 200 mg/kg dose of extract produced a significant reduction in paw volume in comparison with the control group, which was utilized as optimal dose for further studies [Table 1].

Carrageenan-induced paw edema

In the carrageenan-induced edema test, the subplantar injection of carrageenan-induced edema in rats, the paw volumes and percentages of inhibition by the alcoholic extract of *M. philippinensis* fruit and standard drugs are shown in Figure 1. MPE (200 mg/kg) was found to inhibit carrageenan-induced paw edema by 21.9% ($P < 0.05$), 32.5% ($P < 0.01$), 55.3 and 65.8% ($P < 0.001$) at 1st, 2nd, 3rd, and 24th h which was comparable with the effect of diclofenac sodium (DCF).

Turpentine oil-induced granuloma pouch

The subcutaneous dorsal granuloma pouch weight and exudates volume results showed a significant decrease in percent inhibition as compared to control; results are summarized in Table 2. MPE after their 7 days administration, reduced weight of turpentine-induced granuloma pouch by 29.6% and volume of exudates by 26.1% when expressed per 100 g body weight of rats as compared with the standard anti-inflammatory drug, DCF.

Analgesic Activity

Tail flick test

The results of MPE at different doses (100, 200 and 400 mg/kg), for analgesic activity using tail flick method are summarized in Table 3 and found statistically significant ($P < 0.001$) elongation of reaction time at the dose of 200 mg/kg body weight as compared to the control. MPE showed dose-dependently (significant dose, 200 mg/kg) effect, i.e. it increased the latency time during first 180 min during the study and showing peak analgesic effect at 120 min. After 30 min, the extract in doses of 100, 200, and 400 mg/kg ($P < 0.001$) body weight showed a significant elongation. After 120 min, MPE at the doses of 100, 200, and 400 mg/kg showed no significant increase in reaction time. Reaction time data showed a significant increase in case of tail flick method, which indicates the analgesic effect depicting possibly the involvement of central mechanism in analgesic action.

Hot plate test

The result of hot plate test showed no significant difference in mean pre-drug pain reaction time among the groups but after administration, the extract generally significantly ($P < 0.001$) increased the post drug pain reaction time. MPE (200 mg/kg) increased the pain reaction time ($P < 0.001$) similar to the standard antinociceptive agent, PTZ [Figure 2].

Acetic acid-induced writhing

MPE showed a significant antinociceptive activity in terms of acetic acid induced writhes which was decreased significantly with inhibitory rates of 45.7%, while the standard drug, DCF showed a significant antinociceptive activity with the inhibitory rate of 76.2% [Table 4].

Table 1: Anti-inflammatory effect of MPE against formalin-induced paw edema in rats

Oral treatment (mg/kg)	Paw volume (ml)		Inhibition (%)*	
	3 h	7 day	3 h	7 day
Control (0.5% CMC)	1.37±0.09	1.42±0.06	-	-
MPE (100)	1.05±0.08[a]	1.20±0.05[a]	23.4	15.5
MPE (100)	0.80±0.04[c]	0.88±0.02[c]	41.6	38.0
MPE (100)	0.75±0.04[c]	0.83±0.03[c]	45.2	41.5
DCF (10)	0.68±0.03[c]	0.76±0.06[c]	50.4	46.5

Values expressed as mean±SEM (n=6), *Percent inhibition=(1−VT/VC)×100. [a]$P<0.05$, [c]$P<0.001$ compared with respective h/day control group, SEM: Standard error of mean, CMC: Carboxymethylcellulose, MPE: *Mallotus philippinensis* extract

Table 2: Effect of MPE on turpentine oil-induced granuloma pouch in rat

Oral treatment (mg/kg×7 days)	Granuloma pouch		Exudate	
	Weight (g/100 g bw)	*Percent inhibition	Volume (ml/100 g bw)	*Percent inhibition
Control (0.5% CMC)	2.94±0.15	–	0.78±0.04	–
MPE (200)	2.07±0.16[b]	29.6	0.57±0.05[b]	26.1
DCF (10)	1.84±0.12[c]	37.4	0.48±0.03[c]	38.5

Values expressed as mean±SEM ($n=6$) *Percent inhibition=$(1-V_T/V_c)\times100$. [b]$P<0.01$, [c]$P<0.001$ compared to respective control group, CMC: Carboxymethylcellulose, MPE: *Mallotus philippinensis* extract, SEM: Standard error of mean

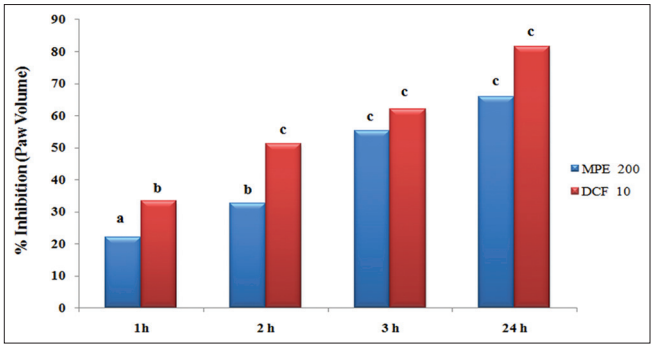

Figure 1: Anti-inflammatory effect of *Mallotus philippinensis* extract against carrageenan-induced paw edema in rats, values expressed as % inhibition paw volume, *P* values: [b]<0.01 and [c]<0.001 compared with respective h control group

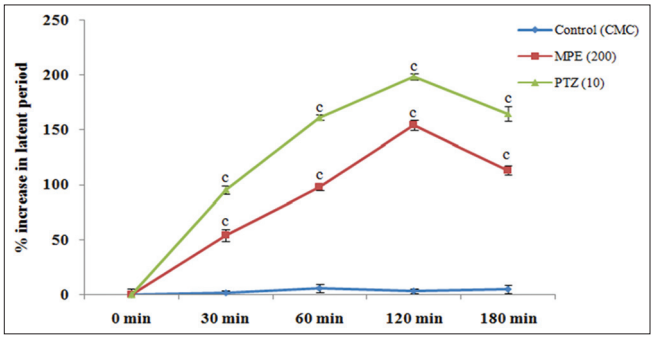

Figure 2: Analgesic activity of *Mallotus philippinensis* extract by hot plate method in rats, values expressed percent mean ± standard error mean of control values. *P* value: [c]<0.001 compared with respective h control group

Table 3: Analgesic activity of MPE by tail flick method in rats

Oral treatment (mg/kg)	Latent period (sec)					
	Basal (T_c)	30 min (T_T)	60 min (T_T)	120 min (T_T)	180 min (T_T)	300 min (T_T)
Control (CMC)	4.51±0.21 (0.0)	4.54±0.21 (0.8)	4.58±0.22 (1.5)	4.51±0.26 (0.0)	4.59±0.20 (1.8)	4.65±0.19 (3.1)
MPE (100)	4.75±0.24 (0.0)	6.05±0.14[c] (27.4)	6.50±0.20[c] (36.8)	6.95±0.18[c] (46.3)	6.36±0.15[c] (33.9)	5.63±0.20[b] (18.5)
MPE (200)	4.78±0.17 (0.0)	7.55±0.21[c] (57.9)	7.80±0.18[c] (63.2)	8.06±0.19[c] (68.6)	7.52±0.23[c] (57.3)	6.75±0.23[c] (41.2)
MPE (400)	4.47±0.16 (0.0)	8.23±0.18[c] (84.1)	8.41±0.24[c] (88.1)	8.68±0.23[c] (92.2)	8.61±0.17[c] (92.6)	7.30±0.19[c] (63.3)
PTZ (10)	4.38±0.19 (0.0)	7.76±0.21[c] (81.3)	8.95±0.25[c] (109.1)	9.41±0.19[c] (119.8)	8.78±0.25[c] (105.2)	7.53±0.22[c] (75.9)

Values expressed as mean±SEM (n=6). Results in brackets represents percentage increase in analgesic time from respective basal value. [c]P<0.001 compared to respective min control group, SEM: Standard error of mean, MPE: *Mallotus philippinensis* extract, PTZ: Pentazocine, [b]P<0.01

Table 4: Effects of MPE and DCF on acetic acid induced writhing and pentobarbitone (PENTO)-induced sleeping time in rats

Acetic acid-induced writhing (mean number of writhing in 15 min)			Pentobarbitone-induced hypnosis (PENTO, 20 mg/kg; intraperitoneal)		
Treatment	Number of writhing	% inhibition*	Treatment**	Sleep latency (min)	Sleeping time (min)
Control (CMC)	53.8±2.8	-	Control (CMC+PENTO)	3.86±0.3	47.2±3.5
MPE (200)	29.2±3.0[c]	45.7	MPE (200+PENTO)	3.53±0.4	65.8±2.2[b]
DCF (10)	12.8±2.5[c]	76.2	-	-	-

Values expressed as mean±SEM (n=6). *Percentage inhibition=$(1-W_T/W_C)\times100$. **Male rats only. [b]P<0.01, [c]P<0.001 as compared to respective control group, CMC: Carboxymethylcellulose, MPE: *Mallotus philippinensis* extract, DCF: Diclofenac sodium, SEM: Standard error of mean

Hypnotic Activity

The hypnotic effect was evaluated by sleep latency and time of duration induced by the subhypnotic dose of pentobarbitone. MPE (200 mg/kg) showed no change in the time of onset of sleep but increased sleeping time induced by pentobarbitone ($P < 0.01$) [Table 4].

DISCUSSION

The process of acute inflammation is mainly initiated by dendritic cells, macrophages, kuppfer cells, histiocytes, and mastocytes. These cells will be activated at the onset of any type of infection, or other injuries, and will release the inflammatory mediators, which are responsible for the clinical signs of inflammation. The aim of the inflammation is to repair the damage or at least to limit it and also to remove the cause [23]. Carrageenan-induced rat paw edema is an acute inflammatory model which involves several mediators released in sequence. It was reported that during the first phase, i.e., 1.5 h, most of the histamine and serotonin were released; while the second phase, i.e., 1.5-2.5 h is mediated by bradykinin and the third phase, i.e., 2.5-6 h after carrageenan injection, in which the mediator is possible as prostaglandin. The carrageenan-induced hind paw edema in rats is known to be sensitive to cyclooxygenase inhibitors, but not to lipoxygenase inhibitors [24]. In this study, MPE significantly decreased the rat paw edema induced by carrageenan in all phases, suggesting the possible mechanism of action of the MPE may involve inhibition of these inflammatory mediators release in all phases.

The formalin-induced paw edema involves the infiltrations of neutrophils, macrophages and proliferation of fibroblasts and this methods resembles with human arthritis and preferred as one of the most suitable assays to identify the antiarthritic and anti-inflammatory agents [25]. The results suggest that MPE significantly decreased the formalin-induced edema, which might be useful for the treatment of chronic inflammatory disease like arthritis. The turpentine oil-induced granuloma pouch model basically evaluates the study with respect to exudative type of inflammation. Kinin is considered as the main mediator of granuloma, as it is responsible for vasodilatation and can further increase the vascular permeability in early stages of inflammation [26]. The MPE showed a significant reduction in the exudative fluid. The anti-inflammatory activity exhibited by MPE showed results similar with the standard drug, which suggests that cyclooxygenase inhibition may partly mediate the plant's activity.

Pain is felt because of inflammation, infection, ischemia, tissue necrosis, chemical, or burn. When an injury occurs, pain is first evoked by stimulation of the nociceptor (Aδ and C fibers), which will release of the kinins and potassium from the injured cells. These stimulate the receptor directly resulting in the release of the neuropeptides such as substance P from nociceptive terminals and the release of the histamine from the mast cells with the production of the platelet-activating factor which in turn releases serotonin from the platelets. Histamine is also released from the mast cells, starting an inflammatory reaction leading to vasodilatation and edema [27]. Opioid receptors of the μ-, δ-, and κ-subtypes mediate the potent analgesic and addictive actions of opioid drugs [28].

Acetic acid test (chemical stimuli) elucidates the peripheral and central mediated analgesic action, while hot plate and tail flick tests (thermal stimuli) elucidate the central mediated analgesic mechanism. The tail flick model is considered as the specific test for evaluation of the central pain at spinal levels. Tail flick model depicts the results of analgesic effect as centrally acting opioid like [29,30], while the hot plate animal test is suggest the central analgesic activity or supraspinal analgesia of any compound [31]. Thermic painful stimuli are known to be selective to centrally, but not peripherally acting analgesic drugs. In the writhing test, acetic acid is used to screen both peripherally and central acting analgesic activity [32, 33]. The writhing test could suggest the possible effective analgesic doses for any test substance

that can be used in humans [33]. Acetic acid cause pain by liberating endogenous substance that includes prostaglandins, serotonin, substance P, histamine, bradykinin, etc. mostly excite the pain nerve ending leading to the abdominal writhing [29]. MPE markedly exhibited a dose-related both peripheral and central mediated analgesic activity, and the potency of MPE (200 mg/kg) was comparable to reference standards, PTZ, and diclofenac. It is possible that MPE might be producing analgesic effects by mechanisms affecting the production of endogenous opioids, prostaglandins, and other mediators important for pain production and sensitizers. *M. philippinensis* is rich in phytochemicals such as phenols, flavonoids, alkaloids, with significant antioxidant activity reported [34] which might be responsible for above biological activities.

MPE was seen to potentiate pentobarbitone induced hypnosis indicating CNS depressant property which could be mediated through gamma-aminobutyric acid or some other mechanism unknown to us. As we have not done the detailed study regarding the involvement of neurotransmitters/modulators so we cannot definitely express their involvements. However, a proven sedative effect could be boon whenever we use the extract for wound healing or for its anti-inflammatory effects. So keeping the diverse ethnopharmacological profile of *M. philippinensis* in view [35-39], we have made an effort to further validate scientifically and found that MPE possessed a significant anti-inflammatory and antinociceptive activity.

CONCLUSION

Thus from the above investigation, it was observed that *M. philippinensis* fruit extract showed significant anti-inflammatory and analgesic activity against all the rat experimental models which indicates its use in the traditional system of medicine in order to support to reduce inflammation and pain in different pathological condition but further studies are required to evaluate the mechanism of action and activity directed bioassay for the new source of drug development. Further isolation and characterization of other active constituents from the extract and the most potent one can be carried further for exploration.

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Original Research

Effects of *Lawsonia inermis* L. (Henna) leaves' methanolic extract on carbon tetrachloride-induced hepatotoxicity in rats

Musab Awad Mohamed[1], Imad Mohamed Taj Eldin[1], Abd-Elwahab Hassan Mohammed[2], Hozeifa Mohamed Hassan[1]*

[1]Department of Pharmacology, Faculty of Pharmacy, University of Gezira, Sudan, [2]Department of Pharmacology and Toxicology, Medicinal and Aromatic Plant Research Institute, National Center of Research, Sudan

Address for correspondence: Hozeifa Mohamed Hassan, Department of Pharmacology, Faculty of Pharmacy, University of Gezira, Sudan. E-mail: hozhooz@hotmail.com

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ABSTRACT

Background: Natural products with therapeutic properties such as plants, minerals, and animal products, for many years, were the main sources of drugs for the treatment of numerous diseases; hence selection of *Lawsonia inermis* L. (Henna) to study its hepatoprotective activity was considered. **Objectives:** This was an attempt to evaluate the hepatoprotective effect of *L. inermis* leaves' methanolic extract on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. **Materials and Methods:** The *L. inermis* leaves' methanolic extract, which obtained by maceration, was orally administered in doses of 100 mg/kg and 200 mg/kg to the tested animals to assess its effects on serum levels of hepatotoxicity parameters, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, and total proteins along with histopathological liver sections examination, while silymarin (25 mg/kg), a potent hepatoprotective drug, was used as standard control. **Results:** The two doses of the plant extract showed dose-dependent hepatoprotective effect, as evident by the significant reduction ($P < 0.05$) in serum levels of AST, ALT, ALP, and bilirubin along with the improvement in histopathological liver sections compared to CCl_4-only treated animals. **Conclusion:** As experimentally evident, it could be concluded that this plant material could provide a hepatoprotective effect that could be attributed to its antioxidant properties.

KEY WORDS: Carbon tetrachloride hepatotoxicity, hepatoprotective, *Lawsonia inermis* leaves, liver enzymes

INTRODUCTION

Because liver plays important functions in the maintenance, performance, and regulating homeostasis of the body, maintenance of a healthy liver is essential for the overall well-being of an individual [1]. In spite of the tremendous advances in modern medicine, there were few drugs available that offer protection to the liver from damage and help to regenerate hepatic cells [2]. Therefore, it is necessary to search for new drugs for the treatment of liver diseases to supplement/replace the currently used ones. Fortunately, the plant kingdom is a valuable source of new medicinal agents, and it has been reported that approximately 25% of modern medications have been derived from plant materials [3,4]. Hepatoprotective plants contain a variety of chemical constituents such as phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids, and xanthenes [3]. However, among numerous plant materials used for liver protection, a considerable number of them lack the scientific prove for these claims [5]. Therefore, it is important to follow systematic research methodology to scientifically evaluate the plant materials that claimed to possess hepatoprotective activity.

Lawsonia inermis (Henna) is a shrub or small tree cultivated in many regions as an ornamental and commercial dye crop [6]. It is mostly found in the tropic, sub-tropic, and semi-arid zones of Africa (tropical Savannah and tropical arid zones), south Asia, and north Australia [7]. As reported by Varghese *et al*., (2010) [8], wide range of chemical constituents have been isolated from Henna which includes naphthoquinone derivatives (lawsone which is the chief ingredient and the coloring matter in the leaves), phenolic derivatives, coumarins, xanthones, tannins, flavonoids, aliphatic components, triterpenes, sterols and other chemical constituents such as glucose, gallic acid, amino acids, mannitol, trace elements and minerals.

As a medicinal plant, Henna has been used in folk remedy as astringent, hypotensive, sedative, and against a headache, jaundice, and leprosy [9]. Leaves were also used for skin diseases, venereal diseases, smallpox, and spermatorrhea. Powered seeds

were effective against dysentery and liver disorders. The bark used in a variety conditions, such as burns, jaundice, spleen enlargement, calculus, leprosy, and skin disorders. Root was considered as a potent medicine for gonorrhea, herpes infection, sore eyes, as an abortifacient, and in the treatment of some nervous disorders [10]. Because, seeds were able to alleviate liver disorders, a question was raised about the protective effects of other plants' parts.

Therefore, this work was aimed to study the hepatoprotective effect of *L. inermis* leaves' methanolic extract on carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats as an initial step that can aid further elucidation of the therapeutic potential of this plant product.

MATERIALS AND METHODS

Plants Materials

Fresh leaves of *L. inermis* were obtained from the local market, Wad Medani, Sudan, which was further identified at the herbarium of the phytochemistry and taxonomy Department, Medicinal and Aromatic Plants Institute, National Center for Research, Khartoum, Sudan, and a voucher specimen was placed for future referencing.

Extraction of Plants Materials

The leaves of *L. inermis* were washed with distilled water; shade-dried at room temperature for 72 h, then grinded into powder by glass mortar and pestle. A 300 g of dry powder were extracted by maceration using 1.5 liters of methanol (99%) as a solvent system in conical flasks for 72 h, with intermittent shaking, and then filtered under vacuum using Buchner funnel. The filtrate was allowed to evaporate at room temperature, in dark, for 7 days, and the greenish-black semisolid extract was collected and stored in an amber glass container in a refrigerator until used for biological testing.

Experimental Animals

Albino rats weighing about 130-200 g (12-15 weeks old) males and females obtained from the animal house, Faculty of Pharmacy, University of Gezira. They were housed in polyacrylic cages and acclimatized to laboratory condition for 14 days before commencement of the experiment (temperature 25 ± 2°C with dark and light cycle 14/10 h). The animals were allowed free access to diet and water. They were divided into five groups as follow:

- Group I (normal control, $n = 5$) received distilled water
- Group II (negative control, $n = 5$) received CCl_4 only
- Group III (standard control, $n = 5$) received silymarin (25 mg/kg)
- Groups IV and V (test control, $n = 10$) received *L. inermis* leaves extract 100 mg/kg and 200 mg/kg, respectively.

Water suspensions of silymarin and *L. inermis* extract were daily orally administered to the animal groups (III, IV, and V), respectively. Carbon tetrachloride (CCl_4) was administered every 72 h with intrapretonial injection in a dose of 2 ml/kg [11] to all rats except the rats of Group I.

This study was ethically approved by the Faculty of Pharmacy, University of Gezira ethical committee, and in compliance with the Faculty of Pharmacy Guiding Principles in the Use of Animals in Toxicology 2006.

Determination of Hepatoprotective Effect

Hepatoprotective effect of *L. inermis* extract on CCl_4-induced hepatotoxicity in rats were carried out for 21 days based on the methods described by Sapakal *et al.*, [12] and Azeem *et al.*, [13].

Blood samples were collected at day 7, day 14, and day 21, allowed to clot for 45 min at room temperature, and serum was separated by centrifugation at 3500 rpm for 15 min at 4°C then used for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, and total proteins levels using an auto-analyzer (Cobas Integra 400, Roche Diagnostics, Switzerland).

Histopathological Studies

Slices of liver from each animal in all groups were preserved in 10% buffered neutral formalin, the tissues were mounted in the laboratory by embedding paraffin sections, then stained with hemotoxyline and eosin and subjected to histopathological examination at the Histopathology Department - Medical Laboratory - University of Gezira.

Data Analysis

The biochemical data were statistically analyzed using paired *t*-test and expressed as mean ± standard error of mean. For comparisons with the CCl_4-only treated group (Group II), differences were considered significant if $P < 0.05$. The percentages of hepatoprotection (H) were calculated as described by Singh *et al.*, [14], using the mean value of each group by the following equation:

$$H = \left(1 - \left(\frac{\text{Group treated with test drug} - \text{Normal control group}}{\text{Group treated with } CCl_4 \text{ alone} - \text{Normal control group}}\right)\right) \times 100$$

RESULTS

The obtained biochemical data showed that administration of CCl_4 to rats significantly elevated the serum levels of ALT and AST compared to the normal group ($P < 0.01$) which indicated acute hepatocellular damage. A significant reduction ($P < 0.001$) in ALT and AST levels were recorded in Silymarin treated group (Group III) compared to CCl_4-alone treated group

(Group II) at day 7 and day 14 of the experimental course. However, at day 21, all treatment groups (Groups III, IV, and V) showed a significant reduction ($P < 0.01$) in ALT and AST levels with the greatest reduction recorded in Group V [Table 1].

The serum levels of ALT, AST, ALP, total bilirubin, and total proteins that obtained from the tested animals at day 21, along with the percentage of hepatoprotection for each biochemical parameter, showed that both plant extract and silymarin decreased the levels of liver enzymes that elevated by CCl_4 administration when compared to CCl_4-only treated group. The two different concentrations that used in this study for the plant extract showed a concentration-dependent effect [Table 2].

In the histopathological analysis, the liver section of Group I (normal control) exhibited normal hepatic cells with distinct hepatic cells, sinusoidal spaces, and central vein [Figure 1], whereas that of CCl_4-only intoxicated animals showed necrosis of hepatic architecture with vacuolization and congestion of sinusoids [Figure 2]. Treatment with silymarin, and the methanolic extract of *L. inermis* leaves, showed minimal necrosis and regeneration of hepatocytes compared to the CCl_4-only intoxicated group which provided supportive evidence for the biochemical analysis [Figures 3 and 4].

DISCUSSION

In animal model studies, induction of liver injury by CCl_4 is commonly used to evaluate the of hepatoprotective agents, because CCl_4 administration significantly elevated the serum levels of ALT, AST, ALP, and bilirubin [15] that considered as mutual signs for liver injury. CCl_4 bio-transformed by cytochrome P_{450} system in the endoplasmic reticulum to produce trichloromethyl free radical which combined with cellular lipids and proteins in the presence of oxygen form trichloromethyl peroxyl radical, and may attack lipids on the membrane

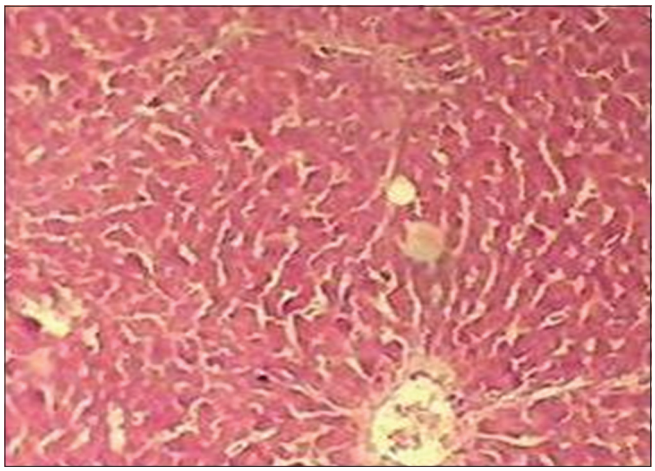

Figure 1: Histopathological liver section of normal control group (H&E, ×10)

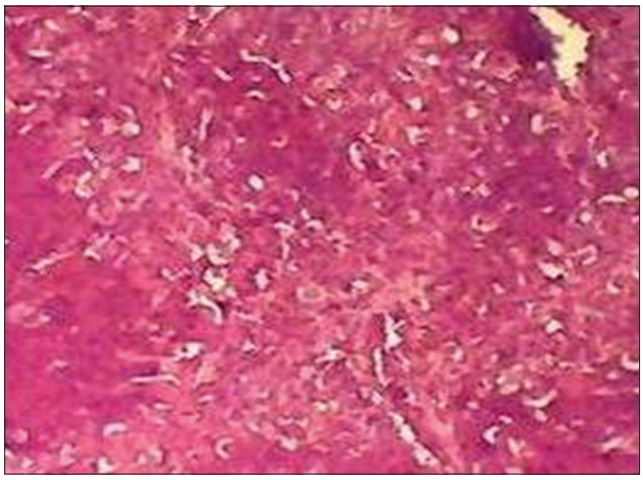

Figure 2: Histopathological liver section of carbon tetrachloride-only intoxicated group (H&E, ×10)

Table 1: Effects of methanolic extract of *L. inermis* leaves and Silymarin on serum levels of ALT and AST in CCl_4-induced hepatotoxicity

Group	Day (7)		Day (14)		Day (21)	
	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)	ALT (U/L)	AST (U/L)
Group I (normal control)	90±12	134±14	63±3	126±9	94±5	119±16
Group II (CCl_4 only)	3165±190	4483±284	1826±367	2897±81	1344±121	2259±117
Group III (CCl_4+Silymarin 25 mg/kg)	791±61	1220±115	1565±178	1985±112	214±26	629±99****
Group IV (CCl_4+*L. inermis* 100 mg/kg)	2536±253	3460±216	1430±176	2800±200	691±51	1417±45**
Group V (CCl_4+*L. inermis* 200 mg/kg)	1874±282	3076±168	955±65	1995±148	341±21	777±49****

P value: ** $P<0.05$, **** $P<0.001$ versus CCL_4 group. CCL_4: Carbon tetrachloride, *L. inermis: Lawsonia inermis,* ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

Table 2: Hepatoprotective percentage of *L. inermis* leaves methanolic extract and Silymarin on CCl_4-induced hepatotoxicity at day 21

Group	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirubin (mg/dL)	Total proteins (g/dL)
Group I (normal control)	94±5	119±16	81±5	0.19±0.02	6.8±0.12
Group II (CCl_4 only)	1344±121	2259±117	383±23	1.4±0.08	5.4±0.2
Group III (CCl_4+Silymarin 25 mg/kg)	214±26 (90%)****	629±99 (76%)***	255±15 (42%)**	0.3±0.06 (79%)***	5.7±0.2 (21%)*
Group IV (CCl_4+*L. inermis* 100 mg/kg)	691±51 (52%)***	1417±45 (39%)*	334±14 (16%)*	0.3±0.07 (79%)***	5.6±0.14 (14%)*
Group V (CCl_4+*L. inermis* 200 mg/kg)	341±21 (80.2%)****	777±49 (69.3%)***	311±12 (24%)*	0.3±0.06 (79%)***	5.6±0.12 (14%)*

P value: * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ versus CCL_4 group, CCL_4: Carbon tetrachloride, *L. inermis: Lawsonia inermis,* ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, ***: ???

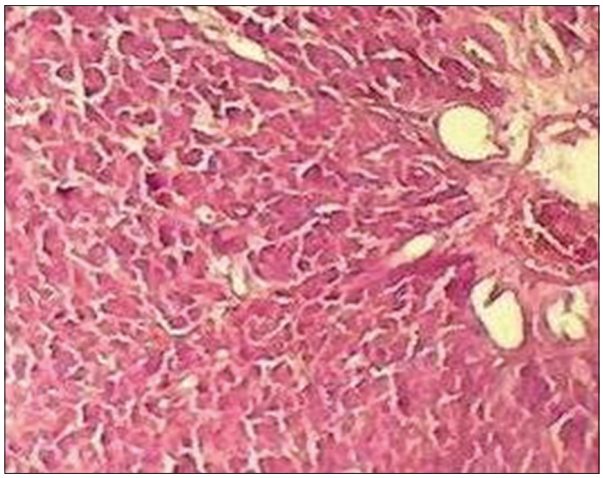

Figure 3: Histopathological liver section of Silymarin treated group (H&E, ×10)

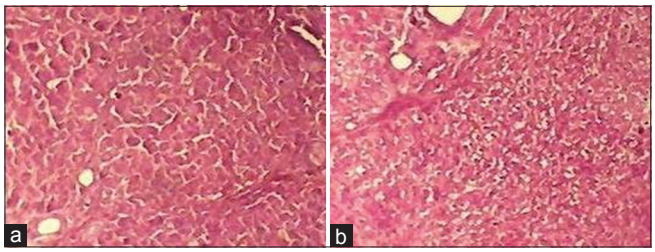

Figure 4: (a and b) Histopathological liver sections of *Lawsonia inermis* treated groups (H&E, ×10)

of endoplasmic reticulum faster than trichloromethyl free radical [16]. The intraperitoneal route was found to be the best route for CCl_4-induced hepatotoxicity in rats, and the optimum dose was found to be 2 ml/kg (dissolved in an equal volume of olive oil), and this significantly increased the level of serum enzymes, without causing the death of the animals [11].

The serum levels of aminotransferases were markedly elevated by hepatocytes damage due to toxins, drugs, or viruses [17], and estimating the activities of the serum marker enzymes could made a useful quantitative biomarker of the extent and type of hepatocellular damage, and the tendency of these enzymes to return to near normal levels considered as a clear manifestation of antihepatotoxic effects of the administered agent [18].

Hepatoprotective agents exert their action against CCl_4-induced liver injury by impairment of CCl_4-mediated lipid peroxidation, either through decreased production of free radical derivatives or due to the antioxidant activity of the protective agent itself [19]. It has been reported that *L. inermis* is a rich plant in phenolic compounds such as phenolic acids, flavonoids, tannins, lignin, and others that possess antioxidant, anticarcinogenic, and antimutagenic effects as well as antiproliferative potentials [20].

The obtained results indicated that the tested plant material could protect the liver against CCl_4 toxicity in a dose-dependent manner as evident from the protection provided when compared to the enzyme levels in CCl_4-only treated rats along with histopathological observations. It has been reported that the plant extract affords hepatoprotective activity due to its antioxidant property attributed to the flavonoids content that are effective scavengers of superoxide anions, peroxynitrite, peroxyl and hydroxyl radicals [21].

CONCLUSION

It could be concluded that biochemical and histopathological alterations induced by CCl_4 administration were improved under the effect of the plant extract. The concentration of 200 mg/kg was found to be more effective than 100 mg/kg. The present study also suggested that the traditional medicine could have the potential to be transposed successfully in the context of modern medical interventions, but additional researches are necessary to characterize the active constituents and to assess their pharmacological properties.

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Modulatory potentials of aqueous leaf and unripe fruit extracts of *Carica papaya* Linn. (*Caricaceae*) against carbon tetrachloride and acetaminophen-induced hepatotoxicity in rats

Olufunsho Awodele[1], Omoniyi Yemitan[2], Peter Uduak Ise[1], Victor Olabowale Ikumawoyi[1]

[1]Department of Pharmacology, Therapeutics and Toxicology, College of Medicine, University of Lagos, Lagos, Nigeria, [2]Department of Pharmacology, College of Medicine, Lagos State University, Lagos, Nigeria

Address for correspondence:
Olufunsho Awodele, Department of Pharmacology, Therapeutics and Toxicology, College of Medicine, University of Lagos, Lagos, Nigeria.
E-mail: awodeleo@gmail.com

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ABSTRACT

Introduction: *Carica papaya* Linn is used in a traditional medicine for hepatobiliary disorders. This study investigated the hepatomodulatory effects of aqueous extracts of *C. papaya* leaf (CPL) and unripe fruit (CPF) at doses of 100 and 300 mg/kg on carbon tetrachloride (CCl_4) and acetaminophen (ACM)-induced liver toxicities in rats. **Materials and Methods:** Rats were administered CCl_4 (3 ml/kg in olive oil, i.p.) followed by oral administration of CPL and CPF at 2, 6 and 10 h intervals. The ACM model proceeded with the same method but inclusive of animals treated with *N*-acetyl cysteine (3 ml/kg i.p). At the end of the study, serum levels of liver biomarkers and antioxidant enzymes were assessed and histology of the liver tissues determined. **Results:** There was a significant ($P < 0.05$) reduction in CCl_4 and ACM-induced increases in serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and direct bilirubin at 100 and 300 mg/kg, respectively. The levels of catalase (CAT), superoxide dismutase and reduced GSH were decreased in both models with corresponding significantly ($P < 0.05$) elevated level of malondialdehyde. However, these antioxidant enzymes were significantly ($P < 0.05$) increased in CPL and CPF-treated rats. Histopathological assessment of the liver confirmed the protective effects of CPL and CPF on CCl_4 and ACM-induced hepatic damage evidenced by the normal presentation of liver tissue architecture. **Conclusion:** These results indicate that aqueous extracts of *C. papaya* may be useful in preventing CCl_4 and ACM-induced liver toxicities.

KEY WORDS: Acetaminophen, antioxidant, carbon tetrachloride, *Carica papaya*, hepatotoxicity, histopathological assessment

INTRODUCTION

Hepato-biliary disorders especially those induced by drugs are a worldwide problem [1]. More than a thousand drugs of the modern pharmacopoeia can induce liver injury with different clinical presentations [2,3]. In the most severe cases, drug-induced liver injury (DILI) can require liver transplantation or lead to the death of the patient [4] and can lead to the withdrawal of drugs from the market or earlier during clinical trials, thus causing huge financial losses [5].

In Nigeria, there is presently very little data about the etiology of DILI, but popular over-the-counter medications (OTC) especially acetaminophen (ACM), is a frequent culprit. Thus, the study of hepatotoxicants as well as the search for hepatoprotectants is on the rise.

Carbon tetrachloride (CCL_4) has been proven to induce toxic effects in various organs on exposure to it. It is actively metabolized in the body tissues to its highly reactive halogenated metabolites (.CCl3 and.Cl) and its metabolic activation is accompanied by the release of reactive oxygen species [6]. The reactive species and the free radicals released subsequently result in the induction of lipid peroxidation leading to array of organ toxicities such as hepatotoxicity, nephrotoxicity, neurotoxicity, cardiotoxicity, and hematotoxicity [7,8].

Carica papaya Linn (Pawpaw plant) is a perennial, herbaceous plant with copious milky latex, broad leaves and grows to heights reaching 6-10 m with large succulent fruits and a complicated means of reproduction [9]. The anti-sickling [10], antihelmintic [11], anti-diabetic [12], and anti-cancer [13] properties of the Pawpaw plant have been demonstrated in previous works.

The boiled leaves of *C. papaya* are used in African traditional medicine in combination with leaves of *Azadirachta indica*, *Cymbopogon citratus*, *Psidium guajava*, and stem bark of *Alstonia boonei* boiled together and the hot infusion drunk as one wine glass full thrice daily in the treatment of malaria [11]. Its fresh leaves are also efficacious in the treatment of gonorrhea, syphilis and amoebic dysentery [11]. The milky juice of the unripe fruit is a powerful abortifacient, antihelmintic for roundworms, stomach disorders and enlargement of liver and spleen [11]. The seeds are also used as a vermifuge [11].

The present study was carried out to investigate the hepatoprotective effects of aqueous extracts of unripe fruit pulp and leaves of *C. papaya* as well as elucidate its possible mechanism of hepatoprotection in rats.

MATERIALS AND METHODS

Plant Material

Collection and identification of plant parts

Freshly harvested leaves and mature, but unripe fruits of *C. papaya* were purchased from the Mushin Market in Lagos, Nigeria. Plant identification was carried out by Oyebanji and authenticated by Odewo, both of the Department of Botany, University of Lagos, Lagos, Nigeria and stored at the University Herbarium with LUH 5748 assigned as the reference number.

Drugs and Chemicals

CCl_4 (JHD chemicals, China), KCl (GPR, Germany), phosphate buffer (BDH, Germany), Sodium carbonate buffer, hydrogen peroxide (Sigma-Aldrich, Germany), Dichromate-acetic reagent, polyethylene glycol (PEG) (Sigma-Aldrich, Germany), Normal saline (Unique pharmaceuticals, Lagos), ACM (May and Baker pharmaceuticals, Nigeria), N-acetyl cysteine (Zhangjiagang Huachang pharmaceuticals, China). All other reagents used were of analytical grade.

Extract Preparation

Leaves of *C. papaya* were air-dried at room temperature (24±2°C) for 3 weeks to remove moisture. The dried leaves were weighed, ground into powder and macerated in distilled water in the ratio: 1:2 (200 g of leaf:400 ml of water). The mixture was then boiled in a beaker placed on a hot plate for 1 h, forming a brownish solution in the process. Percentage yield was found to be 12.5%.

Yield = weight of dry crude extract obtained (g)/weight of initial dry leaves (g) × 100%

Also fresh, mature but unripe fruits of *C. papaya* were washed, peeled, cut to small pieces and soaked in 1 L of distilled water at a ratio of 1:1 (100 g fruit:100 ml water) at room temperature of 25°C, covered with a glass lid according to the method of Oduola *et al.* [10].

After 72 h, the solution was filtered using a clean piece of muslin cloth and filter paper. For the leaf and fruit extracts, the filtrate was oven-dried at 40°C in pre-weighed beakers and the concentrate refrigerated at 4°C until needed.

Experimental Animals

Sprague-Dawley rats of both sexes weighing between 150 g and 180 g were purchased from the Laboratory Animal Centre of the Lagos State University College of Medicine and were acclimatized to the laboratory conditions for 2 weeks. They were housed in clean propylene cages and fed with standard rodent pellets (Nigeria Institute of Medical Research, Yaba) and water was provided *ad libitum*. The experimental protocol adopted in this study was approved by the Experimentation Ethics Committee on Animal Use of the College of Medicine, University of Lagos, Nigeria and was in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research [14].

Experimental Design

CCl_4 hepatotoxicity

Rats (6 groups, 6 per group) were randomly allotted to several groups: Groups I and II served as controls. Group I was administered 10 ml/kg olive oil (vehicle i.p.) plus 1 ml/kg normal saline orally at 2, 6 and 10 h. Rats in Group II were administered 3 ml/kg CCl_4 (20% v/v in olive oil i.p.) plus oral administration of normal saline at 2, 6 and 10 h. Group III were administered 3 ml/kg CCl_4 (20% v/v in olive oil i.p.) plus an oral dose of 100 mg/kg *C. papaya* leaf (CPL) extract at 2, 6 and 10 h. Group IV; 3 ml/kg CCl_4 (20% v/v in olive oil i.p.) plus oral administration of 300 mg/kg CPL extract at 2, 6 and 10 h. Group V; 3 ml/kg CCl_4 (20% v/v in olive oil i.p.) plus oral administration of fruit extract at 100 mg/kg in 2, 6 and 10 h. Group VI; 3 ml/kg CCl_4 (20% v/v in olive oil i.p.) plus oral administration of the fruit extract at 300 mg/kg in 2, 6 and 10 h.

ACM-induced Hepatotoxicity

ACM was dissolved in an equal volume of PEG 400 and normal saline solution. Rats (7 groups, 6 per group) were allotted randomly as follows: Groups I and II served as control. Group I rats were administered 10 ml/kg PEG (400/NS solution, per oral) plus 1 ml/kg NS at 2, 6 and 10 h. Group II; 600 mg/kg ACM per oral, plus 3 ml/kg NS at 2, 6 and 10 h. Groups III and IV were administered 600 mg/kg ACM per oral plus oral administration

of 100 mg/kg and 300 mg/kg leaf extract of *C. papaya* at 2, 6 and 10 h, respectively. Groups V and VI were administered 600 mg/kg ACM a oral plus oral administration of 100 mg/kg and 300 mg/kg fruit extract of *C. papaya* at 2, 6 and 10 h, respectively. Rats in Group VII were administered 10 ml/kg of PEG (400/NS solution, per oral) plus 3 ml/kg N-acetyl cysteine intraperitoneally at 2, 6, and 10 h.

All animals were restricted from feeding for 24 h after oral administration of CCl_4 and ACM solution before being sacrificed for assessment of liver histology, as well as serum biochemical and oxidative stress markers.

Collection of Liver for Hepatic Tissue Antioxidant Enzyme Assay

After blood collection through the cardiac puncture, a deep longitudinal incision was made into the ventral surface of the rat abdomen. The livers were identified and carefully dissected out from each rat. The right lobe of the liver was rinsed in ice-cold 1.15% KCl solution and stored in a clean sample bottle in ice.

Homogenization of Liver Tissue

The liver was washed in an ice cold 1. 15% KCl solution blotted and weighed. It was then homogenized with 0.1 M phosphate buffer (pH 7.2) and blended with laboratory sand (acid washed sand) in a mortar. The resulting homogenate was centrifuged at 2500 rpm for 15 min and the supernatant decanted and stored at −20°C until used.

Assessment of Serum Hepatic Biomarkers

At 24 h post-CCl_4 or ACM administration, experimental rats were anaesthetized by putting each one in a glass jar containing ether-soaked cotton wool for about the 30s. Blood was obtained retroorbitally, through a heparinized capillary tube into a sample bottle. Each sample was centrifuged at 2500 G for 20 min to separate sera.

Samples were analyzed for alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DB), and uric acid level. The activity of serum ALP was determined at 405 nm using a standard method [15], serum ALT at 340 nm [16]. Uric acid levels were measured using the urease cleavage Berthlot's reaction [17]. The colometric method of Malloy and Evelyn [18], (Sulfanilic acid method) was used for the estimation of serum bilirubin.

Serum AST and ALT Determination

The method of Reitman and Frankel [16] was used. Into a test tube, 0.1 ml substrate (D, L-aspartate, 0.2 mol/L and a-ketoglutaric acid, 1.8 mmol/L in phosphate buffer, pH 7.5) solution was pipetted and placed in a 37°C water bath to warm 0.2 ml plasma was added and shaken gently to mix. Exactly 1 h after adding plasma, 1.0 ml color reagent (2, 4-dinitrophenylhydrazine approximately 20 mg/100 ml, in 10% HCl solution) was added and mixed gently and left at room temperature (18-26°C). 20 min after adding color reagent, 10 ml 0.40 N sodium hydroxide solution was added and mixed by inversion. 5 min after, absorbance was read at 340 nm using water as a reference. AST activity in Sigma-Frankel units/ml was determined from the calibration curve. The same procedure was carried out for ALT except that procedures were started 30 min after starting AST. Substrate for ALT was L-alanine (0.2 mol/L) and a-ketoglutaric acid (1.8 mmol/L) in phosphate buffer, pH 7.5.

Serum ALP Determination

ALP was determined using the colorimetric endpoint method of Tietz *et al.* [15] and adapted by Teco Diagnostic Kits. The principle is based on the fact that ALP acts on the AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chorogem, which is measured photometrically. For each sample 0.5 ml of ALP, substrate was dispensed into labeled test tubes and equilibrated to 37°C for 3 min. At timed intervals 0.5 ml of standard, control and sample were added to their respective test-tube and mixed gently deionized water was used as blank. The samples were incubated for exactly 10 min at 37°C following the same sequences, 2.5 ml of ALP color developer at timed intervals were added. The wavelength of the spectrometer was set at 590 nm.

Assessment of Serum and Tissue Antioxidant Enzymes

The reduced glutathione (GSH) content of liver tissue as non-protein sulphydryls was estimated according to the method described by Sedlak and Lindsay [19]. Superoxide dismutase (SOD) activity was determined using the methods of Sun and Zigma [20]. Serum catalase (CAT) activity was determined according to the method of Beers and Sizer as described by Kakker *et al.* [21] and Usoh *et al.* [22]. CAT activity was determined by adopting the methods of Aksenes and Njaa [23]. Malondialdehyde (MDA) production was estimated using the methods as described by Buege and Aust [24].

Determination of Liver Tissue SOD Activity

SOD activity was determined by its ability to inhibit the auto-oxidation of epinephrine by the increase in absorbance at 480 nm as described by Sun and Zigma [20]. The reaction mixture (3 ml) contained 2.95 ml 0.05 M sodium carbonate buffer pH 10.2, 0.02 ml of liver homogenates, and 0.03 ml of epinephrine in 0.005 N HCl was used to initiate the reaction. The reference curette contained 2.95 ml buffer, 0.03 ml of substrate (epinephrine), and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5 min.

Determination of Liver Tissue CAT Activity

Hepatic tissue CAT activity was determined according to Kakkar *et al.* [21] by measuring the decrease in absorbance at

240 nm due to the decomposition of H_2O_2 in UV recording spectrophotometer. The reaction mixture (3 ml) contained 0.1 ml of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM of H_2O_2 in the phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240 nm of 40.0 M 1 cm 1 according to Aebi [25] was used for calculation. The specific activity of CAT was expressed as moles of H_2O_2 reduced per minute per mg protein.

Determination of Liver Tissue Reduced GSH Activity

The reduced GSH content in the liver tissue was estimated according to the method described by Sedlak and Lindsay [19]. To the homogenate 10% TCA was added and centrifuged. About 1 ml of the supernatant was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5-dithiobisnitro benzoic acid in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Determination of Liver Tissue MDA Activity

MDA an index of lipid peroxidation was determined using the method of Buege and Aust [25]. 1 ml of supernatant was added to 2 ml of (1:1:1 ratio) tricarboxylic acid (TCA)-thiobarbituric acid (TBA)-HCl reagent (TBA 0.37%, 0.24 N HCl and 15% TCA) TCA, TBA, reagent boiled at 100°C for 15 min, and allowed to cool. The flocculent material was removed by centrifuging at 3000 rpm for 10 min. The supernatant was removed, and the absorbance was read at 532 nm against a blank. MDA was calculated using the molar extinction for MDA TBA complex of 1.56 × 105/m/cm.

Histopathological Assessment

The method of Baker and Silverston [26] was employed for the preparation of liver tissue before examination under the light microscope at a magnification of ×40.

Statistical Data Analysis

Data were calculated as mean ± standard error of mean. The mean of each treated group was compared for significance at $P < 0.05$, using analysis of variance, followed by Dunnet's *post hoc* multiple comparison tests. The Graph pad prism 6.0 was used for all statistical analysis.

RESULTS

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Serum Hepatic Enzymes in CCl_4-Induced Hepatotoxicity in Rats

The results obtained with the intraperitoneal administration of 3 ml/kg, 20% v/v CCl_4 and aqueous preparations of plant extracts CPL and CPF showed significant ($P < 0.05$) decrease in all the serum enzymes in comparison with the control (CCl_4 alone). There was a decrease in ALT (126±11.92), AST (55.6±1.7), ALP (54.0±10.80), uric acid (138.7±21.80), and DB (1.5±0.20) at 100 mg/kg CPL compared with the control, indicative of attenuation in hepatocyte destruction. However, at 300 mg/kg CPL, serum liver markers did not change significantly for ALT (128.3±1.80), AST (61.6±7.20), ALP (94.8±3.30), uric acid

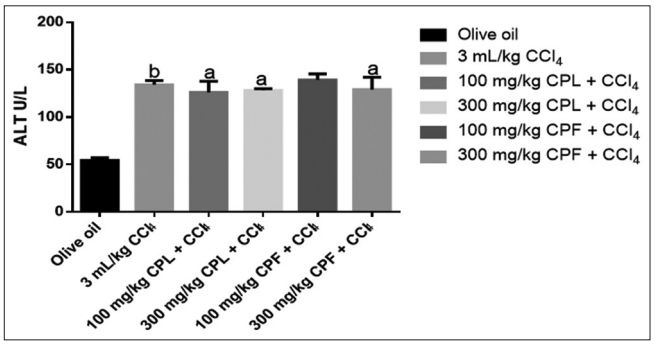

Figure 1: Effects of leaf and fruit extracts of *Carica papaya* L. on levels of alanine aminotransferase in carbon tetrachloride-induced hepatotoxicity in rats. Values are mean±SEM (n=5). [a]$p < 0.05$ vs CCl4, [b]$p < 0.05$ vs olive oil (One Way ANOVA followed by Dunnett's posthoc multiple comparison test

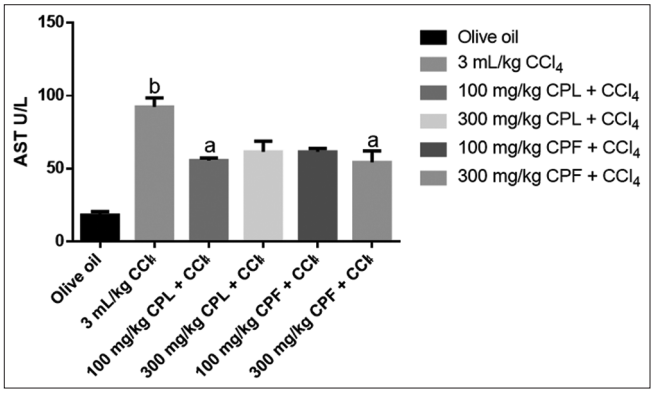

Figure 2: Effects of leaf and fruit extracts of *Carica papaya* L. on levels of aspartate aminotransferase in carbon tetrachloride-induced hepatotoxicity in rats. Values are mean±SEM (n=5). [a]$p < 0.05$ vs CCl4, [b]$p < 0.05$ vs olive oil (One Way ANOVA followed by Dunnett's posthoc multiple comparison test)

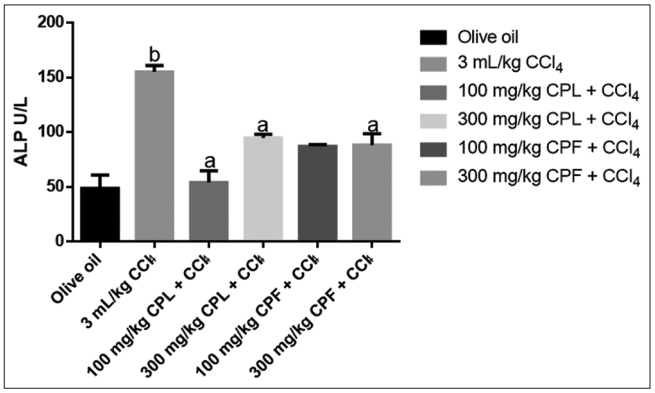

Figure 3: Effects of leaf and fruit extracts of *Carica papaya* L. on levels of alkaline phosphatase in carbon tetrachloride-induced hepatotoxicity in rats. Values are mean±SEM (n=5). [a]$p < 0.05$ vs CCl4, [b]$p < 0.05$ vs olive oil (One Way ANOVA followed by Dunnett's posthoc multiple comparison test).

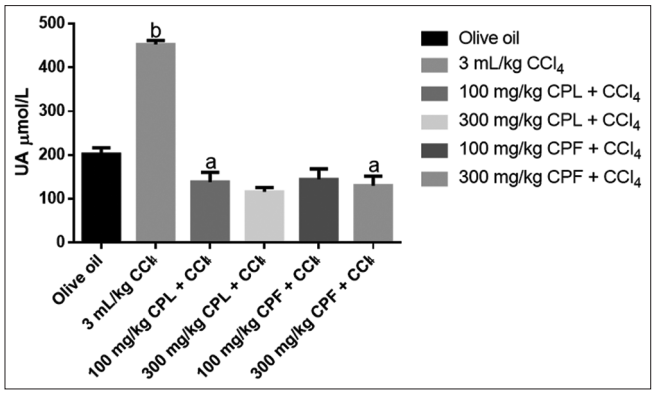

Figure 4: Effects of leaf and fruit extracts of Carica papaya L. on levels of uric acid in carbon tetrachloride-induced hepatotoxicity in rats. Values are mean±SEM (n=5). [a]$p < 0.05$ vs CCl4, [b]$p < 0.05$ vs olive oil (One Way ANOVA followed by Dunnett's posthoc multiple comparison test)

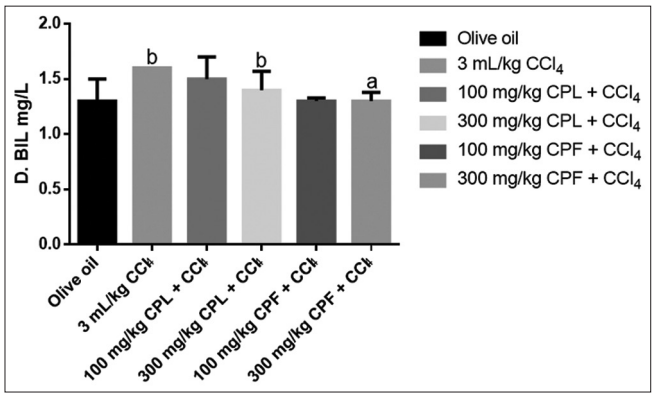

Figure 5: Effects of leaf and fruit extracts of *Carica papaya* L. on levels of direct bilirubin in carbon tetrachloride-induced hepatotoxicity in rats. Values are mean±SEM (n=5). [a]$p < 0.05$ vs CCl4, [b]$p < 0.05$ vs olive oil (One Way ANOVA followed by Dunnett's posthoc multiple comparison test)

(115.9±10.20), and DB (1.3±0.08). There was no significant difference in serum enzyme levels between aqueous leaf extract (CPL) and unripe fruit extract (CPF) of *C. papaya* at all the doses (100 mg/kg and 300 mg/kg) however, at each of the doses, serum enzymes were slightly lower with the leaf extract (CPL) for serum liver markers ALT, AST, ALP, and DB when compared with the fruit extract (CPF) [Figures 1-5].

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Serum Antioxidant Enzymes in CCl_4-Induced Hepatotoxicity in Rats

The results obtained with CCl_4 administration at 3 ml/kg intraperitoneally showed a significant ($P < 0.05$) reduction in serum levels of GSH, SOD and CAT but increased levels of MDA when compared with olive oil treated rats. At 100 mg/kg, CPL showed significant ($P < 0.05$) increase in GSH (1.52 ± 0.20) compared with CCl_4 treated (0.59 ± 0.10), SOD (3.87 ± 1.26) compared with CCl_4 treated (2.53 ± 0.40) as well as lower levels of MDA (0.04 ± 1.50) also compared with CCl_4 treated (0.07 ± 0.00) rats. However, there were no significant ($P < 0.05$) changes in serum antioxidant enzymes in rats treated with leaf

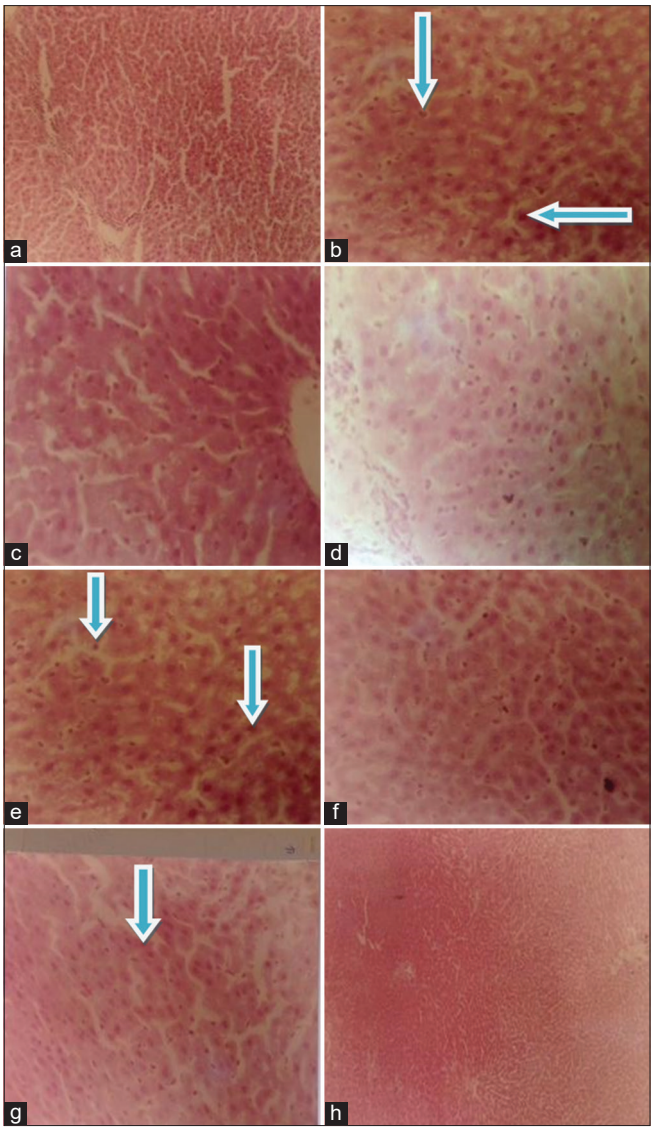

Figure 6: (a). Olive oil with no cellular abnormalities (b). CCl4 showing congested vascular channels, fatty lesions and necrotic formation (c). 300 mg/kg CPL and 20% CCl4 with no cellular abnormalities (d). 300 mg/kg CPF + CCl4 with no cellular abnormalities (e). 600 mg/kg ACM showing fatty lesions and necrotic formation (f). NAC with no cellular abnormalities (g). 600 mg/kg ACM + 300 mg/kg CPL showing mild necrosis (h). 600 mg/kg ACM + 300mg/kg CPF with no cellular abnormalities

(CPL) and fruit extracts (CPF) at 100 mg/kg and 300 mg/kg respectively compared to those that were given CCl_4 alone.

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Liver Antioxidant Enzymes in CCl_4-Induced Hepatotoxicity in Rats

The rats treated with 3 ml/kg CCl_4 in olive oil alone showed significant reductions ($P < 0.05$) in GSH, SOD, CAT but an increase in levels of MDA when compared with olive oil treated rats. At 300 mg/kg, CPL showed significant ($P < 0.05$) increase in GSH (4.7 ± 0.6) compared with CCl_4 treated (1.52 ± 0.17), SOD (3.03 ± 3.17) compared with CCl_4 treated (1.65 ± 0.33)

and CAT (16.05 ± 1.65) also compared with CCl_4 treated rats (3.40 ± 0.81). The same trend was also observed for the fruit extract CPF with GSH compared with CCl_4 treated, SOD compared with CCl_4 treated and CAT compared with CCl_4 treated rats.

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Serum Hepatic Enzymes in ACM-Induced Hepatotoxicity in Rats

The rats administered with 600 mg/kg, p.o. ACM showed significant ($P < 0.05$) increases in serum enzymes ALT, ALP, AST as well as DB when compared to rats who received no extracts. At 300 mg/kg CPL, serum liver markers were less expressed with ALT (53 ± 3.53), AST (15.25 ± 2.62), ALP (79.83 ± 26.40) and DB (1.60 ± 0.07). There was also significant ($P < 0.05$) reduction in serum enzymes in animals treated with the highest dose (300 mg/kg) of CPF with ALT (52.25 ± 5.26), AST (18.0 ± 2.34), ALP (50.41 ± 11.42) and DB (1.47 ± 0.16) compared to the control. There was no significant difference ($P > 0.05$) between rats treated with *N*-acetyl cysteine and the rats who received no extracts [Table 1].

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Serum Antioxidant Enzymes in ACM-Induced Hepatotoxicity in Rats

ACM at 600 mg/kg, caused significant ($P < 0.05$) reduction in the levels of GSH, CAT, and SOD, but increases in MDA when compared with the rats who received no extracts. However, there was no significant difference ($P > 0.05$) in serum oxidative markers in groups treated with plant extracts of *C. papaya* CPL and CPF. However, at 300 mg/kg CPL, there was a slight increase in SOD (3.33 ± 0.13) and CAT (10.78 ± 0.57). These slight elevations were, however, lower compared with the *N*-acetyl cysteine-treated group with GSH (0.52 ± 0.05), SOD (2.32 ± 0.13) and CAT (10.06 ± 0.56) [Table 2].

Effects of Leaf and Fruit Extracts of *C. papaya* L. on Liver Antioxidant Enzymes in ACM-Induced Hepatotoxicity in Rats

ACM at 600 mg/kg, p.o., significantly ($P < 0.05$) decreased organ levels of GSH, SOD, CAT as well as increased MDA in treated rats compared with the rats who received no extracts. *N*-acetylcysteine and CPL and CPF, both at 100 mg/kg and 300 mg/kg reversed this effect significantly ($P < 0.05$). There was, however, no significant difference ($P > 0.05$) between *N*-acetylcysteine-treated rats and the rats who received no extracts. However, the *N*-acetyl cysteine-treated group showed slightly higher values of both GSH (9.71 ± 1.12) and CAT (14.70 ± 4.07) compared with those treated with plant extracts CPL and CPF at all doses [Table 3].

Histology

The histology results revealed the hepatoprotective effect of the liver architecture at all doses in comparison with control (CCl_4) [Figure 6 and Table 4].

DISCUSSION

ACM, a common OTC drug, widely used for its analgesic and antipyretic purposes is a common cause of acute hepatocellular damage which could be lethal if not treated. It is one of the common causes of poisoning worldwide [27]. Oxidative necrosis

Table 1: Effects of leaf and fruit extracts of *C. papaya* on serum hepatic enzymes in ACM-induced hepatotoxicity in rats

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Uric acid (μmol/L)	DB (μmol/L)
PEG	54.25±3.09	18.00±2.61	48.54±12.37	202.00±15.08	1.37±0.22
600 mg/kg ACM	82.50±4.97[b]	31.5±1.32[b]	87.34±2.52[b]	186.20±15.31[b]	24.90±7.44[b]
100 mg/kg CPL+ACM	60±10.29	17.7±1.43	68.22±6.90	173.60±7.94	1.82±0.37
300 mg/kg CPL+ACM	53.00±3.53[a]	15.25±2.62[a]	79.83±26.46[a]	131.30±27.28[a]	1.60±0.07[a]
100 mg/kg CPF+ACM	56.00±7.14[a]	17.75±1.10[a]	58.56±4.9[a]	120.30±21.15[a]	1.25±0.20[a]
300 mg/kg CPF+ACM	52.25±5.26[a]	18.00±2.34[a]	50.41±11.42[a]	126.20±17.64[a]	1.47±0.16[a]
3 ml/kg NAC	54.00±3.87[a]	17.00±3.18[a]	67.00±12.58[a]	120.30±9.62[a]	1.47±0.22[a]

Values are expressed as mean±SEM (n=6); ([a]P<0.05) versus ACM, ([b]P<0.05) versus PEG, (one-way ANOVA followed by Dunnet's *post hoc* multiple comparison tests), SEM: Standard error of mean, PEG: Polyethylene glycol, ACM: Acetaminophen, CPL: *Carica papaya* leaf, CPF: *Carica papaya* fruit, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, DB: Direct bilirubin, *C. papaya: Carica papaya*

Table 2: Effects of leaf and fruit extracts of *C. papaya* on serum antioxidant enzymes in ACM-induced hepatotoxicity in rats

Treatment	GSH (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)
PEG	0.57±0.05	3.22±0.27	16.03±2.01	0.02±0.00
600 mg/kg ACM	0.10±0.03[b]	1.4±0.12[b]	4.2±0.00[b]	0.09±0.00[b]
100 mg/kg CPL+ACM	0.34±0.06[a]	2.38±0.23[a]	10.51±0.99[a]	0.047±0.01[a]
300 mg/kg CPL+ACM	0.10±0.07[a]	1.43±0.12[a]	4.29±0.35[a]	0.09±0.00[aa]
100 mg/kg CPF+ACM	0.28±0.08	3.33±0.13	10.78±0.57	0.03±0.00
300 mg/kg CPF+ACM	0.17±0.02[a]	1.93±0.00[a]	7.02±0.24[a]	0.02±0.00[a]
3 ml/kg NAC	0.52±0.05[a]	2.32±0.13[a]	10.06±0.56[a]	0.03±0.00[a]

Data are expressed as mean±SEM (n=6); ([a]P<0.05) versus ACM, ([b]P<0.05) versus PEG, (One Way ANOVA followed by Dunnet's post hoc multiple comparison tests), SEM: Standard error of mean, PEG: Polyethylene glycol, ACM: Acetaminophen, CPL: *Carica papaya* leaf, CPF: *Carica papaya* fruit, CAT: Catalase, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, *C. papaya: Carica papaya*

Table 3: Effects of leaf and fruit extract of *C. papaya* on liver antioxidant enzymes in ACM-induced hepatotoxicity in rats

Treatment	GSH (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (U/mg protein)
PEG	10.31±1.26	7.07±1.72	18.96±0.05	0.05±0.02
600 mg/kg ACM	1.98±0.16[b]	0.86±0.40[b]	11.50±0.85[b]	0.16±0.85[b]
100 mg/kg CPL+ACM	3.00±0.49[a]	2.28±0.61[a]	13.71±2.75[a]	0.11±0.03[a,b]
300 mg/kg+ACM	5.17±1.26[a]	3.32±0.80[a]	15.83±2.57	3.11±1.52
100 mg/kg CPF+ACM	4.14±0.73[a]	3.03±0-41[a]	11.58±2.20	0.09±0.01[a,b]
300 mg/kg CPF+ACM	4.76±0.14	3.877±0.50	19.48±2.40[b]	0.08±0.01
3 ml/kg NAC	9.71±1.12[a]	6.3±14.70[a]	14.70±4.07[a]	0.05±0.016[a]

Values are expressed as mean±SEM (n=6); ([a]P<0.05) versus ACM, ([b]P<0.05) versus PEG, one-way ANOVA followed by Dunnet's post hoc multiple comparison tests, SEM: Standard error of mean, PEG: Polyethylene glycol, ACM: Acetaminophen, CPL: *Carica papaya* leaf, CPF: *Carica papaya* fruit, CAT: Catalase, SOD: Superoxide dismutase, MDA: Malondialdehyde, GSH: Glutathione, *C. papaya: Carica papaya*

Table 4: Histological presentations of the liver

Olive oil, 10 mL/kg	CCl_4, 3 mL/kg	CPL, 300 mg/kg+ CCl_4 3 mL/kg	CPF, 300 mg/kg+ CCl_4, 3 mL/kg
Normal	Congested vascular channels, fatty lesions and necrotic formation	Mild necrosis	Normal
ACM, 600 mg/kg	NAC	ACM, 600 mg/kg+ CPL, 300 mg/kg	ACM, 600 mg/kg+ CPF, 300 mg/kg
Fatty lesions and necrotic formation	Normal	Mild necrosis	Normal

ACM: Acetaminophen, CPL: *Carica papaya* leaf, CPF: *Carica papaya* fruit, CCl_4: Carbon tetrachloride

and depletion of liver stores of GSH have been implicated in the mechanism of action of ACM poisoning [27-29].

It is important, therefore, to evaluate plant extracts that can be used for the improved treatment of hepatic failure caused by severe oxidative stress and necrosis [30,31]. A estimation of serum enzymes is a useful quantitative marker of the extent and type of hepatocellular damage. Increases in serum AST, ALT, ALP, and lactate dehydrogenase (LDH) levels have been attributed to damage of the structural integrity of the liver because these enzymes are cytoplasmic in location and are released into the circulation after autolytic breakdown or cellular necrosis. Marked release of AST, ALT, ALP, and LDH into the circulation indicates severe damage to hepatic tissue membranes during CCl_4 intoxication [32].

Tarkang *et al.* [33] reported the oral LD_{50} of *C. papaya* aqueous leaf extract as >5 g/kg. Oduola *et al.* [34] had also reported the oral LD_{50} of the unripe fruit extract of *C. papaya* to be 2.52 g/kg. This suggests that the extracts are safe on acute exposure. In this study, extracts of *C. papaya* (leaf and unripe fruits) decreased the serum level of hepatic biomarkers in rats pre-treated with CCl_4 compared to control animals administered CCl_4 alone, indicating the hepato-protective potential of the extracts. This is in agreement with the findings by Adeneye *et al.* [35].

In ACM toxicity, the metabolite NAPQI is known to deplete GSH levels, thereby inducing hepatic necrosis [36]. More so, oxidative stress and lipid peroxidation which are early events of free radical generation have been implicated in ACM metabolism [37]. Extracts of *C. papaya* (CPL and CPF) reversed the depletion of GSH, SOD, CAT induced by ACM toxicity and significantly decreased CCl_4-induced elevation in levels of MDA compared to the control. It has been stated that SOD and CAT are the most important enzymes involved in ameliorating the effects of oxygen metabolism [38,39].

Treatment with the extracts led to significant increases in GSH, SOD and CAT compared to ACM treated rats. Current evidence suggests that intracellular GSH plays an essential role in ACM detoxification and in the prevention of ACM-induced toxicity in the liver and the kidney [40]. The effect of the raised antioxidant enzymes may be responsible for the prevention of tissue damage seen with the presentation of normal architecture in the liver of ACM treated rats. Treatment with *N*-acetyl cysteine, which is the standard for treating ACM toxicity [41,42] showed no significant difference compared to the extracts of *C. papaya* indicating the potential of the extracts (CPL and CPF) in conferring hepatoprotection against ACM toxicity.

Several medicinal plants have been found to inhibit xenobiotic-induced hepatotoxicity as well as nephrotoxicity due to their potent anti-oxidant or free radical scavenging effects [43,44]. The leaves of the pawpaw plant have been shown to contain many active components such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides, and glucosinolates that can increase the total antioxidant power in blood and reduce lipid peroxidation level [45]. Furthermore, alkaloids have been reported to strongly inhibit lipid peroxidation induced in isolated tissues *in-vitro* via their antioxidant activity [46]. The leaves and unripe fruits of *C. papaya* have been found to contain saponins, tannins, flavonoids, cardiac glycosides, alkaloids and anthraquinones [47,48]. The protection offered by the extract may also be due to its high content of flavonoids, ascorbic acid and alkaloids contained in the extract which may account for the reducing and free radical scavenging properties of the extract. The liver protective activity elicited by the extract might be due to its ability to activate antioxidant enzymes suppressed by ACM-induced toxicity [49,50]. The results from this study indicate that extracts of *C. papaya* have hepatoprotective properties which could be harnessed in the treatment of the acute liver injury.

In CCl_4 toxicity, there was marked increase of hepatic enzymes; ALT, AST ALP and uric acid concentration as well as DB [51]. There is a link between hyperuricemia and some hepatic disorders. Serum uric acid level is significantly associated with

non-alcoholic fatty liver disease and elevated serum uric acid level has been shown to be an independent risk factor for non-alcoholic fatty liver disease [52-54]. Rats treated with CCl_4 showed significant increases in ALT, ALP and AST compared to control. The increased serum levels of hepatic biomarkers have been attributed to liver injury because these enzymes are found in the cytoplasmic area of the cell and are released into circulation in the event of cellular damage [55,56]. Zimmerman *et al.* [57] stated that CCl_4 induced the increase of serum ALT and AST levels as a result of cell membrane and mitochondrial damage in liver cells. Khan *et al.* [58], Wang *et al.* [59], Mehmetcik *et al.* [60], Arici and Cetin, [61] had all reported that these enzyme activities were significantly elevated after CCl_4 treatment.

Histological examination of liver tissue in rats treated with CCl_4 alone expressed hepatocellular necrosis, fatty cell accumulation, inflammatory cell infiltration and other manifestations that are consistent with acute liver damage. This is in agreement with the findings of Khan and Al-Zohairy [62]. Extracts of *C. papaya* significantly reversed the increased levels of liver biomarkers at 100 mg/kg and 300 mg/kg compared to animals treated with CCl_4 alone. This hepatoprotective effect, however, was not dose-dependent.

SOD, GSH, CAT were significantly decreased with MDA significantly increased in CCl_4 treated rats when compared with untreated animals. However, in rats treated with aqueous plant extracts of *C. papaya*, there was a significant increase in the levels of these antioxidant enzymes, especially in the liver. This effect is also in coherence with the findings by Wang *et al.* [59] and Slater [63]. The depletion of antioxidant enzymes is an indicator of liver damage in CCl_4 toxicity. Further examination of histological slides prepared from rats treated with CCl_4 confirmed hepatic damage with hepatocellular necrosis and inflammatory lesions compared to animals treated with extracts of *C. papaya* which were seen to be of remarkably less severity with regards to hepatocellular necrosis.

CONCLUSION

Decreased concentration of liver serum biomarkers such as ALP, ALT and AST and increased activities of various antioxidant enzymes indicates that the aqueous extracts of *C. papaya* Linn may be able to protect against drug and chemical induced acute liver pathologies through improvement of antioxidant indices. This may explain its use in traditional medicine for the treatment of hepato-billiary disorders. A further study is required to elucidate the fraction and constituents of the extract that produces this hepatoprotective effect.

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Original Research

Gastrokinetic activity of *Amorphophallus paeoniifolius* tuber in rats

Yadu Nandan Dey[1], Shivani Mahor[2], Dharmendra Kumar[3], Manish Wanjari[4], Sudesh Gaidhani[5], Ankush Jadhav[6]

[1]Department of Pharmacology, National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior, Madhya Pradesh, India and Centre for Advanced Research in Pharmaceutical Sciences, Shobhit University, Meerut, Uttar Pradesh, India, [2]Department of Biotechnology, Government Kamla Raja Girls Post Graduate (Autonomous) College, Gwalior, Madhya Pradesh, India, [3]Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, UCSI University, Kuala Lumpur, Malaysia and Centre for Advanced Research in Pharmaceutical Sciences, Shobhit University, Meerut, Uttar Pradesh, India, [4]Department of Pharmacology, National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior, Madhya Pradesh, India, [5]Department of Pharmacology, Central Council for Research in Ayurvedic Sciences, New Delhi, India, [6]Department of Dravyaguna Vigyan, National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior, Madhya Pradesh, India

Address for correspondence:
Manish Wanjari, National Research Institute for Ayurveda-Siddha Human Resource Development, Gwalior, Madhya Pradesh, India. E-mail: manish.nriashrd@gmail.com

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ABSTRACT

Aim: The tuber of *Amorphophallus paeoniifolius* (Family-Araceae), commonly called suran or jimikand, has medicinal and food value. It is used in ethnomedicinal practices for correction of gastrointestinal disturbances such as constipation and hemorrhoids. The present study evaluated the effect of *A. paeoniifolius* tuber on gastrointestinal motor functions. **Materials and Methods:** The tuber was collected in December 2011, and its methanolic extract was standardized with the major phenolic compound, betulinic acid, by high-performance liquid chromatography. Rats were orally administered methanolic (APME) or aqueous (APAE) extract (250 and 500 mg/kg, each) of tuber for 7 days. Metoclopramide (MET) (3 mg/kg, orally) was used a reference prokinetic drug. The gastrointestinal parameters *viz*. number of feces, wet and dry weight and moisture content of feces, gastric emptying, and intestinal transit were evaluated. The isolated tissue preparations were used to check the effect of the extracts on fundus and intestinal contractility. The glucomannan and total phenolic and flavonoid contents were determined spectrophotometrically. **Results:** The pre-treatment of extracts significantly increased the number of feces, wet and dry weight of feces, moisture content, gastric emptying, and intestinal transit. Results were comparable to MET. Further, APME and APAE showed a contraction of fundus and ileum in isolated preparations. APME and APAE were also found to have fair amount of glucomannan, total phenolics, and flavonoids. The results indicate the gastrokinetic potential of the tuber extracts. This may be attributed to the presence of glucomannan and betulinic acid present in the extracts. **Conclusion:** In conclusion, the tuber of *A. paeoniifolius* exhibits gastrokinetic activity and substantiates its traditional use in gastrointestinal motor disturbances.

KEY WORDS: Betulinic acid, gastric emptying, glucomannan, intestinal transit, jimikand

INTRODUCTION

The disorders associated with bowel movements in gastrointestinal system include constipation, diarrhea, and functional bowel disorders. These diseases are not so severe or life-threatening, however, they badly affect the quality of life [1]. There is a high prevalence of some of the main gastrointestinal disorders and diseases worldwide. According to the survey data, the leading countries with a highest prevalence rate of functional dyspepsia, functional constipation, gastroesophageal reflux disease, and irritable bowel syndrome are Argentina (43.2%), Australia (6.3-10.3%), Argentina (11.9%), and Australia (8.9%), respectively [2]. Plant-based medicines are very popular for the treatment of gastrointestinal disturbances. The Ayurveda, the Indian system of medicine also advocates the use of many plant-based medicines for such disorders. In India, the plants/herbs with medicinal values constitute an integral part of the food to derive therapeutic benefits present in them in addition to source of nutrition [3].

Amorphophallus paeoniifolius (Dennst.) Nicolson (family - Araceae) or Elephant foot yam is a crop of South East Asian origin. In India, it is commonly known as suran or jimikand. The tuber of this plant has high medicinal value and consumed by many people as food. It is an important constituent of many Ayurvedic preparations [4]. The phytoconstituents present in the corms are quercetin, rutin, sitosterol, etc. [5]. A water-soluble polysaccharide containing galactose, glucose, 4-O-acyl-methyl galacturonate, and arabinose was isolated from the aqueous extract of tuber [6]. Glucomannan was also isolated from the tuber and characterized by spectroscopy [7]. High-performance thin layer chromatography analysis showed the presence of quercetin and gallic acid in the tuber [8]. Betulinic acid is a major phytochemical present in the methanolic extract of tuber [9].

The tuber is irritant, digestive, carminative, stomachic, and appetizer and has got remarkable effects on gastrointestinal system. It corrects various abnormalities *viz*. hemorrhoids, vomiting, anorexia, dyspepsia, flatulence, colic, constipation, hepatopathy, etc. [10]. It is used in ethnomedicinal practices for treatment of piles (hemorrhoids), abdominal pain, and constipation [11,12]. It is used for treatment of other conditions such as splenopathy, arthralgia, elephantiasis, tumors, inflammations, hemorrhages, cough, helminthiasis, bronchitis, asthma, amenorrhea, dysmenorrhea, seminal weakness, fatigue, anemia, and general debility. Pharmacologically, it has been demonstrated to exhibit analgesic activity [13], CNS depressant activity [14], anti-inflammatory activity [15], cytotoxic activity [16], antibacterial activity, and antifungal activity [17] in experimental studies.

Despite the myriad of actions of tuber of *A. paeoniifolius* in gastrointestinal system and its disturbances, there are no scientific studies to delineate its influence on the gastrointestinal functions. This study investigated the effect of tuber extract on motor and contractile gastrointestinal function in normal rats.

MATERIALS AND METHODS

Drug and Chemicals

Acetylcholine (Himedia Laboratories Pvt. Ltd. Mumbai), D-mannose, 3,5-DNS (di nitro salicylic acid) and Folin and ciocalteu's reagent (Sisco Research Laboratories Pvt. Ltd., Mumbai), and tannic acid (Merck Chemicals, Mumbai) were procured from local market while quercetin was procured from Sigma-Aldrich, USA. All the reagents and chemical used were of highest purity grade.

Collection and Authentication of the Tuber

The tubers of *A. paeoniifolius* were collected from the local market of Gwalior in December 2011 and identified by Dr. N.K. Pandey, Taxonomist of the Institute. A voucher specimen No. 5-4/10-11/NRIASHRD/Tech/Survey/1611 was deposited in the herbarium of the Institute.

Preparation of Methanolic (APME) and Aqueous (APAE) Extract of *A. paeoniifolius* Tuber

The tubers were chopped into thin pieces, shade dried, and coarsely powdered. The powdered tuber was extracted with methanol in Soxhlet extractor. The marc was finally macerated with distilled water to obtain aqueous extract. The extracts were dried in a rotary evaporator and stored in a desiccator for further use. The methanolic extract of reddish brown semisolid consistency and aqueous extract of brown solid consistency were obtained with percent yield of 9.48% w/w and 6.16% w/w, respectively.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out to detect the presence and absence of various phytoconstituents such as carbohydrates, proteins, steroids, flavonoids, tannins, and other phenolic compounds, glycosides, and alkaloids in the extracts [18].

Quantitative Estimation of Phytoconstituents

The total glucomannan content was determined as described previously [19] and expressed as a gram of glucomannan per 100 gram of extract. The total phenolic content of the extracts was determined spectrometrically [20] and expressed as mg of tannic acid equivalents (TAE) per gram of extract while total flavonoid content was measured by aluminum chloride colorimetric assay [21] and expressed as mg of quercetin equivalent per gram of extract.

Standardization of APME by High-performance Liquid Chromatography (HPLC)

For the standardization, the betulinic acid, a chief constituent of tuber was estimated in the methanolic extract. The estimation of betulinic acid was carried out in APME by HPLC at Natural

Remedies Pvt. Ltd., Bangalore, Karnataka, India. The mobile phase consisted of potassium dihydrogen orthophosphate buffer (0.136 g anhydrous KH_2PO_4 in 900 ml HPLC grade water and 0.5 mL of orthophosphoric acid added to mixture and volume was made up to 1000 ml with water) and acetonitrile in the proportion of 85:15. The column and detector used were phenomenex-luna C-18 (2) of size 250 × 4.60 mm and 5 μm internal diameter and photodiode array detector, respectively. The wavelength, flow rate, and injection volume were 205 nm, 1.5 ml/min, and 20 μl, respectively. Standard of Betulinic acid (Natural Remedy, India, percent purity ≥ 95%) (0.2 mg/ml) or APME (20 mg/ml) was prepared in HPLC grade methanol, and solutions were filtered through 0.2 μ membrane filter. The chromatograms were recorded, and the mean area (n = 3) and relative standard deviation were calculated. The amount of betulinic acid was calculated by the following formula:

Amount of betulinic acid = (area of the sample/area of the standard) × (weight of the standard [mg]/standard dilution [ml]) × (sample dilution [ml]/weight of the sample [mg]) × purity of the standard (%)

Animals

Healthy adult male Wistar rats (8-10 weeks age and 220-250 g weight) were used for the study. The animals were bred and maintained at Central Animal Facility of the Institute under standard experimental conditions of temperature (25 ± 1°C), relative humidity (50 ± 5%), and 12 h light:dark cycle. They were housed in polypropylene cages in a group of 2-3 animals/cage. They were fed standard rodent chow (Ashirwad brand, Chandigarh, India) and water *ad libitum*. Experiments were performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Ministry of Environment and Forest, New Delhi after seeking approval by the Institutional Animals Ethical Committee (IAEC) in the year 2013 (Proposal No. NRIASHRD-GWL/IAEC/2013/01).

Acute Toxicity Study

Acute oral toxicity study was carried out to determine the safe dose by acute toxic class method as per Organization for Economic Cooperation and Development (OECD) 423 guidelines [22]. The overnight fasted rats (n = 3) were orally administered APME and APAE in the limit test dose of 2000 mg/kg and observed continuously for behavioral, neurological, and autonomic profiles for 2 h and after a period of 24-h, 72 h, and thereafter up to 14 days for any lethality, moribund state, or death. The limit test was repeated in another group of rats (n = 3) for confirmation and toxic class of LD_{50} determination.

Grouping and Treatments

The animals (n = 6) were divided into 6 groups. APME and APAE were suspended in 1% tween 80 for the purpose of administration.

Group I: Control, received 1% Tween 80 as vehicle (5 ml/kg/day orally).

Group II-III: Fed orally with methanolic extract of A. *paeoniifolius* at the doses of 250 and 500 mg/kg.

Groups IV-V: Fed orally with aqueous extract of A. *paeoniifolius* at the doses of 250 and 500 mg/kg.

Group VI: Fed orally with metoclopramide (MET) at a dose of 3 mg/kg.

Evaluation of Gastrointestinal Motor Functions

The treatments were given for seven consecutive days and effect on gastrointestinal motor functions was assessed on the 8th day. The following parameters were assessed.

Feces and Stool Consistency

Each animal was kept individually in metabolic cage (Orchid Scientifics, Nasik, India) for four hours every day from 10 am to 2 pm. After 4 h, the number of feces and stool consistency were recorded. The feces were collected and weighed. The wet feces were dried in hot air oven at 105°C until constant weight was acquired. The drying temperature was kept constant in all feces. The drying time taken by feces to dry was different depending on the moisture content of the feces. The dry weight of feces was measured, and the moisture content of feces was then calculated by the following formula:

$$\text{Moisture content} = \frac{\text{Wet weight of feces} - \text{Dry weight of feces}}{\text{Wet weight of feces}} \times 100$$

The average number of feces, wet and dry weight of feces, and moisture content of feces were calculated of each individual rat.

Gastric Emptying and Intestinal Transit

The gastric emptying was assessed in overnight fasted rats by measuring emptying of a non-nutrient solution as described previously [23]. Briefly, each rat received a 1.5 ml test meal consisting of 0.05% phenol red in 1.5% aqueous methylcellulose solution by intragastric route. After 30 min, rats were sacrificed by a high dose of ether. The abdomen was cut opened, and stomach was dissected out after careful ligation at the cardiac and pyloric ends and washed with normal saline. The stomach was cut into pieces and homogenized with 25 ml of 0.1 N NaOH. To this 5 ml homogenate, 0.5 ml of trichloroacetic acid (20% w/v) was added and centrifuged at 3000 rpm for 20 min. To one ml of supernatant, 4 ml of 0.5 N NaOH was added. The absorbance of the pink colored liquid was measured spectrophotometrically at 560 nm. Phenol red recovered from the stomach of rat sacrificed immediately after meal was considered as the average amount of phenol red from standard stomach. The percent gastric emptying was calculated as below.

$$\% \text{ Gastric emptying} = 1 - \frac{\text{Amount of phenol red recovered from test stomach}}{\text{Average amount of phenol red recovered from standard stomach}} \times 100$$

The distance traveled by the phenol red meal in the intestine, from the pylorus to the cecum was measured and expressed as percent intestinal transit [24].

$$\% \text{ Transit} = \frac{\text{Distance travelled by phenol red meal}}{\text{Total lenght of small intestine}} \times 100$$

Fundus and Ileum Contractility [25]

Separate group of adult Wistar albino rats were fasted overnight with free access to water, and then they were sacrificed. The stomach was dissected out and placed in Krebs solution at 37°C. The gray fundal part was separated, cut longitudinally to strips and mounted in the organ bath as per standard procedure. Various concentrations of acetylcholine (1, 2, 4, 6, 8, 16, 32, and 64 μg) from the stock solution (10 μg/ml) or APME or APAE (1, 2, 4, 8, and 16 mg) from the stock solution (10 mg/ml) were injected into the tissue bath until maximum ceiling effect was observed. The concentration-response curves of acetylcholine, APME, and APAE were plotted.

From the same group of rats, the rat ileum preparation was mounted as per standard procedure. Various concentrations of acetylcholine (1, 2, 4, 6, 8, 16, 32, and 64 μg) from the stock solution (10 μg/ml) or APME and APAE (1, 2, 4, 8, and 16 mg) from the stock solution (10 mg/ml) were injected into the tissue bath containing tyrode solution until maximum response was observed. The concentration-response curves of acetylcholine, APME, and APAE were plotted.

Statistical Analysis

The data were analyzed with one-way ANOVA followed by Tukey's multiple comparison post-hoc tests and two-way ANOVA followed by Bonferroni post-hoc tests. A statistical difference of $P < 0.05$ was considered significant in all cases.

RESULTS

Percentage Yield and Preliminary Phytochemical Screening

APME and APAE showed the presence of carbohydrates, proteins, alkaloids, flavonoids, sterols, phenolic compounds, and tannins while glycosides and saponins were found absent.

Quantitative Estimation of Phytoconstituents

The total flavonoid contents of APME and APAE were found to be 92.77 mg and 75.47 mg quercetin equivalents/g of extract, respectively, while the total phenolic contents of APME and APAE were found to be 73.1 mg and 141.5 mg TAE/g of extract, respectively. The glucomannan content of the APME and APAE was found to 1.13 and 9.043 g per 100 g of extract, respectively.

Estimation of Betulinic Acid

HPLC analysis revealed the presence of betulinic acid in APME. The peak of standard betulinic acid and APME solution are shown in Figure 1a and b, respectively. The estimated amount of betulinic acid was found to be 0.08 (% w/w) [Figure 1].

Acute Toxicity Study of APME and APAE

Acute toxicity studies revealed that both APME and APAE were safe up to a dose level of 2000 mg/kg of body weight (limit test) and LD_{50} observed was more than 2500 mg/kg. No lethality or any toxic reactions or moribund state were observed up to the end of the study period [Table 1]. Two-way ANOVA revealed that there were no significant changes observed in weekly body weights of rats treated with APME and APAE at 2000 mg/kg when compared to vehicle.

Effects on Gastrointestinal Functions

Fecal parameters

One-way ANOVA showed that APME and APAE have a significant influence on the number, wet and dry weight, and moisture content of feces. Post-hoc test indicated that APME or APAE at the dose of 250 and 500 mg/kg significantly increased the number ($P < 0.001$), wet weight ($P < 0.05$ and $P < 0.001$, wherever applicable), dry weight ($P < 0.05$ and $P < 0.01$, wherever applicable), and moisture content ($P < 0.05$ and

Table 1: Acute toxicity of APME and APAE

Observations of toxic signs in following parameters	Treatments		
	Control (vehicle)	APME 2000	APAE 2000
Skin and Fur	Normal	No change	No change
Eyes and mucus membrane	Normal	No change	No change
Respiratory system	Normal	No change	No change
Circulatory system	Normal	No change	No change
Autonomic nervous system	Normal	No change	No change
Central nervous system	Normal	No change	No change
Somatomotor activity	Normal	No change	No change
Behavioral pattern	Normal	No change	No change
Tremor	Normal	No change	No change
Convulsions	Normal	No change	No change
Salivation	Normal	No change	No change
Diarrhea	Normal	No change	No change
Lethargy	Normal	No change	No change
Sleep	Normal	No change	No change
Coma	Normal	No change	No change
Body weight (in g)			
Day 1	228.00±2.13	229.67±1.74	228.33±2.85
Day 7	229.83±2.10	231.17±2.04	230.17±2.81
Day 14	231.50±2.11	233.00±1.97	231.33±2.69

Body weights values are mean±SEM (n=6); Doses are expressed as mg/kg, SEM: Standard error of mean

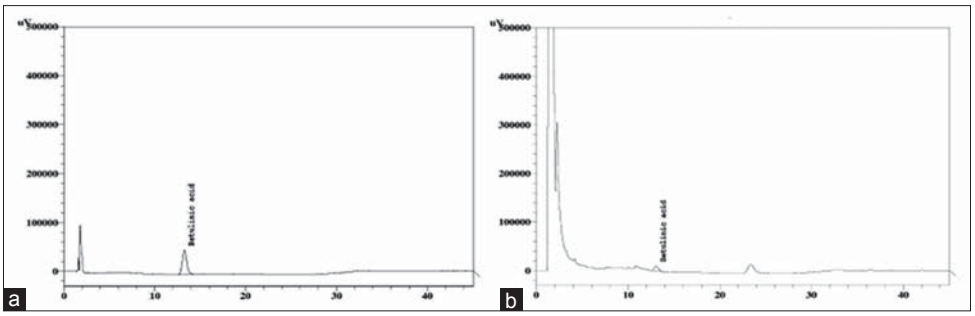

Figure 1: High-performance liquid chromatography (HPLC) fingerprint of administered methanolic, The HPLC fingerprint of (a) reference standard (retention time 13.077) and (b) methanolic extract of tuber (retention time = 13.045) at wavelength of 205 nm for detection of the marker compound, betulinic acid

$P < 0.001$, wherever applicable) of feces of rats as compared to control group [Table 2]. MET (3 mg/kg) also caused significant increase in the number ($P < 0.001$), wet weight ($P < 0.05$), dry weight ($P < 0.05$) of feces without significant change in moisture content.

Gastric Emptying and Intestinal Transit

One-way ANOVA showed that APME and APAE have a significant influence on gastric emptying and intestinal transit. Post-hoc test indicated that APME (250 and 500 mg/kg) or APAE at the dose of 500 mg/kg significantly increased the percent gastric emptying ($P < 0.01$-$P < 0.001$, respectively), whereas APME or APAE at both doses significantly ($P < 0.05$ - $P < 0.001$) increased the percent intestinal transit in rats as compared to control group [Figure 2]. MET (3 mg/kg) also showed significant increase in the gastric emptying ($P < 0.001$) and intestinal transit ($P < 0.05$). The effects of extracts were comparable to MET ($P > 0.05$).

Fundus and Ileum Contractility

The concentration-response curve of acetylcholine or APME or APAE showed concentration-dependent increase in the contractile response on fundus and ileum. The EC_{50} (effective concentration for 50% response) were calculated and shown in Table 3. The EC_{50} of acetylcholine on fundus was 4.17 μg while that of APME and APAE were 779.8 and 2144 μg, respectively. Similarly, EC_{50} of acetylcholine and APME on ileum were 8.35 and 5942 μg, respectively.

DISCUSSION

Acute toxicity study of the APME and APAE revealed that there was no visible toxicity of any nature or moribund stage, and the drug was safe on oral administration. Based on cut-off LD_{50} value of >2500 mg/kg and previous studies [8,13], the dose range of 250 and 500 mg/kg (one tenth of the approximate LD_{50}) was selected for drug administration. The increase in the number of feces as well as wet and dry weight of feces by APME or APAE treatment indicates increased bowel movement (peristalsis) and suggests that extracts have gastrokinetic effect [26]. The increased moisture content of feces due to extract treatment indicates the secretary action of extract in gastrointestinal

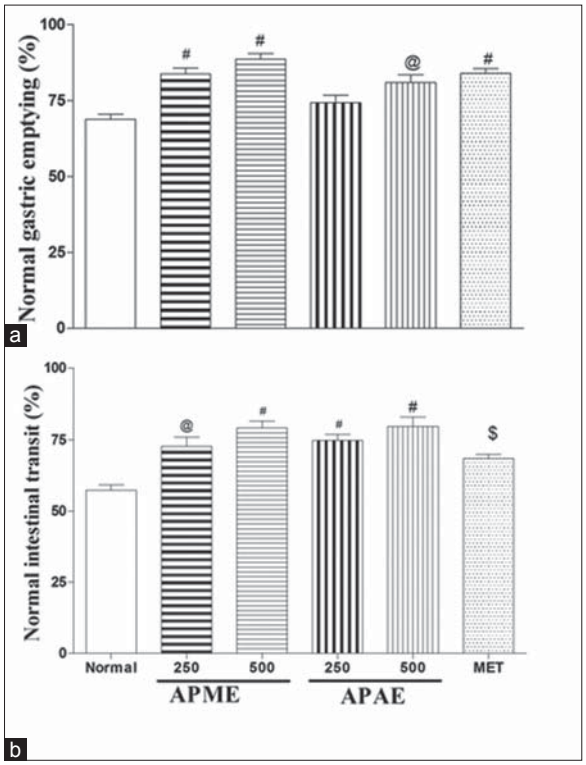

Figure 2: Effect of APME and APAE on Gastric emptying (a) and Intestinal transit (b). Doses are expressed as mg/kg, results are expressed as mean ± SEM (n=6), $^{@}P < 0.01$ and $^{*}P < 0.001$ when compared to control

Table 2: Effects of APME and APAE on fecal parameters

Treatments	Feces content (no. of feces)	Wet weight of feces (g)	Dry weight of feces (g)	Moisture content (%)
Control (vehicle)	4.16±0.54	1.78±0.16	0.91±0.09	48.55±1.85
APME 250	8.33±0.49$^{\#}$	3.37±0.14$^{\$}$	1.62±0.06$^{\$}$	51.25±1.76
APME 500	11.83±0.75$^{\#}$	5.65±0.39$^{\#}$	2.34±0.10$^{\#}$	58.27±1.99$^{\$}$
APAE 250	9.33±0.42$^{\#}$	5.39±0.38$^{\#}$	2.17±0.25$^{\#}$	59.93±2.91$^{\$}$
APAE 500	15.33±0.99$^{\#}$	7.47±0.54$^{\#}$	2.59±0.14$^{\#}$	64.94±0.94$^{\#}$
MET 3	9.83±0.40$^{\#}$	3.21±0.11$^{\$}$	1.58±0.11$^{\$}$	50.64±2.37

Doses are expressed as mg/kg, results are expressed as mean±SEM (n=6), $^{\$}P<0.05$, $^{\#}P<0.001$ when compared to control, SEM: Standard error of mean

tract, which further facilitates the enhanced peristalsis. The dietary fibers are known to affect gastrointestinal transit time

Table 3: Effect of APME and APAE on fundus and ileum contractility

Treatments	EC_{50} (μg) of fundus contractility	EC_{50} (μg) of ileum contractility
Acetylcholine	4.17±0.13	8.35±0.24
APME	779.8±116.80	5942±210.1
APAE	2144±386.6	0

Results are expressed as mean±SEM (n=6), SEM: Standard error of mean

and bulkiness of feces by increasing the water content and their bacterial degradation [27,28]. The tuber is known to contain nearly 70% of carbohydrates [29] and has high glucomannan water-soluble fiber content [7]. The phytochemical studies revealed the presence of fair amount of glucomannan in APAE and APME. Glucomannan has been reported to increase the feces volume and bulkiness of stool [30,31]. This supports and attributes the role of glucomannan in the observed increase in feces number, and weight and moisture content by APME or APAE contributing to prokinetic effect. Several dietary fibers such as *Psyllium* showed gastrokinetic action by increasing the transit, fecal wet and dry weight and moisture content [27,32], and strengthens the findings.

Further, APME and APAE both increased gastric emptying and intestinal transit of non-nutrient meal same as that of standard prokinetic drug-MET ($P > 0.05$). This further supports the gastrokinetic action of the extracts. MET exhibits prokinetic action due to its weak 5-HT3 antagonistic and 5-HT4 agonistic action [33]. Although there are no reports on the interaction of *A. paeoniifolius* with serotonergic system in gastrointestinal neurotransmission, it is possible that APME and APAE may have influence on serotonergic system in exhibiting gastrokinetic effect.

The increased peristalsis, gastric emptying, and intestinal transit suggest that the extracts have an influence on gastric motility or contractile function of the stomach/intestine. In affirmation, APME or APAE showed enhancement in gastric motility as observed by increased fundus and ileum contractility [Table 3]. However, the effect of the extracts was very less potent as indicated by higher EC_{50} values of APME and APAE compared to acetylcholine. It is very difficult to ascertain the role of exact constituent in influencing gastric motility. Previously, ferulic acid, a phenolic acid, showed stimulatory effect on the production of prostaglandins and caused increased gastric motility [26]. The HPLC analysis of APME also revealed the presence of 0.08% betulinic acid, a phenolic acid as chief constituent, in concordance to literature [9]. Though there are no direct evidences of gastrointestinal actions of betulinic acid from *A. paeoniifolius*, Bejar *et al.* [34] has demonstrated that betulinic acid isolated from *Byrsonima crassifolia* exhibited spasmogenic effect on rat fundus preparation. Thus, motility enhancing the effect of the extracts may be attributed to the presence of betulinic acid possibly through spasmogenic effect.

CONCLUSION

In conclusion, the tuber of *A. paeoniifolius* exhibited gastrokinetic action and validates its traditional use in correction of gastrointestinal disturbances. The gastrokinetic effect implicates the therapeutic potential of the plant in the correction of constipation, gastro paresis, and other functional bowel disorders.

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Original Research

ScopeMed

Drug leads agents from methanol extract of Nigerian bee (*Apis mellifera*) propolis

Bashir Lawal[1], Oluwatosin Kudirat Shittu[1], Asmau Niwoye Abubakar[1], Ibrahim Azeez Olalekan[2], Adisa Mohammed Jimoh[3], Adeniyi Kamoru Abdulazeez[4]

[1]Department of Biochemistry, Tropical Disease Research Unit, Federal University of Technology, P.M.B. 65, Minna, Nigeria, [2]Department of Forestry Technology, Federal College of Wild Life Management, New Bussa, Niger, [3]Department of Chemistry, Ibrahim Badamasi Babangida University Lapai, Niger, [4]Department of Biological Science, Applied Parsiitology and Entomology Unit, Federal University of Technology, Minna, Nigeria

Address for correspondence:
Bashir Lawal, Department of Biochemistry, Tropical Disease Research Unit, Federal University of Technology, P.M.B. 65, Minna, Nigeria.
E-mail: bashirlawal12@gmail.com

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ABSTRACT

Background: Propolis is a bee (*Apis mellifera)* product of plant origin with varied chemical composition depending on the ecology of the botanical origin. It has been reported in literature to possess various therapeutic effects both traditionally, clinical trial, and animal study. **Objectives:** In the present study bioactive principle in methanol extract of Nigerian bee (*A. mellifera)* propolis was determined by gas chromatography-mass spectrometry (GC/MS) study. **Materials and Methods:** The methanol extract of Nigerian bee (*A. mellifera*) propolis was characterized for its chemical composition by preliminary phytochemicals screening and GC/MS analysis using standard procedures and methods. **Results:** Phytochemical screening revealed the presence of flavonoids, saponins, alkaloids, tannins, cardiac glycosides, anthraquinones phlobatannins, and steroids while GC/MS chromatogram revealed nineteen peaks representing 60 different chemical compounds. The first compounds identified with less retention time (RT) (13.33s) were methyl tetradecanoate, tridecanoic acid, methyl ester, decanoic acid, methyl ester while squalene, all-trans-squalene, 2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)- and farnesol isomer a took longest RT (23.647s) to identify. Methyl 14-methylpentadecanoate, hexadecanoic acid methyl ester, methyl isoheptadecanoate, and methyl tridecanoate were the most concentrated constituent as revealed by there peak height (26.01%) while eicosanoic acid was the least concentrated (peak height 0.81%) constituent of Nigerian bee propolis. **Conclusion:** The presence of these chemical principles is an indication that methanol extract of Nigeria bee propolis, if properly screened could yield a drug of pharmaceutical importance.

KEY WORDS: Bee, gas chromatography-mass spectrometry, methanol, propolis, phytochemicals

INTRODUCTION

Over the last decades toxins and secretions from poisonous and venomous animals have been used as drugs and drug leads for treatment of numerous untreatable human ailments [1]. Leech salivary secretion exert antimicrobial agents and has been reported to be used in treatments of back pain, Snake Venom serve as anticancer, anti-diabetics and anti-hypertensive agents, secretion from cone snail *Conus magus* used in treatment of chronic pain [1], while hemolymph from African land snail has been reported for their hepatocurative effect against CCl_4 intoxicated rats [2].

Honey bees are members of genus *Apis*, and perennial insect species that can utilize nearly all habitats of the world. They have been in existence for more than 1000 decades. There are about seven species of the honey bees with a total of 44 subspecies [3]. Honeybees produce high-quality foods in the form of honey, building materials in the form of propolis, and chemical defenses inform of bee venom and propolis [4].

Propolis is a sticky honeybee resinous product produced by the honeybees to shut the cracks, and act as moisture and thermo stabilizer in the hive [5]. Bee propolis has been documented for it bacteriostatic, immunomodulatory, anti-inflammatory, anti-tumoral, anti-oxidative, hypotensive [6], hepatoprotective

and pancreato protective [7], and antibacterial properties as well as for the treatments of atherosclerosis among many other uses [8]. Recently, we also reported the hepatocurative [5], hematopoietic [9], and its toxicological effects on serum, and tissues of rats following chronic exposure [10,11].

It is worth noted that despite the high activity, propolis is very stable compounds, retaining their potency up to several years. These stable organic compounds would confer different chemical properties and could be implicated in the biological and toxicological effects of the propolis [10]. However, the chemical composition of bee propolis has been reported to be varied qualitatively and quantitatively, depending on the environmental plant ecology [12]. Since, propolis is a bee product of plant origin, thus at different geographic locations the source plants might vary with respect to the local flora [4]. Literatures have documented the chemical composition of bee propolis from a different region of the word [13-20], however, none of this study was on propolis of Nigerian origin. With the aimed of bridging the gap in knowledge, and the present study sort to evaluate Nigerian bee propolis for its chemical composition.

MATERIALS AND METHODS

Collection of Bee Propolis

Propolis material was collected from an apiary in Akure, Ondo State, Nigeria. The identity of the Propolis was authenticated by an Entomologist in the Biological Sciences Department, Federal University of Technology, Minna, Nigeria. The Propolis material was chopped into small pieces and air dried in the Shade at room temperature for 2 weeks.

Preparation of Propolis Extract

200 g of Propolis pellets were percolated in 1600 mL of absolute methanol and subsequently allowed to stand in the shade for 48 h before filtration, using filter paper (Whatman No. 1). The extract concentrate was stored in air-tight vials in the refrigerator at 4°C, until needed for bioassay.

Phytochemical Analysis

Methanol extract from Nigeria bee propolis was screened preliminary for its phytochemical contents including flavonoids, saponins, alkaloids, tannins, cardiac glycosides, anthraquinones phlobatannins, and steroids according to the methods of Sofowora [21] as described by Lawal [22].

Gas Chromatography Mass Spectrometry (GC/MS) Analysis

The GC/MS analysis of methanol extract from Nigerian bee propolis was perform using GC-MS clarus 500 per kin Elmer system comprising an AOC-20i auto sampler. "The instrument is equipped with a VF 5 ms fused silica capillary column of 30 m length, 0.25 mm diameter and 0.25 μm film thickness." The temperatures employed were; column oven temperature 80°C, injection Temp 250°C at a pressure of 108.0 kPa, with total flow and column flow of 6.20 ml/min and 1.58 ml/min, respectively. The linear velocity was 46.3 cm/s and a purge flow of 3.0 ml/min. The GC program ion source and interface temperature were 200.00°C and 250.00°C, respectively, with solvent cut time of 2.50 min. The MS program starting time was 3.00 min which ended at 30.00 min with event time of 0.50 s, scan speed of 1666 μl/s, scan range 40-800 u, and an injection volume of 1 μl of the propolis extract (split ratio 10:1). The total running time of GC-MS was 30 min. The relative percentage of the extract was expressed as percentage with peak area normalization as previously reported by Lawal *et al.* [23].

Identification of the Components

Interpretation on the mass spectrum was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The fragmentation pattern spectra of the unknown components were compared with those of known components stored in the NIST library. The relative percentage amount of each bio-component was calculated by comparing its average peak area to the total area. The name, molecular weight, and structure of the components of the test materials were ascertained.

RESULTS

Table 1 shows the phytochemical composition of methanol extract from Nigeria bee propolis. The result revealed the presence of flavonoids, saponins, alkaloids, tannins, cardiac

Table 1: Phytochemical compositions of methanol extract of Nigerian bee propolis

Phytochemicals	Inference
Alkaloids	+
Flavonoids	+
Saponins	+
Alkaloids	+
Steroids	+
Anthraquinone	+
Tannins	+
Glycosides	+
Phlobatannins	+

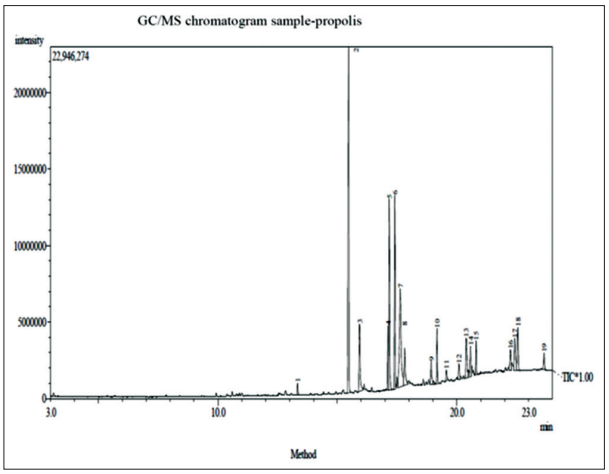

Figure 1: Gas chromatography mass spectrometry chromatogram of methanol extract of Nigerian bee (*Apis mellifera*) propolis

Table 2: Bio-active components identified in methanol extract of Nigerian bee (*Apis mellifera*) propolis using GCMS

Peak no	RT	Compound	MF	MW (g/mol)	Peak area (%)	Peak height (%)
1	13.333	Methyl tetradecanoate	$C_{15}H_{30}O_2$	242	0.63	0.89
1	13.333	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228	0.63	0.89
1	13.333	Decanoic acid, methyl ester	$C_{11}H_{22}O_2$	186	0.63	0.89
2	15.468	Methyl 14-methylpentadecanoate	$C_{17}H_{34}O_2$	270	22.39	26.01
2	15.468	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	22.39	26.01
2	15.468	Methyl isoheptadecanoate	$C_{18}H_{36}O_2$	284	22.39	26.01
2	15.468	Methyl tridecanoate	$C_{14}H_{28}O_2$	228	22.39	26.01
3	15.930	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.97	5.04
3	15.930	Octadecanoic acid	$C_{18}H_{36}O_2$	284	5.97	5.04
3	15.930	Pentadecanoic acid	$C_{15}H_{30}O_4$	242	5.97	5.04
3	15.930	Heptadecanoic acid	$C_{17}H_{34}O_2$	270	5.97	5.04
4	17.118	9,12-Octadecadienoic acid, methyl ester, (E, E)	$C_{19}H_{34}O_2$	294	3.79	4.80
4	17.118	11,14-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322	3.79	4.80
4	17.118	7,10-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	266	3.79	4.80
4	17.118	9,12-Hexadecadienoic acid, methyl ester	$C_{17}H_{30}O_2$	266	3.79	4.80
5	17.174	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	11.53	14.33
5	17.174	13,16-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294	11.53	14.33
5	17.174	6-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	11.53	14.33
5	17.174	7-Hexadecenoic acid, methyl ester	$C_{17}H_{32}O_2$	268	11.53	14.33
5	17.174	9,12-Octadecadienoyl chloride, (Z, Z)-	$C_{18}H_{31}ClO$	298	11.53	14.33
6	17.398	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	11.18	14.61
6	17.398	Hexadecanoic acid, 15-methyl-, methyl ester	$C_{18}H_{36}O_2$	284	11.18	14.61
7	17.630	Oleic Acid	$C_{18}H_{34}O_2$	282	14.70	7.38
7	17.630	9-Hexadecenoic acid	$C_{16}H_{30}O_2$	254	14.70	7.38
7	17.630	Erucic acid	$C_{22}H_{42}O_2$	338	14.70	7.38
7	17.630	Z-10-Pentadecen-1-ol	$C_{14}H_{26}O_2$	226	14.70	7.38
7	17.630	E-9-Tetradecenoic acid	$C_{14}H_{26}O_2$	226	14.70	7.38
8	17.817	Aqua Cera	$C_{22}H_{44}O_4$	372	3.68	2.81
9	18.925	2-Methylnonadecane	$C_{20}H_{42}$	282	2.09	1.72
9	18.925	n-Cetane	$C_{16}H_{34}$	226	2.09	1.72
9	18.925	2-Methyleicosane	$C_{21}H_{44}$	296	2.09	1.72
9	18.925	2,6,10,14-Tetramethylheptadecane	$C_{21}H_{44}$	296	2.09	1.72
9	18.925	7-n-Hexyleicosane	$C_{26}H_{54}$	366	2.09	1.72
10	19.168	Eicosanoic acid, methyl ester	$C_{21}H_{42}O_2$	326	3.40	4.17
10	19.168	Heneicosanoic acid, methyl ester	$C_{22}H_{44}O_2$	340	3.40	4.17
10	19.168	Docosanoic acid, methyl ester	$C_{23}H_{46}O_2$	354	3.40	4.17
10	19.168	Methyl 15-methylhexadecanoate	$C_{18}H_{36}O_2$	284	3.40	4.17
11	19.558	Eicosanoic acid	$C_{20}H_{40}O_2$	312	0.81	0.81
12	20.085	Chalcone, 2′,6′-dihydroxy-4′-methoxy	$C_{16}H_{14}O_4$	270	1.40	1.17
13	20.386	13-Tetradece-11-yn-1-ol	$C_{14}H_{24}O$	208	3.84	3.02
13	20.386	2-Octylcyclopropene-1-heptanol	$C_{18}H_{34}O$	226	3.84	3.02
13	20.386	(6Z,9Z)-6,9-Pentadecadien-1-ol	$C_{15}H_{28}O$	224	3.84	3.02
13	20.386	2-(7-Octenyl)oxirane	$C_{10}H_{18}O$	154	3.84	3.02
14	20.575	Eicosane	$C_{20}H_{42}$	282	2.10	2.27
14	20.575	Tetracosane	$C_{24}H_{50}$	338	2.10	2.27
14	20.575	Pentadecane	$C_{15}H_{32}$	212	2.10	2.27
15	20.804	Heptacosanoic acid, methyl ester	$C_{28}H_{56}O_2$	424	2.12	2.54
16	22.245	2,6,10-Trimethyltetradecane	$C_{17}H_{36}$	240	1.87	1.55
16	22.245	Octacosane	$C_{28}H_{58}$	394	1.87	1.55
16	22.245	Heptacosane	$C_{27}H_{56}$	380	1.87	1.55
16	22.245	Tetratetracontane	$C_{44}H_{90}$	618	1.87	1.55
16	22.245	11-Butyldocosane	$C_{26}H_{54}$	366	1.87	1.55
17	22.421	Phenol, 3-pentadecyl	$C_{21}H_{36}O$	304	3.76	2.41
17	22.421	Benzene, 1-methyl-4-(2-pentenyloxy)	$C_{12}H_{16}O$	176	3.76	2.41
17	22.421	Oxalic acid, dodecyl 2-methylphenyl ester	$C_{21}H_{30}O_4$	348	3.76	2.41
18	22.547	Triacontanoic acid, methyl ester	$C_{31}H_{62}O_2$	466	3.35	3.25
19	23.647	Squalene	$C_{30}H_{50}$	410	1.36	1.23
19	23.647	All-trans-Squalene	$C_{30}H_{50}$	410	1.36	1.23
19	23.647	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (E, E)-	$C_{15}H_{26}O$	222	1.36	1.23
19	23.647	Farnesol isomers	$C_{15}H_{26}O$	222	1.36	1.23

MF: Molecular formula, MW: Molecular weight, GCMS: Gas chromatography mass spectrometry

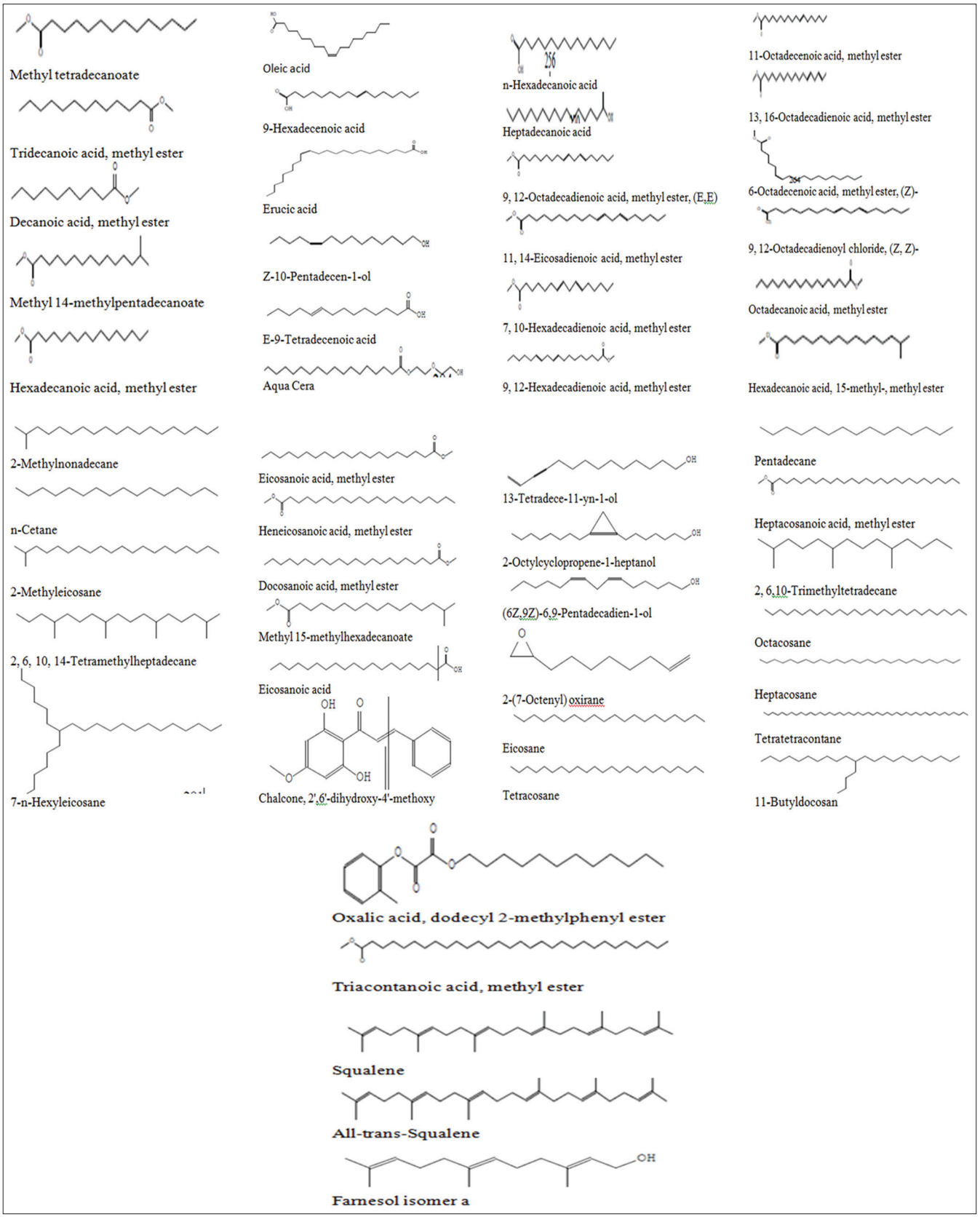

Figure 2: Structures of some chemical compounds identified from methanol extract of Nigerian bee propolis by gas chromatography mass spectrometry

glycosides, anthraquinones phlobatannins, and steroids in methanol extract of Nigeria bee propolis. The GC/MS chromatogram revealed nineteen peaks [Figure 1] representing 60 different chemical compounds. The chemical compounds with their molecular formula, molecular weight, retention time (RT), and % peak area are presented in Table 2 while the chemical structures as revealed by the GC/MS were shown in Figure 2.

DISCUSSIONS

The use of and search for, plant-derived drugs have accelerated in recent years. Biochemist, pharmacologists, botanists, microbiologists, and natural-products chemists globally are continuously in search of natural-products for bioactive phytoconstituents that could serve as a drug lead for treatment of various human ailments [24].

Phytochemicals elicit varied pharmacological and biochemical effects when administered by animals [25]. The present study revealed the present of various important phytochemicals in methanol extract from Nigerian bee propolis [Table 1]. Flavonoids are phenolic compounds with important roles in scavenging free radicals and thus play vital roles in preventing oxidative stress associated disorder. Alkaloids also possess a significant pharmacological property [26]. Tannin is non-toxic compounds that are known for there antidiarrheal, antifungal, antihemorrhoidal, and antioxidant agents [27]. Saponin has also been reported for there anti-inflammatory, cardiac depressant, and hypercholesterolemic [25].

GC/MS chromatogram of methanol extracts of Nigerian bee (*Apis mellifera)* Propolis shows nineteen peaks [Figure 1]. The gas chromatogram shows the relative concentrations of various compounds getting eluted as a function of RT. The heights of the peak indicate the relative concentrations of the components present in the sample. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds are giving rise to the appearance of peaks at different m/z ratios. These mass spectra are fingerprint of that compound which can be identified from the data library [28].

When the mass spectra of the constituents from the propolis were compared with the NIST library, a total of 60 different chemical compounds were characterized and identified. The first compounds identified with less RT (13.33s) were methyl tetradecanoate, tridecanoic acid, methyl ester, decanoic acid, methyl ester while squalene, all-trans-squalene, 2,6,10-dodecatrien-1-ol, 3,7,11-trimethyl-, (E,E)- and Farnesol isomer a [Figure 2] took longest RT (23.647s) to identify.

Methyl 14-methylpentadecanoate, hexadecanoic acid methyl ester, methyl isoheptadecanoate, and methyl tridecanoate were the most concentrated constituent as revealed by there peak height (26.01%) while eicosanoic acid was the least concentrated (peak height 0.81%) constituent of Nigerian bee propolis. The presence of these chemical principles is an indication that in methanol extract of Nigeria bee propolis if properly screened could yield a drug of pharmaceutical importance.

CONCLUSIONS

In the present study 60 compounds have been identified. The presence of various bioactive principles in Nigerian Bee propolis extract is an indication that Nigerian propolis extract, if properly screened could yield a drug of pharmacological significance. However, the isolation of individual constituents and subjecting it to biological activity will be of medical significant.

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Original Research

ScopeMed

Ethnomedicinal studies on plants used by Yanadi tribe of Chandragiri reserve forest area, Chittoor District, Andhra Pradesh, India

Nataru Savithramma, Pulicherla Yugandhar, Koya Siva Prasad, Sade Ankanna, Kummara Madhava Chetty

Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India

Address for correspondence:
Pulicherla Yugandhar, Department of Botany, Sri Venkateswara University, Tirupati - 517 502, Andhra Pradesh, India.
E-mail: yugandharbotany@gmail.com

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ABSTRACT

Aim: Ethnomedicinal studies on medicinal plants used by Yanadi tribe of Chandragiri reserve forest area are documented during the period of 2014-2015. The study is mainly focused on medicinal importance of plants used by Yanadi tribe to treat various ailments. **Materials and Methods:** The information collected on treated ailments, part used, preparation, combination, and addition of ingredients to prepare herbal medicines with the help of standard questionnaire. **Results:** During the study, 53 types of ailments were treated using 48 medicinal plants belongs to 26 families were documented. Among the medicinal plants, shrubs (15) were most using life form of plants for the preparation of herbal medicines. Leaf part (40%), paste form (33%), and oral administration (63%) of herbal medicines were most preferable. The documented ethnomedicinal importance of this tribe was cross-checked with Dr. Duke's Phytochemical and Ethnobotanical database shows most of the plants were correlated with this database. **Conclusion:** There is no record of traditional medicinal knowledge of these villages so far, hence the present study is aimed to document the information on medicinal plants used by Yanadi tribe in Chandragiri reserve forest area. The correlation of ethnomedicinal uses with Dr. Duke's Phytochemical and Ethnobotanical database clearly indicates the high medicinal significance of claimed data of this Yanadi tribe.

KEY WORDS: Ethnomedicinal studies, Yanadi tribe, Chandragiri reserve forest, Dr. Dukes ethnobotanical database

INTRODUCTION

Since time immemorial herbal systems of medicines are the major curatives in traditional system of medicine have been used in ancient medicinal practices. The importance of the traditional herbal medicinal system has gained vital importance in developed and developing countries [1]. These practices are continuing until today because of its biomedical benefits as well as its cultural belief in many parts of the world [2]. According to the World Health Organization about 80% of people in developing countries are still depending on traditional medicine and currently the demand is increasing eventually [3]. Medicinal plants are an important source of bioactive compounds and 25% of pharmaceutical prescriptions in the United States contain at least one plant-derived ingredient [4].

India is an oldest, the richest and most diverse cultural traditions associated with the use of medicinal plants in the form of traditional systems of medicine including Ayurveda, Homeopathy, Siddha and Unani [5]. India is a botanical garden of the world and a goldmine of well recorded and traditionally well-practiced knowledge of herbal medicine [6]. More than 6000 plants in India are use in traditional folk and herbal medicine representing about 75% of the medical needs of third world countries [7]. Indians rely chiefly in these systems of medicine and had been practiced for 5000 years. It is officially recognized that 2500 plant species have medicinal value while over 6000 plants are estimated to be explored in traditional, folk and herbal medicine. Presently, this plant based traditional medicinal systems continue to provide the primary health care to more than three-quarters of the world population [8].

Ethnobotany is the study of the interaction between plants and people with a particular emphasis on traditional tribal cultures [9]. Which play an important role on a collection of medicinal use of plants, based on the knowledge on plants by the local people and their usefulness by a particular ethnic group and information concerning particular plant varies from one ethnic group to another [10]. The use of medicinal herbs is still a tradition adopted by ethnic communities who are living in undulating plains and at the foothills of dense forest [11]. These types of ethnomedicinal studies play an important role for the conservation and documentation of sustainable use and importance of medicinal knowledge of

particular area/civilization [12]. Previously, ethnomedicinal studies or documentation of traditional knowledge of tribal people on medicinal plants from surrounding areas of Chittoor District was carried out by a number of ethnobotanists [13-18]. However, omitted the Chandragiri and Gopalapuram villages of Chandragiri reserve forest area. Because of its high altitudinal geography, strict tribal beliefs and lack of proper transport. In these villages, medicinal knowledge on herbal treatments is passed from generation to generation from their ancestors. Nowadays lack of interest among younger generations to carry out the same is due to attraction towards modern medicine. These two villages have a high abundance of flora and disappearance traditional healers dwindling rapidly. This is the reason behinds and the right time to document at least the remaining knowledge of this tribe. Hence, the present objective of this study is to document the medicinal knowledge of Yanadi tribe, explore and support their medicinal significance to compare their claims with Dr. Duke's phytochemical and ethnobotanical database.

MATERIALS AND METHODS

Study Area

The Chadragiri and Gopalapuram village forest areas are belonging to Chadragiri reserve forest area of Chandragiri mandal, Chittoor District, Andhra Pradesh, India with the geographical coordination's like 13° 33' 37.62" N, 79° 20' 51.87" E, 975 feet. elevation from sea level, having 31 ± 2°C day temperatures with 900 mm of average annual rainfall [Figure 1]. The protected area is proclaimed national land with distinct boundaries they are managed under the authority of Vana Samrakshana Samithi. This reserve forest area comes under the vegetation of dry deciduous forest having timber yielding, thorny, bushy trees along with herbaceous flora, covers 202 acres of land comes under Seshachalam hill region of Eastern Ghats.

Data Collection

To document the ethnomedicinal knowledge, the inhabitants belongs to the Yanadi tribe of Chandragiri reserve forest area is selected for documentation of their traditional knowledge on medicinal plants during the months of July to February of 2014-2015 period. The information obtained from traditional herbal healers like Muniah (60 Y), Subramanyam (44 Y), Nagaraju (56 Y) and Rajendra (40 Y), who are the people practicing traditional herbal treatments is interviewed and short discussion was made in their dialect. Chandragiri and Gopalapuram are medium sized tribal villages in the Chandragiri reserve forest area with the inhabitants of nearly 70 families and have a population of 190 people, of which 55 males, 69 females and 66 children's are residing. The families of these tribal peoples lived in thatched houses, thatched huts and as well as roofed houses. The tribal people in these villages depend mainly on agriculture, dairy and get wage from surrounding villages for doing labor works. A very little quantum of people practicing herbal treatments to cure their daily ailments mainly the knowledge gets from their ancestors. A structured questionnaire was used to elicit information from them and methodology used based on the methods available in the literature [19]. During the study the information collected with the help of structured questionnaire with main headings like details of tribe, name of the disease, number of diseases cured, data on the plant, description of the drug, therapeutic indications, and reasons of the plant for considering as medicine were recorded along with many more sub headings [Figure 2].

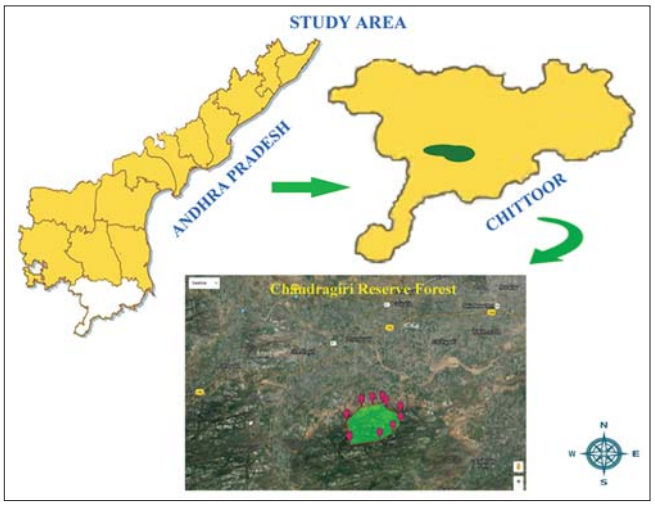

Figure 1: Study area

PROFORMA FOR COLLECTING FIELD DATA ON MEDICINAL PLANTS

I. Tribe: Name of the TMP
Gender: Male/Female Age: below 15/15-40/ 40 above
Experience: below 5/15-10/10 above Locality: Altitude:
Knowledge gained from: Knowledge transferred to:
Occupation:
II. Name of the Diseases:
III. Number of diseases cured:
IV. Data on the plant:
a) Scientific Name: b) Vernacular Name (s) (Specify the dialect):
c) Family: d) Habitat: H/S/C/T
e) In case of Tree Species: Height and Girth and Bark nature f) flower color:
g) Fruit characteristics: h) Small: i) Latex Present: Yes/No
j) Collection and identified: k) Photograph: l) availability:
V. Description of the drug:
a) Time of the collection: Morning/Afternoon/Evening/Night
b) Method of preparation of the drug: (1) Natural form (2) Crushed (3) Juice
(4) Decoction (5) Poultice (6) Soft paste (7) Solid preparation (8) Powder
i) Internal application (Chewing, Ingestion, Inhalation, Ticking)
ii) External application (Lotion, Bath Ointment, Poultice)
c) Ingredients used: single/mixed
d) Mode of administration:
e) Preservation of the drug: Y/N (Duration----------)
f) Plant part used as Medicine: (i) Root (ii) Stem (iii) Leaf
(iv) Flower (v) Fruit (vi) Seed (vii) Root bark (viii) Stem bark
(ix) Latex (x) Gum.
Percentage of the plant parts used for the preparation of drugs for 100 gr
V. Therapeutic indications:
a) Dosage b) Person

Content	Duration			Child	Adult	Old
	day	Month	Year			

c) Diet restrictions: Y/N
d) Patient Treatment With in the Tribal /other than Tribal
e) Side effects: Y/N/Unknown
VI. Reason of the plant for considering as medicine
a). Magico – religious belief b) traditional c) personal experience of healers
d) Strong belief on herbal drug e) Tales f) proverbs
g) Satisfaction level on particular drug: satisfied/ partially satisfied/ not satisfied
h) No. of persons treated:
i) No. of persons cured:
j) Other information:

Figure 2: Proforma for collecting field data on medicinal plants

Identification of Plants

The collected plant species and claimed medicinal values of Yanadi tribe is cross checked with Gamble volumes [20], local floras as well as herbaria deposited in Dept. of Botany, Sri Venkateswara University, Tirupati. The information collected from healers is documented on data sheets and herbariums were prepared with a voucher specimen number, deposited in Sri Venkateswara University, Tirupati. The medicinal values claimed by these healers were cross checked with ethnomedicinal data on medicinal plants from Dr. Duke's Phytochemical and Ethnobotanical database [21].

RESULTS

The study revealed that the Yanadi tribal practitioners were using 48 medicinal plants belonging to 26 families to treat 53 types of ailments [Table 1 and Figure 3]. The medicinal data collected and a brief discussion was made in their local language. The herbalists prepare most of their medicines in fresh

Table 1: Enumeration of ethnomedicinal data from Yandi tribe of Chandragiri reserve forest area

Scientific, vernacular name and voucher specimen no.	Family	Life from and part used	Mode of preparation and administration	Uses
Abrus precatorius L. (Guravindha) NP 117	Fabaceae	Climber (S)	Powder (O)	1-2 spoonful of powder with glass of hot water taken orally for 2 weeks to get relief from intestinal ulcers
Abutilon indicum (L.) Sweet. (Thutturubenda) NP 121	Malvaceae	Herb (R)	Powder (O)	Spoonful of powder taken orally once per day to continue up to 1 month to act as energy stimulant
Andrographis paniculata (Burm. F.) (Nelavemu) NP 111	Acanthaceae	Herb (W)	Juice (O)	10-20 ml of juice taken two times per day, continue to 3 days for diabetes, malaria and fever
Andrographis serpyllifolia (Rottl. Ex Vahl) (Pamu nelavemu) NP 127	Acanthaceae	Climber (RT)	Juice (O)	Spoonful juice with admixture of jaggery taken orally at early mornings up to 3 days to cure stomachache
Asparagus racemosus Willd. (Pilli teegalu) NP 149	Liliaceae	Shrub (RT)	Powder (O)	A pinch of powder with sugar taken once per day and continue up to 3 months to delay aging
Bauhinia racemosa Lam. (Arichettu) NP 154	Caesalpiniaceae	Tree (R)	Capsule (O)	Oral administration of two capsules per day, continue up to 3 days at the time of menstrual cycle to cure menstrual pains
Caralluma attenuata (Wt.) Grav. and Mayur. (Sanna Kundetikommulu) NP 181	Asclepiadaceae	Herb (S)	Natural form (O)	Consumption of handful of stem cuttings to enhance the appetite
Cassia auriculata L. (Thangedu) NP 152	Caesalpiniaceae	Shrub (F)	Paste (T)	Handful of flowers with pinch of turmeric powder, ground to prepare paste and applied externally 3 days to cure pimples and skin disorders
Cassia hirsuta L. (Pydee tanghadu) NP 167	Caesalpiniaceae	Shrub (L)	Paste (T)	Topical poultice of paste form of leaf act as bone strengthener
Cassytha filiformis L. (Seethamma savaralu) NP 169	Lauraceae	Climber (R)	Powder (O)	Daily intake of a pinch of powder with glass of hot water/milk to act as memory booster and reduce worm infestations
Ceropegia juncea Roxb. (Bellagadda) NP 183	Asclepiadaceae	Climber (S)	Crushed form (O)	Daily intake of spoonful of crushed from of leaf acts as an alterative
Cissus quadrangularis L. (Nalleru) NP 145	Vitaceae	Climber (R)	Powder (O)	Oral administration of 1-2 spoons of root powder with the admixtures of turmeric powder and gingelly oil once per day continue up to 7 days to cure jaundice and enhance the appetite
Clerodendrum phlomidis L.f (Takkili) NP 142	Verbenaceae	Shrub (L)	Powder (O)	Oral administration of 1-2 spoons of leaf powder with glass of hot water two times per day up to 7 days to cure psoriasis and insect bites
Croton bonplandianum Baill. (Gali vana mokka) NP 129	Euphorbiaceae	Herb (Fr)	Paste (O)	Licking of 50-100 g of fruit paste with admixture of jaggery twice per day up to 1 month to cure nervous disorders and mental illness Precaution: Avoid intake of curd at the time of treatment to mental illness
Datura stramonium L. (Ummetta) NP 120	Solanaceae	Herb (Fr)	Paste (T)	External application of paste form of fruit to reduce foot palm and rheumatic pains
Decalepis hamiltonii Wt. and Arn. (Maredu kommulu) NP 161	Asclepiadaceae	Liana (RT)	Powder (O)	1-2 spoons of powder taken orally thrice per day up to 7 days to improve muscle contraction, delay ageing and for scorpion stinging/snake bites
Dichrostachys cinera (L.) (Veluthuru chettu) NP 174	Mimosaceae	Tree (SB)	Juice (O)	Oral administration of diluted 10-20 ml of juice in glass of hot water given once per day to cure indigestion in children
Digera arvensis Forsk. (Chenchellaku) NP 187	Amaranthaceae	Herb (L)	Paste (O)	Licking of paste form of leaf with admixture of jaggery to cure scorpion stinging and reduce digestive problems
Diplocyclos palmatus (L.) Jeffrey (Linga donda) NP 221	Cucurbitaceae	Climber (L)	Capsule (O)	Daily intake of one capsule per day up to exoneration of ailments like asthma and gastric ulcers
Dodonaea viscosa (L.) Jacq. (Banderu) NP 247	Sapindaceae	Shrub (L)	Capsule (O)	100 g of tender leaves with the admixtures of cup of curd ground to made capsules and intake two per day with glass of hot milk to cure leucorrhea and increases bone strength
Euphorbia antiquorum L. (Bontha jamudu) NP 257	Euphorbiaceae	Shrub (F)	Latex (T)	External application of 10 ml of latex with pinch of turmeric powder twice per day continued up to 1-2 weeks for removal of warts

(*Contd...*)

Table 1: (Continued)

Scientific, vernacular name and voucher specimen no.	Family	Life from and part used	Mode of preparation and administration	Uses
Flacourtia indica (Burm. f.) Merr. (Pulleruka) NP 222	Flacourtiaceae	Shrub (W)	Powder (O)	Daily licking of 2 spoons of powder with one spoon of honey acts as bone strengthener and enhance the appetite
Glycyrrhiza glabra L. (Atimaduram) NP 194	Fabaceae	Herb (R)	Powder (O)	Daily intake of a spoonful of powder with glass of hot water to control diabetes and up to 2 weeks to reduce intestinal disorders like stomachache and indigestion
Gmelina asiatica L. (Adavi gummadi) NP 172	Verbenaceae	Shrub (Fr)	Natural form (T)	Topical rubbing of fruit once per day up to 3-5 days to cure dandruff and reduce rheumatic pains
Gynandropsis pentaphylla DC. (Vominta) NP 126	Capparidaceae	Herb (L)	Fumes (I)	Daily twice inhalation of leaf fumes up to 3-4 days to cure a migraine headache
Hemidesmus indicus (L.) R. Br. (Sugandapala) NP 131	Asclepiadaceae	Climber (RT)	Powder (O)	Daily intake of 1-2 spoonful of powder with glass of water up to 1-2 months to acts as cooling agent, controls over sweating and acts as energy stimulant
Hugonia mystax L. (Kakibeera) NP 136	Linaceae	Shrub (L)	Juice (O)	Oral administration of 4-6 drops of juice up to 7-15 days to cure mental disorders and scorpion stinging/snake bites
Indigofera aspalathoides Vahl. Ex DC. (Nela vempali) NP 197	Fabaceae	Shrub (W)	Powder (O)	Daily intake of 1-2 spoonful of powder to reduce nerve disorders and brushing of mouth up to 3 days to cure toothache
Jatropha gossypifolia L. (Seemanepalamu) NP 248	Euphorbiaceae	Shrub (RT)	Capsule (O)	Oral intake of 2 capsules per day up to 2-3 months to relief from skin cancers
Justicia tranquebariensis L.f. (Pindikonda) NP 175	Acanthaceae	Shrub (L)	Paste (T)	External application of leaf paste with admixture of turmeric powder to get relief from rat bite and skin diseases
Lawsonia inermis L. (Gorinta) NP 173	Lythraceae	Tree (L)	Paste (T)	External application of paste and take head bath twice per week to increase hair growth
Maerua oblongifolia (Forsk.) (Bhoochakra gadda) NP 186	Capparidaceae	Climber (RT)	Natural form (O)	Daily intake of 100-150 g of root tuber with jaggery to acts as alterative and energy stimulant
Malvastrum coromandelianum (L.) NP 125	Malvaceae	Shrub (L)	Paste (T)	100 g of leaves with 10-15 g of turmeric power is ground to prepare paste is applied externally for 3-4 days to cure any type of skin diseases
Melhania incana Heyne. (Choklamaram) NP 199	Sterculiaceae	Herb (L)	Paste (T)	External application of leaf paste for 3-4 days to cure boils and burns
Martynia annua L. (Telikondikaya) NP 135	Martyniaceae	Herb (L)	Crushed from (T)	External application/poultice of crushed form of leaf with admixture of pinch of lime stone to cure rheumatic pains, scorpion stinging and skin diseases
Pachygone ovata Miers. (Pedda dhusara teega) NP 182	Menispermaceae	Climber (L)	Paste (T)	External application of leaf paste for 3-5 days to cure cuts and boils
Pandanus fascicularis Lam. (Mogali) NP 246	Pandanaceae	Shrub (R)	Juice (O)	Daily intake of 2-3 drops of leaf juice to control diabetes, blood pressure and acts as astringent
Pithecellobium dulce (Roxb.) Benth. (Seemachintha) NP 202	Mimosaceae	Tree (L)	Paste (O)	Daily licking of paste form of leaf with admixtures of honey and turmeric powder up to 4-7 days to cure fever and the paste prepared with admixtures of 5 year old children urine poultice to stomach to cure nerve disorders in the case of postpartum mothers
Polygala arvensis Willd. NP 128	Polygalaceae	Herb (L)	Paste (T)	External application of paste form of leaf with admixtures of lime stone and turmeric powder for 3-5 days to cure pimples and skin diseases
Sansevieria roxburghiana Schult. and Schult.f. (Jaga) NP 143	Vitaceae	Herb (L)	Juice (T)	Oral intake of 3-5 drops of leaf juice to reduce cold, cough and pouring into ear to cure earache
Scutia myrtina (Burm.f.) Kurz. (Budidhapallu) NP 184	Rhamnaceae	Shrub (R)	Paste (T)	Licking of root paste with admixture of gingelly oil thrice per day up to 3 days to cure scorpion stinging/snake bites
Sida acuta Burm.f. (Medabirusaku) NP 245	Malvaceae	Herb (L)	Paste (T)	External application of leaf paste prepared with the help of castor oil for 7-10 days cure psoriasis and rheumatic pains
Strychnos nux-vomica L. (Mushti) NP 165	Strychnaceae	Tree (S)	Paste (T)	Oral intake of paste form of stem thrice per day up to 3 days to cure scorpion stinging/snake bites
Syzygium cumini (L.) Skeels. (Nerudu) NP 132	Myrtaceae	Tree (SB)	Decoction (O)	Oral intake of 10-15 ml of decoction twice per day up to 3 days to cure diarrhea and gastric ulcers
Tragia involucrata L. (Nosintaku) NP 178	Euphorbiaceae	Climber (L)	Capsule (O)	Oral intake of 2 capsules per day up to 3 days to get relief from scorpion stinging/snake bites and continued up to 5-6 days with a glass of milk and honey acts as aphrodisiac
Tribulus subramanyamii Singh *et al.* (*Peddanugu palleru*) NP 214	Zygophyllaceae	Herb (Fr)	Powder (O)	Daily intake of pinch of powder with glass of hot water up to 10-15 days to reduce bladder stones
Wattakaka volubilis (L.f.) Stap f. (Kalisaku) NP 217	Asclepiadaceae	Climber (L)	Paste (T)	100 g of leaves with 10 g of camphor, 5 g of turmeric powder and ground to prepare paste, rubbed thrice per day up to 1 week to reduce rheumatic pains
Ziziphus xylopyrus (Retz.) Willd. (Gotti) NP 254	Rhamnaceae	Tree (R)	Decoction (O)	Oral intake of 10-15 ml of root decoction prepared with admixtures of *Curcuma aromatica* tuber and *Piper longum* fruit powder for 3 days for snake bite Precaution: Avoid head bath and taken curd meals at the time of treatment

L: Leaf, R: Root, RT: Root Tuber, S: Stem bark, W: Whole plant, F: Flower, Fr: Fruit, T: Topical, O: Oral, I: Inhalation

Figure 3: List of important medicinal plants used by Yanadi tribe of Chandragiri reserve forest area

form like juice, paste and some of the medicines were stored as capsules, decoctions, dried root bulbs, seeds and powders. Among the documented data most of the medicinal plants materialized from Asclepiadaceae by 05 species followed by Euphorbiaceae by 04 species, Acanthaceae, Caesalpiniaceae, Fabaceae, Malvaceae by 03 species and finally Capparidaceae, Mimosaceae, Rhamnaceae, Verbenaceae, Vitaceae by 02 species. While rest of the families represented only by 01 species each. The collected materials from medicinal plants, most of the herbal medications prepared from shrubs (15) followed by herbs (14), climbers (11), trees (07), and lianas (01) [Figure 4]. These medicines were prepared mostly from leaf (40%) part of the plant followed by root (17%), root tuber (12%), stem bark (13%), flower (8%), whole plant (6%), and fruit (4%) [Figure 5]. They commonly prepared herbal medicines in the form of a paste (33%) followed by powder (25%), juice (15%), capsule (10%), natural form (6%), crushed form (4%), decoction (4%), fumes (2%), and latex (2%) [Figure 6]. Tribal people of the Chandragiri reserve forest area preferred the administration of their herbal medicines through oral (58%) followed by topical (40%) and inhalation (02%) through the nostrils [Figure 7]. This documented ethnomedicinal data were cross-checked with Dr. Duke's phytochemical and ethnobotanical database, most of the plants, i.e., 30 are correlated out of 48 plants at least by one ethnomedicinal use.

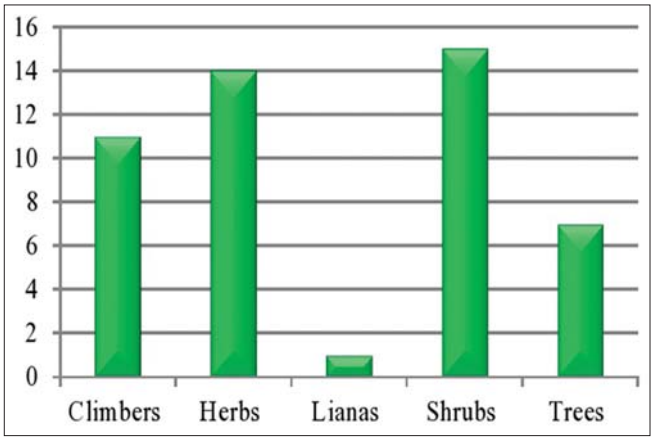

Figure 4: Life form of medicinal plants used for the preparation of herbal medicines by Yanadi tribe

DISCUSSION

Previously a number of ethnobotanists studied ethnomedicinal knowledge of the Yanadi tribe in different parts of Chittoor District [22,23]. However, no more ethnomedicinal study is carried out in Chadragiri and Gopalapuram village areas of Chadragiri reserve forest area. The usage of medicinal plants from plant families like Asclepiadaceae is the most preferred by this tribe. Our findings regarding most utilization of this family are due to wide distribution of these family plants in the study area and known number of traditional uses. This type of wide documentation of Asclepiadaceae members was observed in Yanadi tribe of Kavali district [24]. Shrubs are most utilized plants in these areas for preparation of herbal formulations due to easily available and made easy for collection of these plants. This type of results was found in medicinal plants used by the

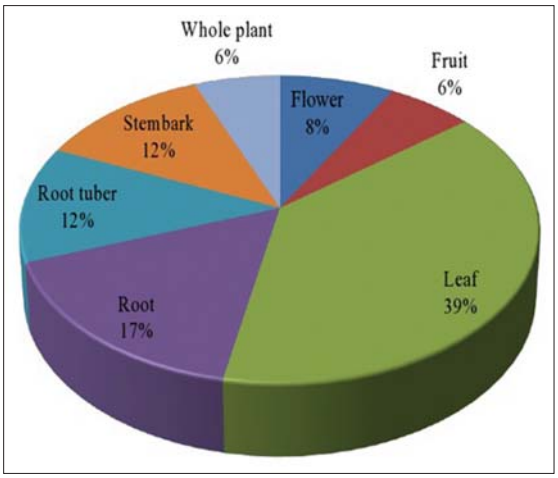

Figure 5: Percentage of plant parts used for preparation of herbal medicines by Yanadi tribe

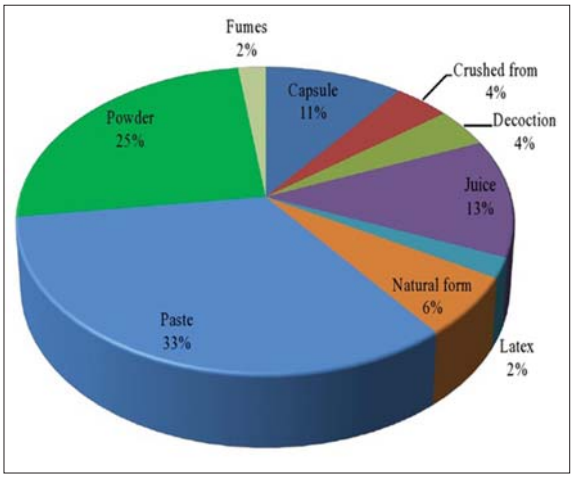

Figure 6: Percentage of different forms of medicines used for preparation of herbal medicines by Yanadi tribe

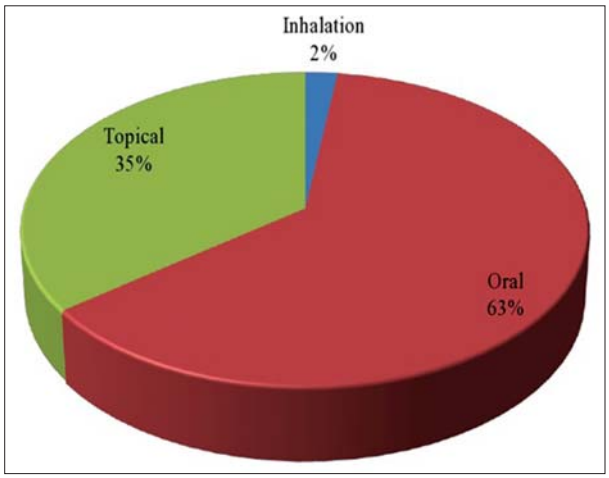

Figure 7: Percentage of administration of herbal medicines by Yanadi tribe

local people in Kailasakona sacred grove area of Chittoor District [18]. Leaf part of plants is most preferable for preparation of herbal medicine in this areas. The common use of leaf in the preparation of remedies could partly due to the relative ease of finding of this plant part. The leaves remain green and available in plenty throughout the year. The same type of results was observed in Yanadi tribe and local villagers of Veyilingalakona sacred grove of Chittoor District [25]. The herbal preparation in the form paste and oral administration of medicine is the most preferable in these areas; it may due to most of the plant parts were brought fresh from nearby forests and taken orally may be due to the effective exoneration of ailments. This type of results was observed in the tribal people of East Godavri District [26] and Japali Hanuman Theertham sacred grove area of Chittoor District [27].

The way of disease treatment, formulation, combination, ingredients used and administration of herbal medicines in these areas claimed was entirely different from the previous studies. The use of hot water for oral administration of medicines prepared from *Abrus precatorius*, *Clerodendrum phlomidis*, *Dichrostachys cinerea*, *Glycyrrhiza glabra*, *Hemidesmus indicus* and *Tribulus subramanyamii*. Use of hot water is due to easy uptake for oral administration and it avoids any microbial organisms which contaminate the medicine. In the case of *Cassytha filiformis*, *Dodonaea viscosa*, *Tragia involucrata* milk is preferred. Milk is an excellent medium for oral administration of drugs and strengthens the patients as nutritionally. Admixtures of sweeteners like jaggery and sugar in the case of *Andrographis serpyllifolia*, *Asparagus racemosus*, *Croton bonplandianum*, *Digera arvensis*, and *Maerua oblongifolia*. These sweeteners avoid the bitterness of medicines and provide cumulative interest to patients to take medicines at regular intervals. An addition of spices like turmeric powder in the case of *Cassia auriculata*, *Cissus quadrangularis*, *Euphorbia antiquorum*, *Justicia tranquebariensis*, *Malvastrum coromandelianum*, *Pithecellobium dulce*, *Polygala arvensis* and *Wattakaka volubilis*. As the turmeric powder elevates the performance of actual drug and its acts as excellent antimicrobial agent on different disease causative microorganisms. Addition of lubricants like castor oil, gingelly oil and honey was in the case of *Sida acuta*, *C. quadrangularis*, *Scutia myrtina*, *Flacourtia indica*, *P. dulce*, and *T. involucrata*. These lubricants are helpful to patients taken oral medicines in an easiest way. Admixture of curd in the case of *D. viscosa*, limestone in the case of *Martynia annua*, *P. arvensis*, camphor in the case of *W. volubilis*, urine in the case of *P. dulce*. Admixtures like curd and limestone improves overall performance of actual medicine, camphor provides excellent relief from rheumatic pains. There is no scientific reason behind the amalgamation of urine in the case of *P. dulce*, only the people of these areas believes about to heal the disease. In the preparation of medicines they admixture, not only the ingredients, but also combination of medicinal plants were used to prepare medicines such as *Curcuma aromatica* and *Piper longum*. This type of utilization of the ingredients or combination of medicinal plants for the preparation of herbal formulations was recorded in Chenchu tribe of Mahabubnagar district [28]. This type of combination and admixture of ingredients is used in preparation of herbal medicines are recorded in our study area is very scant. This documented information is transferred from generations to generations,

especially to the elder son of their family. The younger generations are not interested to learn and practice this system of traditional medicine system. They are inconvenient with this system and desires immediate relief from their afflictions, due to this cumulative reduction of interest on herbal treatments, which become extinct nearby future.

The ethnomedicinal data of 48 medicinal plants enumerated from the Yanadi tribe of Chadragiri reserve forest area, among them medicinal uses of 30 plants are correlated to Dr. Duke's phytochemical and ethnobotanical database at least by one medicinal value. But the medicinal plants like *C. phlomidis*, *Gynandropsis pentaphylla*, *Pachygone ovata* and *S. myrtina* having medicinal value in the database but it's not correlated with this study. The medicinal plants like *A. serpyllifolia*, *Bauhinia racemosa*, *Caralluma attenuata*, *Cassia hirsuta*, *Ceropegia juncea*, *C. bonplandianum*, *Diplocyclos palmatus*, *M. oblongifolia*, *Melhania incana*, *Pandanus fascicularis*, *P. arvensis*, *Tribulus subramayamii*, *W. volubilis* and *Ziziphus xylopyrus* are not appearing at least by one ethnomedicinal use in the database. Based on this database, we concluded that the medicinal values claimed by the Yanadi tribe of Chadragiri reserve forest area are high significance towards curing of different ailments in a traditional way with their unique nature of selection and preparation of herbal medicines from medicinal plants.

CONCLUSION

The tribal people of Chadragiri reserve forest area have vast knowledge on medicinal values of plants in their surrounding forest. The study revealed that 48 medicinal plants belonging to 26 families are used to treat 53 types of ailments. The plant species belonging to Asclepiadaceae are most used for the preparation of herbal medicine and shrubs of life form, leaf part of plant, paste form of medicine, oral administration of medicines are most preferred characters of these study areas. Selection, preparation, addition of ingredients, and combination of medicinal plants for the preparation of herbal formulations is unique and no more documentation was made previously in these areas. Tribal people along with local people of surrounding villages attracted to this traditional medicine, because of its cost-effective and easy to get from local traditional healers. Most of the data claimed in these areas is only get from the above age of 40-60 years and the people of younger generations are not interested in practicing the same. Hence, this is the right time to document the disappearing ethnomedicinal uses of this tribe and to explore this data to globe. The claimed ethnomedicinal data of this tribe is correlated to Dr. Dukes phytochemical and ethnobotanical database concluded that high significance of this tribal claims. Novel information on the preparation of herbal medicines from medicinal plants in this study will be useful for future generations to discover novel drugs.

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Original Research

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Spirulina versicolor improves insulin sensitivity and attenuates hyperglycemia-mediated oxidative stress in fructose-fed rats

Walaa G. Hozayen[1], Ayman M. Mahmoud[2], Hanan A. Soliman[1], Sanura R. Mostafa[1]

[1]Department of Chemistry, Division of Biochemistry, Faculty of Science, Beni-Suef University, Egypt, [2]Department of Zoology, Division of Physiology, Faculty of Science, Beni-Suef University, Egypt

Address for correspondence:
Ayman M. Mahmoud, Division of Physiology, Department of Zoology, Faculty of Science, Beni-Suef University, Egypt.
E-mail: ayman.mahmoud@science.bsu.edu.eg

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ABSTRACT

Aim: The current study aimed to investigate the anti-hyperglycemic, anti-hyperlipidemic and insulin sensitizing effects of the cyanobacterium *Spirulina versicolor* extract in fructose-fed rats. **Materials and Methods:** Rats were fed 30% fructose solution in drinking water for 4 weeks. Animals exhibited hyperglycemia and hyperinsulinemia were selected for further investigations. Diabetic and control rats were orally supplemented with 50 mg/kg body weight *S. versicolor* extract for 4 weeks. **Results:** At the end of 8 weeks, fructose-fed rats showed a significant increase in serum glucose, insulin, cholesterol, triglycerides, cardiovascular risk indices and insulin resistance. Treatment of the fructose-fed rats with *S. versicolor* extract improved this metabolic profile. Fructose feeding produced a significant increase in serum tumor necrosis factor alpha and a decrease in adiponectin levels. In addition, fructose-fed rats exhibited a significant increase in liver, kidney and heart lipid peroxidation levels, and declined antioxidant defenses. Supplementation of the fructose-fed rats with *S. versicolor* extract reversed these alterations. **Conclusion:** *S. versicolor* attenuates hyperglycemia-mediated oxidative stress and inflammation, and is thus effective in improving insulin sensitivity in fructose-fed rats.

KEY WORDS: Diabetes, fructose, inflammation, insulin resistance, oxidative stress, *Spirulina*

INTRODUCTION

Type 2 diabetes mellitus is a metabolic disease characterized by the presence of chronic hyperglycemia that results from defective or deficient insulin [1,2]. It accounts for more than 90% of all diabetic patients [3]. According to the International Diabetes Federation, the number of patients with diabetes mellitus in 2015 was estimated to be 415 million, and is expected to increase to 642 million by 2040 [4]. Type 2 diabetes and its complications constitute a major public health problem [5]. Several lifestyle factors such as physical inactivity [6], sedentary lifestyle [7], alcohol consumption [8] and smoking [9] are of key importance to the development of Type 2 diabetes. In addition, diet is a modifiable risk factor for Type 2 diabetes. The consumption of fructose has been enormously increased in the last few centuries because of the high increase in using sucrose and high fructose syrup [10]. Previous studies have demonstrated that high fructose intake is hazardous for human beings and animals [11,12], and results in hyperlipidemia, fatty liver, and insulin resistance [13]. The metabolism of fructose in the liver increases *de novo* lipogenesis [14], and an increase in high fructose corn syrup consumption has been linked to a rise in obesity and metabolic disorders [11]. Fructose feeding has also been shown to provoke oxidative damage and exert disturbing effects by diminishing antioxidant defenses, and increasing generation of free radicals [15]. Thus, the use of antioxidants could offer protection against fructose-induced metabolic alterations.

Currently, there is growing interest in the usefulness of algae for the treatment of diabetes. The cyanobacterium *Spirulina* is gaining a more attention as a nutraceutical and as a source of potential pharmaceutical. Studies have revealed the potential properties of *Spirulina* including antigenotoxic, anti-carcinogenic, immunostimulants, anti-inflammatory, anti-hepatotoxic, anti-diabetic and anti-hypertensive. *Spirulina* is a well-known source of anti-oxidant and anti-inflammatory molecules [16] such as c-phycocyanin, vitamins, β-carotene, phenolic compounds γ-linolenic acid and minerals [17,18]. *Spirulina maxima* (*Arthrospira maxima*), *Spirulina platensis* (*Arthrospira platensis*) and *Spirulina fusiformis* (*Arthrospira fusiformis*) are the most intensively investigated species of

Spirulina [17,19,20]. Recently, the preliminary anti-diabetic effect of *Spirulina versicolor* was reported in the study of AbouZid *et al*. [21] in streptozotocin/nicotinamide-induced diabetic mice. The authors reported that *S. versicolor* exerts anti-hyperglycemic effect, depending on assaying fasting and postprandial blood glucose levels in diabetic mice. To the best of our knowledge, nothing has yet been reported on the beneficial effects of *S. versicolor* in fructose-fed rats. Therefore, the current study was undertaken to investigate the anti-hyperglycemic, insulin sensitizing, anti-hyperlipidemic and antioxidant effects of *S. versicolor* in high fructose-fed rats. This investigation could provide an understanding of the anti-diabetic mechanism of *S. versicolor*.

METHODS

Preparation of *S. versicolor* extract

S. versicolor was purchased from Harraz medicinal plant company, Cairo, Egypt (www.harrazegypt.com). The algae was ground to a fine powder and extracted by 80% aqueous ethanol. Following filtration, the filtrate was concentrated under reduced pressure in a rotary evaporator and was stored at −20°C until use.

Experimental Animals

Male Wistar rats weighing 130-150 g, obtained from animal house of the National Research Centre (El-Giza, Egypt), were included in the present investigation. The animals were housed in plastic well-aerated cages at a normal atmospheric temperature (25±2°C) and normal 12 h light/dark cycle. Rats had free access to water and were supplied daily with laboratory standard diet of known composition. All animal procedures were undertaken with the approval of Institutional Animal Ethics Committee of Beni-Suef University (Egypt).

Experimental Design

About 24 rats were allocated into 4 groups, each consisting of six ($n = 6$) animals and were subjected to the following treatments:

Group 1 (Control): Received the vehicle 1% carboxymethylcellulose (CMC) and served as control rats.

Group 2 (Control + *S. versicolor*): Received 50 mg/kg b.wt. *S. versicolor* extract suspended in 1% CMC and served as drug control.

Group 3 (Diabetic): Received 30% fructose in tap water.

Group 4 (Diabetic + *S. versicolor*): Received 30% fructose in tap water and 50 mg/kg b.wt. *S. versicolor* extract suspended in 1% CMC.

Rats were fed 30% fructose solution in drinking water for 4 weeks, and biochemical parameters were estimated. Rats exhibited hyperglycemia and hyperinsulinemia were selected for further subsequent studies. *S. versicolor* extract has been administered by oral gavage for 4 weeks. The doses were balanced consistently as indicated by any change in body weight to keep up the comparable dosage for every kg body weight over the entire period of study.

Samples Preparation

By the end of the experiment, overnight fasted animals were sacrificed, and blood samples were collected, left to coagulate and centrifuged at 3000 rpm for 15 min to separate serum. Liver, kidney, and heart samples were immediately excised and perfused with ice-cold saline. Frozen samples (10% w/v) were homogenized in chilled saline, and the homogenates were centrifuged at 3000 rpm for 10 min. The clear homogenates were collected and used for subsequent assays.

Biochemical Study

Oral glucose tolerance test (OGTT)

On the day before sacrifice, OGTT was performed using blood samples obtained from lateral tail vein of rats deprived of food overnight. Successive blood samples were then taken at 30, 60, 90 and 120 min following the administration of glucose solution (3 g/kg b.wt.). Blood samples were left to coagulate, centrifuged, and clear sera were obtained for determination of glucose concentration according to the method of Trinder [22] using reagent kit purchased from Spinreact (Spain).

Determination of Serum Insulin, Adiponectin and Tumor Necrosis Factor Alpha (TNF-α)

Serum levels of insulin, adiponectin and TNF-α were determined using specific ELISA kits (R&D systems) following the manufacturer's instructions. The concentrations of assayed parameters were measured specrophotometrically at 450 nm. Standard curves were constructed by using standard proteins and concentrations of the unknown samples were determined from the standard plots.

Determination of Homeostasis Model of Insulin Resistance (HOMA-IR)

The insulin resistance was evaluated by homeostasis model assessment estimate of insulin resistance (HOMA-IR) [23] as follows:

$$\text{HOMA} - \text{IR} = \frac{\text{Fasting insulin } (\mu\text{U/ml}) \times \text{Fasting blood glucose} (\text{mmol/L})}{22.5}$$

Determination of Lipid Profile and Cardiovascular Risk Indices

Serum total cholesterol [24], triglycerides [25] and high density lipoprotein (HDL)-cholesterol [26] were assayed

using commercial diagnostic kits (Spinreact, Spain). Serum very low density lipoprotein (vLDL)-cholesterol concentration was calculated according to the following formula [27]: vLDL-cholesterol = triglycerides/5. Serum LDL-cholesterol level was calculated from the formula [28]: LDL-cholesterol = Total cholesterol - ([Triglycerides/5] + HDL-cholesterol]. Cardiovascular risk indices were calculated according to Ross [29] as follows: Cardiovascular risk index 1 = Total cholesterol/HDL-cholesterol and cardiovascular risk index 2 = LDL-cholesterol/HDL-cholesterol. Antiatherogenic index (AAI) was determined according to the following equation [30]: AAI = HDL-cholesterol × 100/Total cholesterol - HDL-cholesterol.

Assay of Serum Enzymes

Serum aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK-MB) activities were assayed using reagent kits purchased from Biosystems (Spain) following the methods of Schumann and Klauke [31], Teitz and Andresen [32] and Kachmar and Moss [33], respectively.

Assay of Lipid Peroxidation and Antioxidant Defenses

Lipid peroxidation levels in liver, kidney, and heart homogenates were assayed by measurement of malondialdehyde (MDA) formation according to the method of Preuss *et al*. [34]. Reduced glutathione (GSH) content and activity of the antioxidant enzymes superoxide dismutase (SOD) and GSH peroxidase (GPx) were measured according to the methods of Beutler *et al*. [35] Marklund and Marklund [36] and Matkovics *et al*. [37], respectively.

Statistical Analysis

Data were analyzed using Graph Pad Prism 5 software and all statistical comparisons were made by means of the one-way ANOVA test followed by Tukey's test *post hoc* analysis. Results were articulated as mean ± standard error of the mean (SEM) and a P value < 0.05 was considered significant.

RESULTS

S. versicolor Represses Hyperglycemia and Insulin Resistance in Fructose-fed Rats

OGTT of the fructose-induced diabetic rats showed significantly ($P < 0.001$) elevated glucose levels and at all points of the OGTT when compared with the normal control rats [Figure 1a]. Oral supplementation of *S. versicolor* extract to fructose-fed rats significantly alleviated the blood glucose levels. The OGTT area under the curve (AUC) showed non-significant ($P > 0.05$) difference between the control and *S. versicolor* supplemented control rats. On the other hand, fructose-induced diabetic rats exhibited a significant ($P < 0.01$) increase in AUC when compared with the control rats. Treatment of the diabetic rats with *S. versicolor* markedly ($P < 0.01$) decreased OGTT AUC when compared with the diabetic control rats, as depicted in Figure 1b.

Serum insulin level was significantly ($P < 0.001$) increased in fructose fed rats compared with the control group as depicted in Figure 1c. Oral treatment with *S. versicolor* markedly ameliorated serum insulin levels in the fructose-induced diabetic rats. Similarly, diabetic rats exhibited a significant ($P < 0.001$) increase in HOMA-IR, an effect that was reversed by oral administration of *S. versicolor* to fructose-induced diabetic rats [Figure 1d].

S. versicolor Exerts Anti-hyperlipidemic, Cardioprotective and Anti-atherogenic effects in Fructose-fed Rats

Data represented in Table 1 show the effect of *S. versicolor* on lipid profile, cardiovascular risk indices, heart marker enzymes and antiatherogenic index of control and diabetic rats. Compared with the control group, rats supplemented with *S. versicolor* exhibited non-significant ($P > 0.05$) changes in all lipid profile parameters. On the other hand, fructose-induced diabetic rats exhibited significant increase in serum total cholesterol ($P < 0.001$), triglycerides ($P < 0.01$), LDL-cholesterol ($P < 0.001$) and vLDL-cholesterol ($P < 0.01$) when compared with the control group. Serum levels of HDL-cholesterol showed a non-significant ($P > 0.05$) difference between all studied groups. In addition, diabetic rats showed significantly ($P < 0.001$) increased HDL-cholesterol/T. cholesterol and LDL-cholesterol/HDL-cholesterol. In addition, the antiatherogenic index was significantly ($P < 0.05$) declined in diabetic rats. By comparison, the oral supplementation of *S. versicolor* extract to diabetic rats potentially ameliorated the altered serum lipid profile as well as cardiovascular risk indices.

Serum AST, CK-MB and LDH activities were significantly increased in the fructose-induced diabetic rats when compared with the control group [Table 1]. Treatment of the diabetic rats with *S. versicolor* extract significantly ameliorated serum activities of AST ($P < 0.05$), CK-MB ($P < 0.05$) and LDH ($P < 0.001$).

S. versicolor Increases Circulating Adiponectin and Decreases TNF-α in Fructose-fed Rats

Fructose-fed rats exhibited markedly ($P < 0.01$) declined serum adiponectin levels when compared with the control group, as represented in Figure 2a. Treatment of the fructose-induced diabetic rats with *S. versicolor* extract significantly ($P < 0.01$) alleviated serum adiponectin levels. The effect of *S. versicolor* on serum levels of TNF-α in control and fructose-induced diabetic rats showed a significantly ($P < 0.01$) increased levels of TNF-α ($P < 0.001$) in diabetic rats and potential ($P < 0.05$) alleviation following treatment with *S. versicolor* extract [Figure 2b].

S. versicolor Attenuates Hyperglycemia-induced Oxidative Stress in Liver, Kidney and Heart of Fructose-fed Rats

Fructose-induced diabetic rats showed significantly increased MDA levels in liver ($P < 0.001$), kidney ($P < 0.01$) and heart

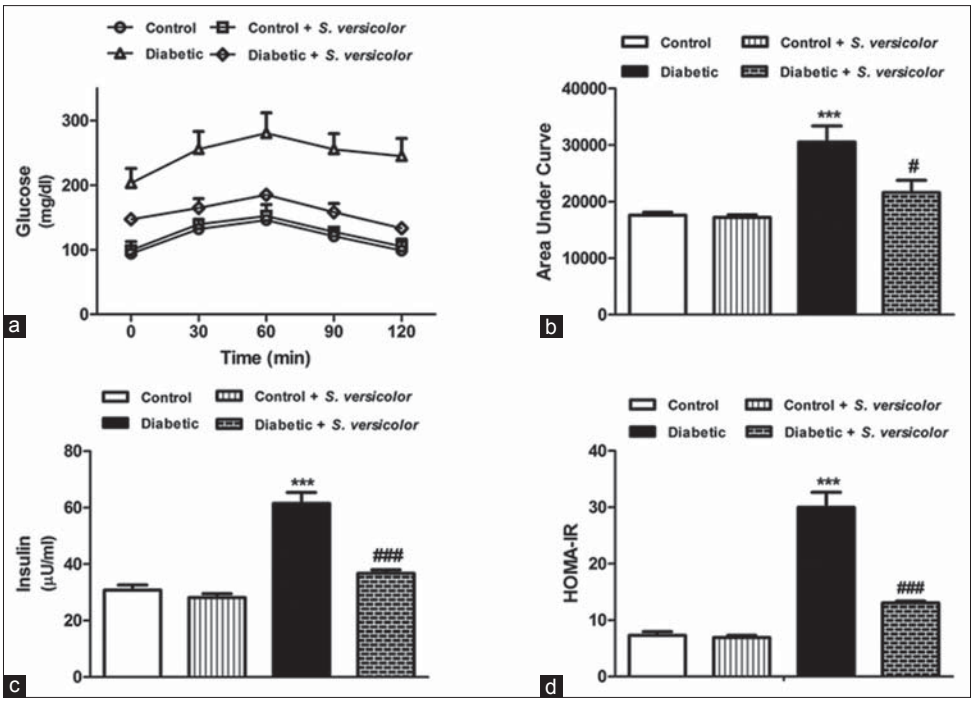

Figure 1: Effect of *Spirulina versicolor* on (a and b) glucose tolerance, (c) serum insulin and (d) homeostasis model of insulin resistance. Results are mean ± standard error of the mean ($n = 6$). ***$P < 0.001$ versus control, and $^{\#}P < 0.05$, and $^{\#\#\#}P < 0.001$ versus diabetic group

Table 1: Effect of *S. versicolor* on serum lipid profile, cardiovascular risk indices and antiatherogenic index in control and fructose-fed rats

Parameter	Control	Control+*S. versicolor*	Diabetic	Diabetic+*S. versicolor*	*P* value
Total cholesterol (mg/dl)	83.62±6.70	83.85±8.88	171.80±8.04***	111.90±3.36$^{\#\#\#}$	$P<0.001$
Triglycerides (mg/dl)	110.40±5.71	95.40±6.08	249.8±41.19**	110.60±8.37$^{\#\#}$	$P<0.001$
HDL-cholesterol (mg/dl)	39.25±3.44	38.23±2.05	29.22±4.63	44.88±1.39$^{\#}$	$P<0.05$
LDL-cholesterol (mg/dl)	27.85±6.34	26.54±6.65	92.64±6.87***	44.92±3.65$^{\#\#\#}$	$P<0.001$
vLDL-cholesterol (mg/dl)	22.07±1.14	19.08±1.22	49.96±8.24**	22.12±1.67$^{\#\#}$	$P<0.001$
Total cholesterol/HDL-cholesterol	2.18±0.23	2.18±0.16	6.32±0.79***	2.49±0.05$^{\#\#\#}$	$P<0.001$
LDL-cholesterol/HDL-cholesterol	0.61±0.21	0.67±0.15	3.39±0.40***	1.00±0.08$^{\#\#\#}$	$P<0.001$
Antiatherogenic index (%)	98.80±6.95	94.41±14.79	20.90±3.71*	67.21±2.56$^{\#}$	$P<0.01$
AST (U/L)	19.20±1.35	19.77±0.79	30.45±1.09***	24.22±0.69$^{\#}$	$P<0.001$
LDH (U/L)	56.95±4.53	21.14±3.17	138.50±10.54**	18.27±2.67$^{\#\#\#}$	$P<0.001$
CK-MB (U/L)	185.70±8.40	234.79±17.84	290.00±8.02**	268.91±11.86$^{\#}$	$P<0.01$

Data are expressed as mean±SEM. Number of rats in each group is six, *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ versus diabetic, SEM: Standard error mean, CK: Creatine kinase, LDH: Lactate dehydrogenase, AST: Aspartate aminotransferase, LDL: Low density lipoprotein, HDL: High density lipoprotein, vLDL: Very low density lipoprotein, *S. versicolor: Spirulina versicolor*

($P < 0.01$) when compared with the control rats [Figure 3a]. Treatment of the fructose-induced diabetic rats with *S. versicolor* extract significantly alleviated liver ($P < 0.01$), kidney ($P < 0.001$) and heart ($P < 0.001$) lipid peroxidation levels. Oral supplementation of *S. versicolor* to normal rats produced significant ($P < 0.05$) decrease in kidney MDA content, with no effect exerted on liver and heart.

On the contrary, fructose supplementation significantly decreased liver ($P < 0.001$), kidney ($P < 0.05$) and heart ($P < 0.001$) GSH content when compared with the control group, as represented in Figure 3b. Similarly, SOD activity was significantly decreased in the liver ($P < 0.05$), kidney ($P < 0.05$) and heart ($P < 0.01$) of fructose-induced diabetic rats when compared with the control group [Figure 3c]. GPx activity showed a similar pattern where it was significantly decreased in the liver ($P < 0.01$), kidney ($P < 0.001$) and heart ($P < 0.05$) of fructose-induced diabetic rats, as depicted in Figure 3d. On the other hand, treatment of the fructose-induced diabetic rats with *S. versicolor* extract potentially ameliorated GSH content as well as activities of SOD and GPx in the liver, kidney and heart.

DISCUSSION

Several studies have demonstrated the deleterious effects of fructose on insulin sensitivity and glucose metabolism [38]. In the present study, fructose-fed rats showed significantly impaired glucose tolerance accompanied with hyperinsulinemia and increased HOMA-IR. Therefore, it is suggested that insulin resistance has been developed in these animals. This would closely reflect the natural history and metabolic characteristics of human diabetes, and it is further sensitive to pharmacological

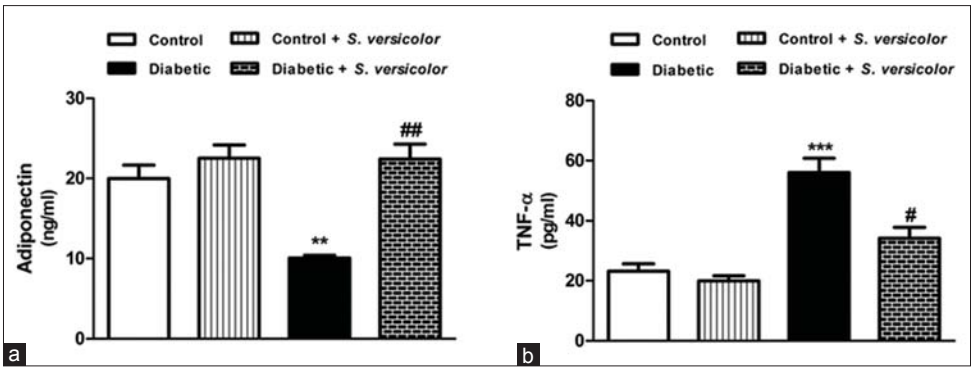

Figure 2: Effect of *Spirulina versicolor* on (a) serum adiponectin and (b) tumor necrosis factor alpha. Results are mean ± standard error of the mean ($n = 6$). $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ versus diabetic group

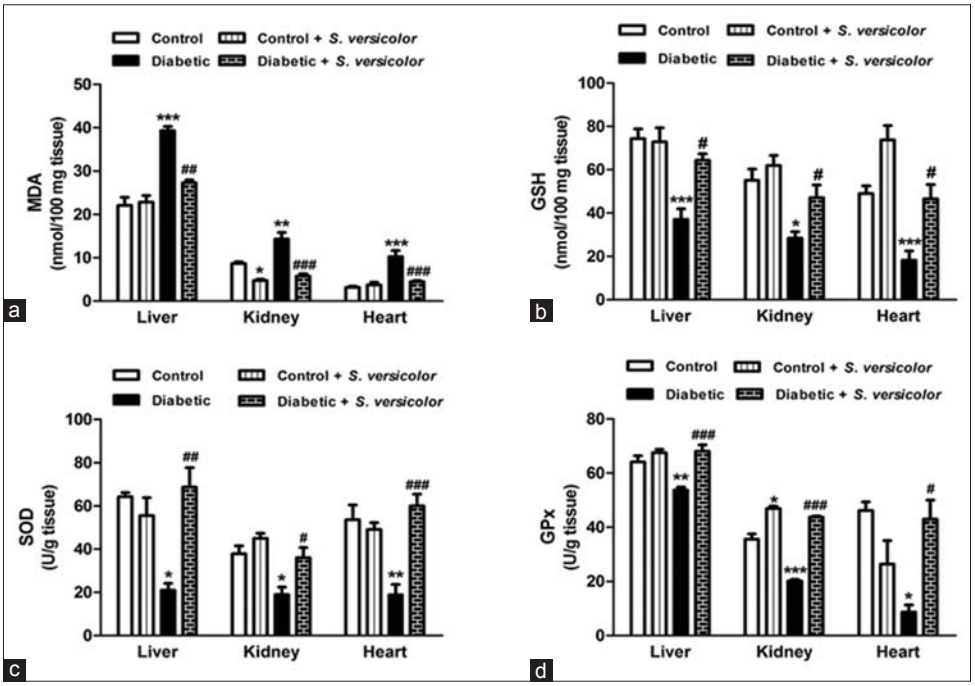

Figure 3: Effect of *Spirulina versicolor* on (a) lipid peroxidation, (b) reduced glutathione, (c) superoxide dismutase and (d) glutathione peroxidase in liver, kidney and heart. Results are mean ± standard error of the mean ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control, and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus diabetic group

testing [2]. Long term fructose feeding has been demonstrated to induce diabetes associated with insulin resistance in experimental animals [38-41]. The fructose-induced insulin resistance may be linked to alteration of insulin signaling. In this context, high fructose feeding has been reported to decrease insulin receptor substrate (IRS)-1 phosphorylation in rat skeletal muscles [42]. In addition, fructose-induced hyperlipidemia [43] and fat deposition [44] may generate lipid-derived metabolites which reduce insulin signaling via increasing serine/threonine phosphorylation of IRS-1 [45]. Oral supplementation of *S. versicolor* extract markedly reduced blood glucose and improved insulin sensitivity in fructose-fed rats. Although the anti-hyperglycemic effect of different *Spirulina* species has been previously reported, studies demonstrating the anti-diabetic efficacy of *S. versicolor* are scarce. In this context, Mani *et al*. [46] showed a significant decrease in the fasting blood sugar level of patients received 2 g/day *Spirulina* for 21 days, and Layam *et al*. [47] proved the same effect in diabetic rats treated with 15 mg/kg *Spirulina* for 45 days. The hypoglycemic effect of *Spirulina* could perhaps attributed to its high fiber content that diminish glucose absorption [48], or to the possible action of peptides generated by the digestion of *Spirulina* proteins [49].

Insulin resistance in Type 2 diabetes is also associated with hyperlipidemia and atherosclerosis [50]. Fructose-fed rats in the present investigation exhibited hypercholesterolemia and hypertriglyceridemia. The fructose-induced hyperlipidemia may be attributed to the increased *de novo* hepatic lipogenesis through providing large amounts of hepatic triose-phosphate for fatty acid synthesis [14]. In addition, fructose increases the expression of key lipogenic enzymes and induces the expression of sterol regulatory element binding protein-1c which is the principal inducer of hepatic lipogenesis [51,52]. Moreover,

fructose has been demonstrated to activate carbohydrate-responsive element binding protein (ChREBP), leading to up-regulated expression of hepatic fatty acid synthase and acetyl-CoA carboxylase [53]. Activation of ChREBP may be attributed to the fructose-induced expression of glucose-6-phosphate dehydrogenase and intermediary substrates of the hexose-monophosphate shunt [54].

The elevated triglycerides and cholesterol levels in the fructose-induced diabetic rats represent atherogenic lipid profile. The recorded values of atherogenic indices in the present study showed the bad impact of fructose-induced dyslipidemia on the cardiovascular system. These findings were confirmed by the elevated serum levels of AST, CK-MB, and LDH. Treatment of the diabetic rats with *S. versicolor* extract significantly ameliorated the altered lipid profile and atherogenic indices. Reduction of these indices in treated fructose-fed rats strongly supported the notion that dietary supplementation with *S. versicolor* may reduce the risk of developing heart diseases. These findings were further confirmed by the significantly decreased serum activities of the cardiac markers, CK-MB, LDH and AST, in *S. versicolor* treated fructose-fed rats. The anti-hyperlipidemic effects of *Spirulina sp.* have been demonstrated in animal [55,56] and human studies [57-59].

The beneficial effects of *S. versicolor* extract in fructose-induced diabetic rats might be explained, at least in part, through its ability to increase serum adiponectin levels. Serum level of adiponectin is in agreement with insulin sensitivity and its reduced levels are associated with insulin resistance [60]. Adiponectin regulates glucose metabolism [61], increases muscle fat oxidation and glucose transport mediated [62], inhibits hepatic gluconeogenesis [63] and activates peroxisome proliferator activated receptor-α leading to decreased triglyceride content in skeletal muscles and liver [64]. We also assume that suppression of the release of TNF-α following *S. versicolor* administration could be a direct result of increased serum adiponectin levels. Adiponectin is well known to inhibit the expression of the pro-inflammatory cytokine TNF-α in various tissues [65]. TNF-α diminishes the ability of insulin to stimulate peripheral glucose uptake and to suppress hepatic glucose production [66], and increases circulating free fatty acids; thus contributes to the pathogenesis of insulin resistance [67]. In the present study, treatment of the fructose-induced diabetic rats with *S. versicolor* markedly decreased serum levels of TNF-α, confirming its anti-inflammatory efficacy.

Oxidative stress has been implicated in fructose-induced insulin resistance and Type 2 diabetes in rats [13]. Oxidative stress can cause oxidation and damage to many cellular components such as DNA, lipids and proteins [68]. Reactive oxygen species (ROS) in diabetes could react with polyunsaturated fatty acids leading to lipid peroxidation [69]. In addition, high levels of free radicals and the simultaneous decline in endogenous antioxidants can lead to damage of cellular organelles, and development of insulin resistance [70]. Hence, it was recommended by Mahmoud *et al.* [2] that therapy with antioxidants represents a useful pharmacologic overture to the management of diabetes. The present findings showed significant elevation in lipid peroxidation levels in liver, kidney and heart of fructose-administered rats. Treatment of the fructose-fed rats with *S. versicolor* extract significantly decreased lipid peroxidation levels, reflecting its radical scavenging property.

In contrary, GSH and the antioxidant enzymatic defenses showed a simultaneous decrease in the liver, kidney and heart of fructose-induced diabetic rats. Antioxidant defenses are known to decrease under hyperglycemia [71] and oxidative stress [72]. Treatment of diabetic rats with *S. versicolor* significantly increased levels of GSH and activity of the antioxidant enzymes SOD and GPx. GSH is an endogenous antioxidant that protects against oxidative stress-induced cellular damage by reacting with oxidants or as a substrate for GPx. SOD and GPx provide a defense system against ROS-induced cellular damage [73]. The antioxidant effect of *Spirulina* and their constituents has been previously demonstrated. Ahmed *et al.* [74] reported that *S. versicolor* extract protected against diethylnitrosamine-induced hepatocarcinogenesis through potentiating the antioxidant defense system.

Several studies have reported the *in vitro* and *in vivo* antioxidant and/or anti-inflammatory efficacies of *Spirulina* and its extracts, suggesting the beneficial effects of *Spirulina* in managing insulin resistance and diabetes. The antioxidant and anti-inflammatory effects of *Spirulina Sp.* could be attributed to its active constituents. *Spirulina* contains a relative high concentration of β-carotene, provitamin A, vitamin B, vitamin C, vitamin D, vitamin E, ω-3 and ω-6 polyunsaturated fatty acids, and phycocyanin [75]. Phycocyanin has the ability to scavenge free radicals, decrease nitrite production, suppress inducible nitric oxide synthase expression, and inhibit liver microsomal lipid peroxidation. In addition, phycocyanin has been reported to inhibit pro-inflammatory cytokine formation, suppress cyclooxygeanase-2 expression and decrease prostaglandin E2 production [76-78]. Another constituent, β-carotene, has been reported to have antioxidant and anti-inflammatory activities [79,80].

CONCLUSION

The current findings provide new information on the antidiabetic mechanism of *S. versicolor* in fructose-fed rats. High fructose feeding induces insulin resistance, inflammation and oxidative stress. Oral administration of *S. versicolor* ameliorates insulin sensitivity, increases serum adiponectin, and attenuates oxidative stress and inflammation in diabetic rats. Our findings suggest that *S. versicolor* extract could be used as a dietary supplement in diabetes management, pending further studies to trace out its exact mechanistic pathways.

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Original Research

Hepatoprotective effects of parsley, basil, and chicory aqueous extracts against dexamethasone-induced in experimental rats

Hanan A. Soliman[1], Mohamed A. El-Desouky[2], Walaa G. Hozayen[1,3], Rasha R. Ahmed[4], Amal K . Khaliefa[1]

[1]Division of Biochemistry, Department of Chemistry, Faculty of Science, Beni-Suef University, Beni Suef, Egypt, [2]Division of Biochemistry, Department of Chemistry, Faculty of Science, Cairo University, Giza, Egypt, [3]Department of Biotechnology, Faculty of Postgraduate Studies for Advanced sciences, Beni-Suef University, Beni Suef, Egypt, [4]Department, of Zoology, Faculty of Science, Beni-Suef University, Beni Suef, Egypt

Address for correspondence:
Walaa G. Hozayen, Department of Biotechnology, Faculty of Postgraduate Studies for Advanced sciences, Beni-Suef University, Beni Suef, Egypt.
E-mail: Walaahozayen@hotmail.com

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ABSTRACT

Aim: The objective of this study is to investigate the hypoglycemic, hypolipidemic, and hepatoprotective effects of the aqueous extract of parsley, basil, and chicory whole plant in normal and dexamethasone (Dex) rats. **Materials and Methods:** 50 female albino rats were used in this study and divided into 5 groups (for each 10). Group (1) fed basal diet and maintained as negative control group. Group (2) received Dex in a dose of (0.1 mg/kg b. wt.). Groups 3, 4, and 5 were treated with Dex along with three different plant extracts of parsley, basil, and chicory (2 g/kg b. wt.), (400 mg/kg b. wt.), and (100 mg/kg b. wt.), respectively. **Results:** All these groups were treated given three times per week for 8 consecutive weeks. Dex-induced alterations in the levels of serum glucose, triglyceride, cholesterol, low-density lipoprotein-cholesterol levels and cardiovascular indices and serum alanine aminotransferase, aspartate aminotransferase and lactate dehydrogenase activities, liver thiobarbituric acid (TBARS) levels increased, while high-density lipoprotein-cholesterol, total protein, albumin, and liver glutathione (GSH) levels decreased. On the other hand, plant extracts succeeded to modulate these observed abnormalities resulting from Dex as indicated by the reduction of glucose, cholesterol, TBARS, and the pronounced improvement of the investigated biochemical and antioxidant parameters. **Conclusions:** It was concluded that probably, due to its antioxidant property, parsley, basil, and chicory extracts have hepatoprotective effects in Dex-induced in rats.

KEY WORDS: Antioxidants, dexamethasone, hyperglycemia, hyperlipidemia

INTRODUCTION

Dexamethasone (Dex) has potent immunosuppressant and anti-inflammatory properties but therapeutic benefits of this drug limited as it presents several side effects such as insulin resistance and skeletal muscle atrophy and generation of the free radicals which may contribute to oxidative stress as long-term treatment [1]. Natural products have been examined for promising new source pharmaceutical and therapeutic agents in plant secondary metabolites that characterize definite plants [2].

It is thought that the health promoting effect of *Petroselinum crispum* (parsley) may be due to its flavones components as ascorbic acid, carotenoids, flavonoids, coumarins, myristicin, apiol, various terpenoid compounds, phenylpropanoids, phthalides, furanocoumarins, and tocopherol [3].

Ocimum basilicum (basil) had been found to contain linalool, eugenol, methyl chavicol, methyl cinnamate, ferulate, methyl eugenol, triterpenoids, and steroidal glycoside known to exhibit antioxidant, chemopreventive, anti-inflammatory, bactericidal, antiulcer activities, a nervous system stimulant effect,

modulatory effect on glutathione and antioxidant enzymes, antidiarrheal and hypoglycemic effects [4,5].

Cichorium intybus L. (chicory) own excessive medicinal importance as it has alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterols, flavonoids, saponins, and tannins [6].

The aim of this study was both to investigate the hepatoprotective effect of *P. crispum*, *O. basilicum*, and *C. intybus L.* aqueous extracts against Dex-induced in rats and to explore the antioxidant capability of the extracts.

MATERIALS AND METHODS

Chemicals

Dex ([Fortecortine® 8 mg - Mono ampule) Manufactured by Sigma - Tec Pharmaceutical industries - Egypt - S. A. E. under Licence of: Merck KGaA, Darmstadt, Germany).

Plant Materials

Parsley (*P. crispum*), basil (*O. basilicum*), and chicory (*C. intybus L.*) leaves were collected from herbal medicine market (Cairo, Egypt) and identified by an ecologist in plant department, Faculty of Science, Beni-suef University. The leaves of these plants were cleaned, shade dried for 30 days at room temperature, crushed to a coarse powder and preserved for further processing.

Preparation of Plant Extract

Preparation of aqueous parsley extract

The 100 gram dried parsley leaves were extracted by adding 1000 ml of distilled water and boiled for 30 min. The extract was then filtered, and then the filtrate was evaporated, using rotary evaporator under reduced pressure to dryness (at 45°C). The extract was dissolved in distilled water before the administration to rats [7].

Preparation of aqueous basil extract

The 300 g ground powder of dried basil leaves was infused for 30 min in 200 ml of distilled water at 100°C followed by filtration. The solution obtained was concentrated rotary evaporator under a vacuum at 65°C. The resulting crude extract was suspended in 30 ml sterile distilled water, and aliquots were stored at −20°C until use [8].

Preparation of aqueous chicory extract

The powdered chicory leaves were added to the already boiling distilled water and infused for 15 min. Then, the infusion (2% w/v) was filtered, and the filtrate was freshly used [9].

Animals

Female albino rats (Rattus norvegicus) weighing about 120-150 g were used for the study and were kept in the animal house at 26 ± 2°C with relative humidity 44% to 56% along with light and dark cycles of 12 h. Animals were provided with standard diet and water *ad libitum*.

Animal Groups

For the achievement of the objectives of this study, 50 female albino rats were randomly divided into 5 groups including the normal groups and the diabetic groups each group consists of 10 rats. Group 1: Served as normal control. Group 2 was considered as Dex-control was given Dex subcutaneously 0.1 mg/kg/day [10], and this group was considered as control for the Groups 3, 4, and 5. Group 3 (Dex-treated with parsley): The rats in this group were given Dex and also were administrated aqueous parsley at dose level of 2 g/kg/b. wt. [7]. Group 4 (Dex-treated with basil): The animals in this group were Dex and also were given aqueous basil at dose level 400 mg/kg/b. wt. [4]. Group 5 (Dex-treated with chicory): The animals in this group were Dex and also were treated with aqueous chicory at dose level 100 mg/kg/b. wt. [11]. All these groups were treated for three times per week for 8 consecutive weeks and the treatments with parsley, basil, and chicory were performed orally between 7.00 and 9.00 a.m.

Biochemical Assay

At the end of the experimental period (8 weeks), rats were sacrificed under diethyl ether anesthesia. Blood samples were collected from each rat, allowed to coagulate at room temperature then centrifuged at 3000 r.p.m. for 20 min. The clear, non-hemolyzed supernatant sera were quickly removed and kept at −20°C until examined.

The serum samples were analyzed for glucose according to the method of [12] using standard diagnostic kits from Spinreact and serum cholesterol [13], triglycerides [14], high-density lipoprotein-cholesterol (HDL-C) [15], total protein [16], albumin [17] using the commercial assay kit provided from Bioscope Diagnostics, beta lab, Egypt, while very low-density lipoprotein-cholesterol (VLDL-C), LDL-C, and cardiovascular indices were calculated mathematically according to [18,19] using the following formula:

VLDL-C = Triglycerides/5

LDL-C = Cholesterol - (Triglyceride/5) - HDL-C

Cardiovascular index 1 = Total cholesterol/HDL-C.

Cardiovascular index 2 = LDL-cholesterol/HDL-C.

The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was determined kinetic at 546 nm using

a standard method [20], respectively, using kit provided from Bioscope Diagnostics, betalab, Egypt. Lactate dehydrogenase (LDH) was measured using the kinetic method described by Young [21] using reagent kits obtained from BioSystems, Barcelona, Spain.

Hepatic Oxidative Stress and Antioxidant Enzymes Assay

Liver tissues were homogenized in cold 0.9% NaCl with a glass homogenizer to make up to 10% homogenate (w/v). The homogenates were centrifuged, and the clear supernatants were used for lipid peroxidation (LPO), glutathione (GSH), and antioxidant enzymes. The supernatant was used for the estimation of malondialdehyde (MDA) [22]; GSH [23]; GSH s-transferase (GST) [24]; GSH peroxidase (GPx) [25]; GSH reductase (GR) [26]; and catalase [27] levels using the reagent kits purchased from Biodiagnostic Company, Giza, Egypt.

Histological Examination of Liver

Liver specimens were fixed in 10% formal saline for 24 h. 5% formic acid was used for decalcification, processed, and embedded in paraffin. Thin paraffin sections (4 μm) were stained by H and E [28].

Statistical Analysis

The data were analyzed using the one-way analysis of variance [29] followed by least significant difference test to compare various groups with each other. Results were expressed as mean ± standard deviation (SD), and values of $P > 0.05$ were considered non-significantly different, while those of $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered significantly, highly, and very highly significantly different, respectively.

RESULTS

Data in Table 1 show treatment of diabetic rats induced by Dex with parsley, basil, and chicory extracts caused considerable positive effects on the investigated parameters as compared to their corresponding control rats where $P < 0.001$ in all compared groups.

Data presented in Table 2 demonstrated that treatment of diabetic rats induced by Dex with parsley and chicory extracts cause a significant increase in serum total protein and albumin while treatment with basil cause a non-significant increase in total protein and a significant increase in albumin level when compared with diabetic rats.

Results showed that the treatment of rats with parsley, basil, and chicory extracts produced a profound, significant decrease of the LPO products in the liver and also, produced an increase of glutathione level and antioxidant enzymes when compared to their control group as shown in Table 3.

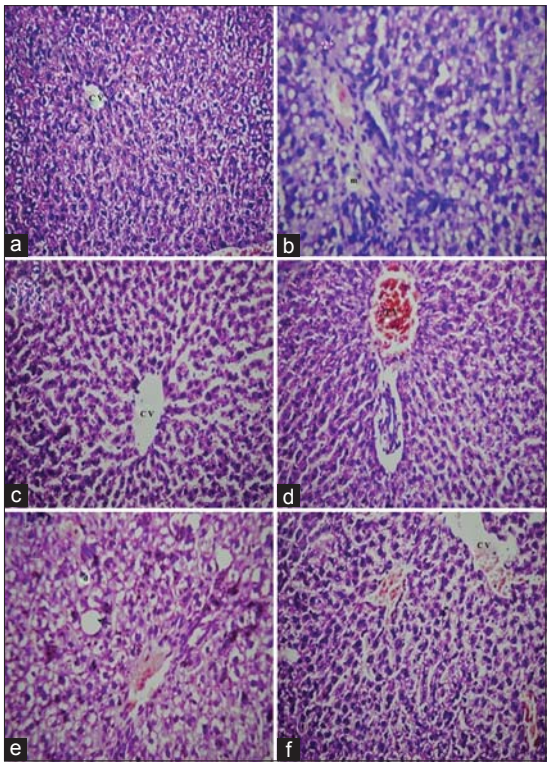

Figure 1: (a-f) Photomicrographs of rat liver sections of different experimental groups stained with hematoxylin and eosin, (a and b) liver sections of normal (G1) and dexamethasone (G2) groups, (c-d) liver sections of diabetic rats treated with parsley extract group (G3), (e) Liver section of diabetic rats treated with basil extract group (G4), and (f) liver section of diabetic rats treated with chicory extract group (G5)

Table 1: Serum biochemical parameters in control and different treated groups

Parameters	Norm	Dex	Dex+parsley	Dex+basil	Dex+chicory	P_{ANOVA}	LSD at 5%	LSD at 1%
Glucose (mg/dl)	123±12.3^{d}	218±11.7^{a}	170±4.48^{b}	143±21.8^{c}	160±1.95^{b}	$P<0.001$	14.92	20.19
Triglyceride (mg/dl)	44.7±4.43^{c}	90.1±7.51^{a}	61.1±7.37^{b}	54.1±8.60^{b}	53.0±7.46^{bc}	$P<0.001$	8.575	11.60
Cholesterol (mg/dl)	101±10.2^{c}	189±7.96^{a}	118±2.83^{bc}	135±3.52^{b}	153±11.7^{bc}	$P<0.001$	30.54	41.32
VLDL-C (mg/dl)	8.94±0.88^{c}	18.0±2.05^{a}	12.2±1.47^{b}	10.8±1.72^{b}	10.6±1.49^{bc}	$P<0.001$	1.714	2.319
LDL-C (mg/dl)	47.8±10.5^{e}	139±8.97^{a}	63.5±5.71^{d}	84.9±4.78^{c}	107±14.7^{b}	$P<0.001$	11.46	15.50
HDL-C (mg/dl)	44.2±3.59^{a}	32.0±3.35^{d}	42.3±3.06^{ab}	39.3±3.81^{bc}	36.0±4.01^{c}	$P<0.001$	4.269	5.775
Cardiovascular index 1	2.28±0.24^{d}	5.90±0.69^{a}	2.78±0.23^{d}	3.43±0.39^{c}	4.25±0.76^{b}	$P<0.001$	0.608	0.823
Cardiovascular index 2	1.08±0.25^{d}	4.39±0.65^{a}	1.50±0.22^{d}	2.16±0.35^{c}	2.90±0.71^{b}	$P<0.001$	0.573	0.775

Data are expressed as mean±SD $n=6$ animals per group. LSD: Least significant difference, mean with the different letters in the row are significantly different, VLDL-C: Very low-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol, SD: Standard deviation

Table 2: Serum liver function markers in control and different treated groups

Parameters	Norm	Dex	Dex+parsley	Dex+basil	Dex+chicory	P_{ANOVA}	LSD at 5%	LSD at 1%
Total protein (g/dl)	10.4±0.90^{a}	7.54±0.28^{c}	8.86±0.98^{b}	8.47±1.33^{bc}	8.72±1.09^{b}	$P<0.01$	1.223	1.654
Albumin (g/dl)	6.63±0.37^{a}	4.52±0.19^{d}	5.58±0.33^{b}	5.31±0.44^{bc}	5.48±0.46^{c}	$P<0.001$	0.600	0.812
ALT (IU/L)	52.4±5.74^{c}	76.5±10.3^{a}	61.7±6.37^{b}	58.1±4.19^{bc}	58.3±3.28^{bc}	$P<0.001$	7.654	18.50
AST (IU/L)	179±10.4^{c}	240±20.9^{a}	187±11.7^{c}	190±17.7^{c}	214±14.8^{b}	$P<0.001$	18.51	25.03
LDH (IU/L)	2481±332^{d}	3530±258^{a}	2813±246^{c}	2925±211^{bc}	3143±165^{b}	$P<0.001$	295.4	399.7

Data are expressed as mean±SD $n=6$ animals per group, mean with the different letters in the row are significantly different, ALT: Alanine transaminase, AST: Aspartate transaminase, LDH: Lactate dehydrogenase, LSD: Least significant difference, SD: Standard deviation

Table 3: Liver oxidative stress marker and antioxidant parameters in control and different treated groups

Parameters	Norm	Dex	Dex+parsley	Dex+basil	Dex+chicory	P_{ANOVA}	LSD at 5%	LSD at 1%
TBARS (nmol/g T)	18.2±1.41^{d}	29.6±2.19^{a}	21.6±2.11^{b}	19.7±1.77^{bc}	18.7±2.13^{c}	$P<0.001$	2.313	3.127
GSH (mg/g T)	27.3±3.86^{a}	13.1±2.64^{c}	21.6±2.11^{b}	20.9±3.40^{b}	19.9±3.79^{b}	$P<0.001$	3.844	5.201
GST (U/g T)	4180±396^{a}	2108±209^{c}	3532±202^{b}	4059±65.9^{a}	3627±182^{b}	$P<0.001$	280.8	379.9
GPx (U/g T)	181±23.1^{a}	61.7±16.6^{c}	170±7.56^{a}	91.0±13.9^{b}	92.6±12.9^{b}	$P<0.001$	18.60	25.17
GR (U/g T)	328±15.4^{a}	170±37.5^{c}	209±22.5^{b}	307±20.9^{a}	314±20.2^{a}	$P<0.001$	29.07	39.37
Cat (U/g T)	0.80±0.09^{a}	0.30±0.06^{d}	0.39±0.08^{c}	0.36±0.06^{cd}	0.60±0.07^{b}	$P<0.001$	0.075	0.101

Data are expressed as mean±SD $n=6$ animals per group, mean with the different letters in the row are significantly different, TBARS: Thiobarbituric acid reactive substance, GSH: Glutathione reduced, GST: Glutathione s-transferase, GPx: Glutathione peroxidase, GR: Glutathione reductase, SD: Standard deviation

Histopathological Results

Microscopically, liver of rat from negative control revealed no histopathology change [Figure 1a]. Meanwhile, section of liver of rat from positive control group (rats administrated with Dex) revealed fatty change was detected in diffuse manner all over the hepatocytes in association with inflammatory cells infiltration in the portal area [Figure 1b]; a section of liver of parsley extract treated rats showed there was no histopathological alteration [Figures 1c and d]: The liver of rats from group supplemented with aqueous basil extract showed normal liver with few individual hepatocytes [Figure 1e]. Moreover, the liver of rats from group supplemented aqueous chicory revealed no histopathological changes except mild congestion was observed in the central veins [Figure 1f].

DISCUSSION

The causes for diabetes after chronic Dex-treatment explained by reduced insulin sensitivity, impaired α and β-cell functions, increased hepatic gluconeogenesis, by stimulating amino acids releasing from skeletal muscle, fatty acids and glycerol from adipose tissue and enhance gluconeogenic enzymes expression as phosphoenolpyruvate carboxykinase [30].

Hyperlipidemia caused in Dex-induced diabetes is due to excess mobilization of fat from the adipose tissue, increase VLDL secretions by the liver and stimulate VLDL formation by the intestine, decreasing hepatic lipoprotein lipase activity that inhibit the removal of plasma VLDL and leading to an increase plasma VLDL level [31].

A decline in serum total protein level in diabetics due to decrease in protein synthesis, increase in catabolic processes, and reduction of protein absorption as inhibition of oxidative phosphorylation [32].

Corticosteroids therapy associated with several forms of liver injury leads to increase the liver function enzymes. ALT only significantly elevated in hepatobiliary disease and is liver specific enzyme AST level increased with damages of heart or skeletal muscle as well as of liver parenchyma. Changes in LDH may be attributed to severe damage to heart tissue as myocardial necrosis [33-35].

The Dex-induced oxidative stress and altered the antioxidant status in several tissues. The reduction of GSH contents corresponds to cellular oxidative damage and death as the cellular energy is impaired or due to the fast oxidation of GSH-induced by steroidal drug Dex. Depletion in the activity of antioxidant enzymes can be due to an enhanced radical production during Dex metabolism [36,37].

The current biochemical alterations were coincided with the present histological investigation for liver section, so the negative results associated with Dex may be attributed to the hepatic injury induced by superoxide anions and hydroxyl radicals which cause oxidative damage to cell membrane resulted in fatty change in diffuse manner all over the hepatocytes in association with inflammatory cells infiltration in the portal area. In agreement with our result [38] showed the treatment of rats with glucocorticoids led to the accumulation of lipids in the liver.

Parsley extract possess antihyperglycemic by facilitating glucose usage via extra-pancreatic ways as inhibition of gluconeogenesis and may be stimulate glycolysis process directly [6]. Aqueous extract of parsley possesses hypocholesterolemic and hypotriglyceridemic activities due to it has flavonoids that possess the bioactivity to beneficially affect the cardiovascular risk factors such as lipoprotein oxidation, dyslipidemia, endothelial dysfunction, and blood platelet aggregation and regulation of lipid levels in plasma or tissue leads to a decrease

in the risk of micro or macro vascular disease and related complications [39]. Flavonoids decreased blood cholesterol levels by decreasing the biosynthesis of cholesterol, enhancing the phosphorylation of 3-hydroxy-3-methylglutaryl coenzyme A reductase indirectly [40].

Phytochemical screening of parsley has revealed the presence of flavonoids (apiin, luteolin, and apigenin-glycosides) and a considerable elevation in albumin concentration was recorded in apigenin-treated mammary adenocarcinoma group [41]. The parsley showed a significant decrease in the serum activity of AST, ALT, and LDH this result indicated that parsley had able to regenerate liver after liver cell damage and reduced incidence of heart disease in diabetes mellitus as it contains flavonoids, particularly the quercetin (as a flavonol) [42]. A variety of flavonoids, lignans, alkaloid, bisbenzyl, coumarins, and terpenes were tested for their antioxidant activity as showed they able to prevent the oxidative damage in epithelial cells kidney and liver [43]. Parsley extract has the ability to reduce the toxic effects may be recognized to the high nutritive value that determined too high percent of vitamins (A, C, riboflavin, and niacin) and minerals (Fe, Mg, P, K, Ca, Na, and Zn) [44].

O. basilicum decreased glucose levels in blood which act via inhibition renal glucose reabsorption and/or inhibition of hepatic glucose production, improving insulin action or stimulate peripheral tissues to utilize glucose [45]. The reduction of plasma total cholesterol and its LDL fraction by the *O. basilicum* extract was associated with an increase in plasma HDL-cholesterol, and the activity of *O. basilicum* extract to decrease cholesterol concentration by stimulating the elimination of cholesterol in the form of bile acids by promoting cholesterol mobilization from peripheral tissues to the liver [46]. There is a significant increase in the amount of protein and globulin levels as *O. basilicum* is immunostimulant herbals incorporated diets helped to increase the humoral elements in the serum [47]. Basil extract decreases the activities of serum AST and ALT due to it increase the level of antioxidant enzymes that may protect liver against the damaging effects and inhibit LPO [48]. Basil is a rich source of flavonoids, and the hepatoprotective effect of *O. basilicum* may be attributed to the antioxidant activity of its flavonoids [4], so the liver of rats from the group treated with aqueous basil extract showed normal liver.

Water-soluble extract of chicory reduced serum glucose may be due to the presence of inulin, oligofructose, and esculetin [49,50]. The decrease in blood cholesterol level might be due to chicory has the ability to stimulate lactic acid producing bacteria which secret the hydrolase enzyme that in-turn converts bile salts into deconjugated bile acids and ultimately resulted in the reduced serum cholesterol level, and the insulin decrease the expressions of acetyl-coenzyme A carboxylase and fatty acid synthase messenger RNA [49,51]. Inulin and oligofructose decrease the synthesis of triglycerides and fatty acids in the liver and decreasing their level in serum by inhibiting hepatic lipogenesis and reducing the risk of atherosclerosis [34]. Significant improvement in albumin serum level of chicory protected rats compared to nitrosamine precursors-treated rats [52]. The administration of chicory supplemented diet resulted in an improvement of protein pattern by preventing protein oxidation and improves liver and other organs functions which synthesized plasma protein [34,50]. The antihepatotoxic effect of chicory due to it contains isoflavones, polyphenols, and other antioxidants that can reduce the activity of serum ALT and AST, and it was noticed that chicory significantly lowers serum activity of AST and ALT enzymes in carbon tetrachloride (CCL_4) intoxicated rats. Liver function was improved due to protective activity of antioxidants components in chicory extract, and it was shown that chicory decreased levels of MDA and increased GSH, antioxidant enzymes in (CCL_4) intoxicated rats [52-55]. Chicory cause elevation of intracellular antioxidant enzyme activities and decreased oxidative stress in tissues due to chicory extract enhance endogenous antioxidant defense status [49,56]. This hepatoprotection of chicory prevent liver damage, and no histopathological changes occur.

CONCLUSION

The parsley, basil, and chicory extracts offered a hepatic protection against Dex-induced in rats. In a comparison of our result, we found that parsley has a potent hepatoprotective effect more than basil and chicory. All the aforementioned effects of extracts may explain their ameliorative impact on Dex changes in our study.

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Original Research

Modification of sleep-waking and electroencephalogram induced by vetiver essential oil inhalation

Dania Cheaha[1], Acharaporn Issuriya[2], Rodiya Manor[3], Jackapun Kwangjai[4], Thitima Rujiralai[5], Ekkasit Kumarnsit[4]

[1]Faculty of Medicine, Princess of Naradhiwas University, Khok Khian, Muang, Narathiwat, 96000, Thailand, [2]Faculty of Veterinary Science (The Establishment Project), Prince of Songkla University, Songkhla 90110, Thailand, [3]Department of Pre-clinic, Faculty of Science and Technology, Prince of Songkla University, Pattani campus, Thailand, Pattani, 94000, Thailand, [4]Department of Physiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand, [5]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand

Address for correspondence:
Ekkasit Kumarnsit, Department of Physiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.
Tel.: +66 74 288210,
Fax: +66 74 446680,
E-mail: Kumarnsit.e@gmail.com

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ABSTRACT

Background: Essential oils (EOs) have been claimed to modulate mental functions though the most of data were obtained from subjective methods of assessment. Direct effects of EO on brain function remained largely to be confirmed with scientific proof. This study aimed to demonstrate quantifiable and reproducible effects of commercial vetiver (*Vetiveria zizanioides*) EO inhalation on sleep-waking and electroencephalogram (EEG) patterns in adult male Wistar rats. The experiments were conducted during November 2013 - February 2014. **Materials and Methods:** The following electrode implantation on the skull, control, and treated animals were subjected for EEG recording while inhaling water and vetiver EO (20 and 200 μl), respectively. Fast Fourier transform was used for analysis of EEG power spectrum. **Results:** One-way ANOVA analysis confirmed that vetiver EO inhalation significantly increased total waking and reduced slow-wave sleep time. Moreover, EO inhalation decreased alpha and beta1 activity in both frontal and parietal cortices and increased gamma activity in the frontal cortex. Changes in these frequencies began almost from the start of the inhalation. **Conclusion:** These data suggest refreshing properties of vetiver EO on electrical brain activity and alertness.

KEY WORDS: Electroencephalogram, essential oil, sleep-waking, vetiver, *Vetiveria zizanioides*

INTRODUCTION

Essential oils (EOs) have been used for the mental improvement since ancient times. However, most of the initial researches have focused mainly on antimicrobial activity [1,2]. Therefore, various physiological effects of EOs have also been consistently confirmed [3,4]. There are many contemporary applications of EOs which are still controversial especially for mood

enhancements. To validate these applications, scientific proof with objective assessments is needed. Most of the direct EO mechanisms in the central nervous system (CNS) have been obtained from animal studies. These included the effects of lavender oil inhalation on rat brain areas [5]. In addition, the effects of long-term lemon oil exposure on behavioral, hormonal, and neuronal parameters were demonstrated in rats [6]. In particular, the induction of dopamine release by green odor from rat striatal brain slices may suggest the underlying mechanisms of the EO that regulate reward, mood and attention [7]. Treatment with EO from *Arachis hypogaea* L. stem and leaf extract led to the increase in adenosine triphosphate levels in the brain of sleep-deprived rats [8]. This might explain central mechanism of sedative effects of the EO.

Recently, the application of EOs for therapeutic purposes has been progressively increasing. Significant improvement in sleep quality was confirmed in postpartum women [9]. Similar results in postpartum women were also seen for lavender fragrance EO [10]. Moreover, increased quality of sleep and reduced anxiety level were observed in patients with coronary artery disease who received lavender EO inhalation [11]. In nursing care for residents who suffered from dementia, anxiety and disturbed sleep patterns, beneficial effects of lavender EO were reported [12]. However, these findings were not well-accepted because aromatherapy has not been well evidence-based practice. Hence, further research with more scientific methodology is, in particular, needed in terms of efficacy confirmation of EO application.

Vetiveria zizanioides, an aromatic perennial grass, is widely grown in many tropical countries. Vetiver EO can be extracted using hydrodistillation of its roots. Vetiver EO exhibited an *in vitro* scavenging activity with β-vetivenene, β-vetivone, and α-vetivone as strong antioxidative constituents [13]. Until recently, vetiver EO was found to produce antioxidant activity against some oxidative stress effects [14].

However, effects of vetiver EO remained to be elucidated in terms of psychological and behavioral activities. The present study aimed to examine the effects of vetiver EO inhalation on sleep-waking and EEG patterns in rats. To mimic the realistic condition, extremely low amounts of vetiver EO were blown with controlled flow rate of ambient air for the inhalation. Following electrode implantation on the skull of rats, electroencephalography was used to record electrical brain wave of animals while receiving vetiver EO inhalation. For analysis, fast Fourier transform (FFT) was used to calculate EEG power to reflect the oscillatory rhythmicity of the brain as a function of frequency. In general, brain wave synchronization was expressed in power of either broad frequency spectrum or discrete frequency bands.

MATERIALS AND METHODS

Animal Surgery

Male Wistar rats weighing 300-350 g (approximately 3-month-old) provided by the Southern Laboratory Animal Facility (Prince of Songkla University, Songkhla, Thailand) were housed in standard environmental conditions (23-25°C, 50-55% humidity and 12/12 hrs light/dark cycle) with freely access to food pellets and water *ad libitum*. The experiments were conducted during November 2013 - February 2014. All the tests were performed during 9.00 AM - 3.00 PM. The experimental protocols in the present study were approved by the Animals Ethical Committee of PSU (MOE 0521.11/1079).

A surgery process was performed as previously described [15]. Briefly, animals were anesthetized with an intramuscular injection of 60 mg/kg Zoletil® 100 (Virbac, Thailand Co. Ltd.). Stainless steel screw electrodes were stereotaxically implanted in the frontal cortex (AP; +3, ML; 3) and the parietal cortex (AP; –4, ML; 4) on the left side skull. The reference and ground electrodes were placed at midline over the cerebellum. Bipolar electromyogram (EMG) electrodes were inserted into bilateral dorsal neck muscles. All electrodes were secured in place with acrylic resin (Unifast Trad, Japan).

Vetiver EO Analyses

The vetiver EO was purchased from the Doi Kham shop, the Royal Project Foundation in 2012. It was diluted with dichloromethane and analyzed by a DSQII gas chromatograph equipped with a quadrupole mass analyzer (ThermoScientific, USA). Thermo Xcalibur 2.2 software was used for data acquisition. The analysis was performed using a ZB-5MS, 5% diphenyl-95% polydimethylsiloxane capillary column (30 m × 0.25 mm I. D., 0.25 μm film thickness). The column temperature was initially held at 40°C and then increased to 230°C at 4°C min^{-1} with a final hold time of 5 min. Helium was used as a carrier gas at a constant flow rate of 1 mL min^{-1}. About 1 μl of the diluted sample was injected in the split mode at a ratio of 1:10. Injector, MS transfer line, and ion source temperatures were set at 230°C, 240°C and 220°C, respectively. Ionization mode was electron impact (70 eV) in the m/z range 40-300.

Retention indices (RI) of all constituents were calculated by the Kováts indices with references to C_8-C_{25} *n*-alkanes. The oil components were identified by comparison of their RI and mass spectra with data published by Adams [16] and also by matching their mass spectra with reference spectra from The national institute of standards and technology (NIST) database. Gas chromatograph-mass spectrometer (GC-MS) chromatograms and the identified dominant volatile components, as well as their relative peak area percent values, are shown in Figure 1 and Table 1, respectively.

Vetiver EO Inhalation

Inhalation chamber was modified from airtight cylindrical plastic chamber with 25 and 50 cm in diameter and height, respectively. This chamber contains 2 opposite openings for connections with inlet and outlet air tubes (2.5 cm in diameter) at 5 cm above the bottom. The inlet tube was connected with a blowing apparatus for delivering ambient air or smell into the chamber and the tube on the other side was for outflow

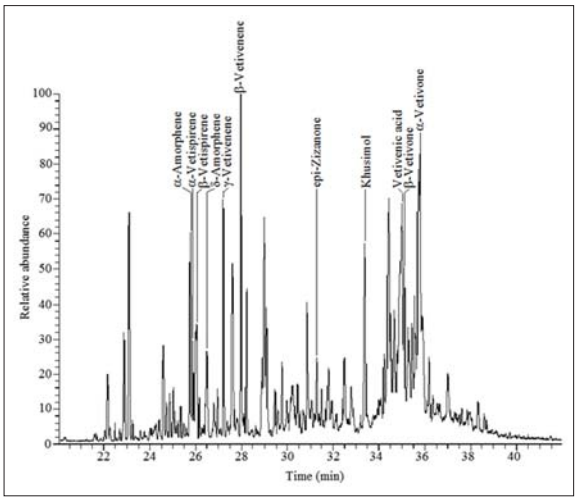

Figure 1: Gas chromatograph-mass spectrometer chromatogram of vetiver essential oil

Table 1: Chemical compositions of vetiver essential oil by GC-MS analysis

Retention time (min)	Chemical composition	RI[a]	Area (%)
22.87	91, 105, 119, 175, 190	1385	1.47
23.09	91, 105, 119, 175, 190	1392	2.94
24.60	105, 119, 161, 175, 190	1440	1.21
25.76	α-Amorphene	1477	2.70
25.89	α-Vetispirene	1481	1.42
26.00	δ-Selinene	1485	1.37
26.05	β-Vetispirene	1486	1.35
26.50	δ-Amorphene	1500	1.27
27.21	γ-Vetivenene	1525	3.08
27.60	81, 95, 109, 157, 191, 220	1538	2.60
27.98	β-Vetivenene	1551	4.45
28.21	Vetivenene derivative	1559	1.95
28.98	55, 81, 91, 123, 177, 220	1584	4.00
29.10	81,95, 109, 177, 205, 220	1588	2.12
29.76	40, 91, 187, 202	1610	1.19
31.29	epi-Zizanone	1665	1.28
32.78	Vetiselinenol	1719	1.20
33.38	Khusimol	1741	3.89
34.43	91, 131, 145, 191, 219	1780	5.56
34.52	41, 81, 95, 109, 137, 218	1784	1.85
34.67	41, 81, 95, 108, 175, 218	1789	1.99
34.99	Vetivenic acid	1801	7.65
35 11	β-Vetivone	1806	2.31
35.80	α-Vetivone	1832	9.04

[a]Retention indices calculated by Kovats indices, GC-MS: Gas chromatograph-mass spectrometer

ventilation to prevent carbon dioxide buildup. The blowing apparatus was made from electric fan with the flow rate at 0.41 m^3/min.

Animals were divided into control ($n = 6$) and 2 treated groups to receive water and vetiver EO (20 and 200 μl, $n = 6$ each), respectively. In general, olfactory perception is essential for detection of the olfactory stimulus but not for discrimination of olfactory intensity. Dose management of vetiver EO inhalation remained to be explored. To examine sleep response to vetiver EO inhalation, a broad range of vetiver EO quantity was chosen to ensure that it is sufficient to have CNS effects.

Animals were individually placed into the inhalation chamber. A baseline recording was performed with ambient air inhalation for 30 min and followed with either water or vetiver EO inhalation for 1 h. The EO was pipetted onto a cotton wool inside the blowing apparatus and remained for the whole period of recording. Chambers were cleaned up with 70% ethanol solution between trials to prevent transmission of olfactory cues.

EEG Recording and Data Processing

EEG signals were amplified and digitized at 400 Hz (sampling rate) by a PowerLab/4SP system (AD Instruments, Australia) with 12-bit A/D, and stored in a PC through the LabChart program software version 7.3.7 (AD Instruments, Australia). EEG signals were processed through 1.25-45 Hz digital bandpass filter. The signals were converted to power spectra by the FFT algorithm which embedded in LabChart software (Hanning window cosine transform, FFT size = 1024-point, 50% overlap). Then, the power spectra of 2.56 s sweeps of selected periods were averaged to give the power spectra of the period. The power spectra were divided into 7 frequency bands according to previous report [15,17]: Delta, 0.8-4.3 Hz; Theta, 4.7-8.2 Hz; Alpha, 8.6-12.1 Hz; Beta1, 12.5-18.0 Hz; Beta2, 18.4-30.1 Hz; and Gamma, 30.5-45 Hz. EEG powers in each frequency band of each group were averaged and expressed as percent baseline power.

Statistical Analysis

Percent baseline power of EEG signal was calculated from pre- and post-treatment periods. Baseline values were calculated from pre-treatment EEG and set to 100%, whereas posttreatment values were referenced with that of baseline to obtain percent baseline values. Data of frontal and parietal EEG were expressed as mean ± standard error of mean. Significant differences between treatments were considered using one-way ANOVA, followed by Student-Newman-Keuls method ($P < 0.05$, 0.01, and 0.001 for *, **, and ***, respectively). All statistics were calculated using Microsoft Excel version 2013 (Microsoft Corporation).

RESULTS

Chemical Analysis of Vetiver EO

Compositions in vetiver oil identified by GC-MS were listed in Table 1. β-vetivenene, khusimol, vetivenic acid, α-vetivone were found to be the major components of vetiver oils [Figure 1].

Effects of Vetiver EO Inhalation on Sleep-Waking State

Frontal and parietal EEG and EMG were collectively analyzed for classification of brain states [Figure 2]. Waking periods were characterized by overall desynchronized brain wave as seen in low power. In contrast, high amplitudes of low-frequency power were criteria of slow-wave sleep. Rapid eye movement (REM) sleeps were identified with the dominant parietal theta wave. EMG activity was obviously high during waking period

but almost absent during REM sleep. Therefore, fragments of each brain state were summed and shown in 3 categories of brain state [Figure 3]. One-way ANOVA confirmed that EO inhalation significantly increased total awake period (F [2, 17] = 11.48, $P < 0.001$). The inhalation was found to result in 2 folds of waking time for both doses of vetiver EO. In contrast, the inhalation significantly decreased total slow wave sleep (F [2, 17] = 11.125, $P = 0.001$]. However, no significant change in REM sleep was detected.

Effects of Vetiver EO Inhalation on EEG Power Spectrum

Power spectrum of EEG signals following the inhalation was shown in frequency domain from 1 to 45 Hz. In frontal EEG, vetiver EO inhalation was found to significantly reduce power of slow frequency range and enhance that of high-frequency range [Figure 4a]. When each frequency band was individually analyzed, significant increases were found in alpha (F [2, 19] = 3.67, $P = 0.047$) and beta1 (F [2, 19] = 4.33, $P = 0.030$) bands [Figure 4b]. It was contrast for high frequency band as gamma power was significantly increased by the inhalation (F [2, 19] = 6.149, $P = 0.010$).

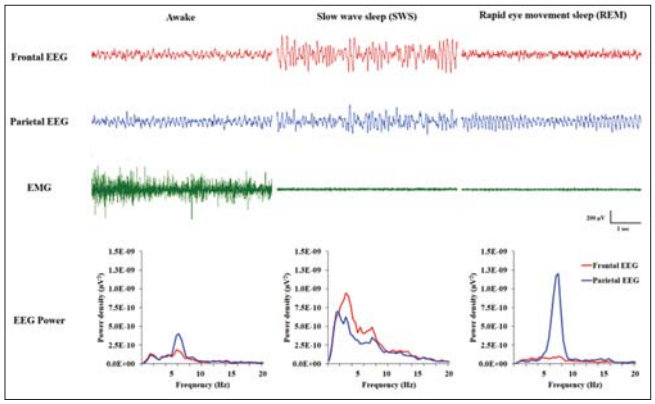

Figure 2: Representative electroencephalogram (EEG) and electromyogram signals from awake, slow-wave sleep and rapid eye movement sleep periods. EEG powers of signals from frontal and parietal cortices were analyzed to identify brain states

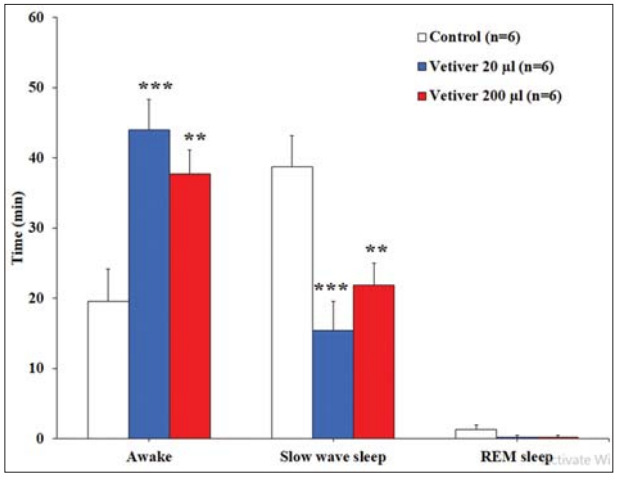

Figure 3: Effects of vetiver essential oils inhalation on sleep-waking. **, *** = $p < 0.01$, $p < 0.001$, respectively

Similar findings were found in parietal EEG [Figure 4c]. Vetiver EO inhalation significantly reduced powers of alpha (F [2, 19] = 6.57, $P = 0.008$) and beta1 (F [2, 19] = 5.902, $P = 0.011$) waves [Figure 4d].

Time-course Effects of Vetiver EO Inhalation

In frontal EEG, the effect of vetiver EO inhalation on slow wave activity (0.8 - 18 Hz) was particularly monitored. The results showed that the inhalation of 200 µl appeared to decrease slow wave power within a few minutes from the start [Figure 5]. The decrease of slow wave activity persisted until 1 h. On the other hand, the increase in gamma activity induced by vetiver EO inhalation was immediately observed and also remained for the whole period of recording [Figure 6].

DISCUSSION

Most of previous EO studies have confirmed the effects of EOs on the CNS by measuring behavioral, hormonal, cellular, and molecular parameters. These investigations used relatively indirect techniques that detected some physiological changes following EO administration. However, they revealed the potential of EOs in modifying CNS functions that regulate reward [18], mood [19-21], attention [22], and memory and learning [23]. In particular, there are only few studies that confirmed EO effects on the CNS by using electrical brain oscillations. Recently, the findings from our previous work clearly indicated the modification of electrical brain activity induced by orange (*Citrus* sp.) EO inhalation [24]. Increasing of slow wave oscillation (4-12 Hz) by orange EO suggested possible anxiolytic or sedative effects reported previously [24]. These data appeared to indicate that brain oscillations reflect spontaneous and direct activity of neural network circuits associated with behavioral status. Therefore, recording of EEG or electrocorticography might be the highly sensitive method to monitor effects of EOs on neural network activity.

In folklore medicine, *V. zizanioides* Linn or vetiver. has been used in the treatment of various illnesses including mental and emotional symptoms. In modern scientific studies, it was claimed to reduce oxidative stress according to its antioxidative [14,25] and scavenging [26] activities. The present study demonstrated the stimulating effects of vetiver EO inhalation with increased total awake period, gamma EEG power and decreased slow wave EEG power. Alterations of electrical brain wave by EOs have been reported in animal models. Previously, inhalation of citrus EO produced EEG patterns in both frontal and parietal cortices which might be associated with anxiolytic-like effect [24]. In addition, dose-dependent increase in the power of fast wave in the hippocampus and the cerebral cortex was also produced following intraperitoneal injection of bergamot EO [27]. Altogether, these findings collectively suggest that EOs are capable of modifying CNS functions. However, EOS are supposed to be applied through inhalation as the most common

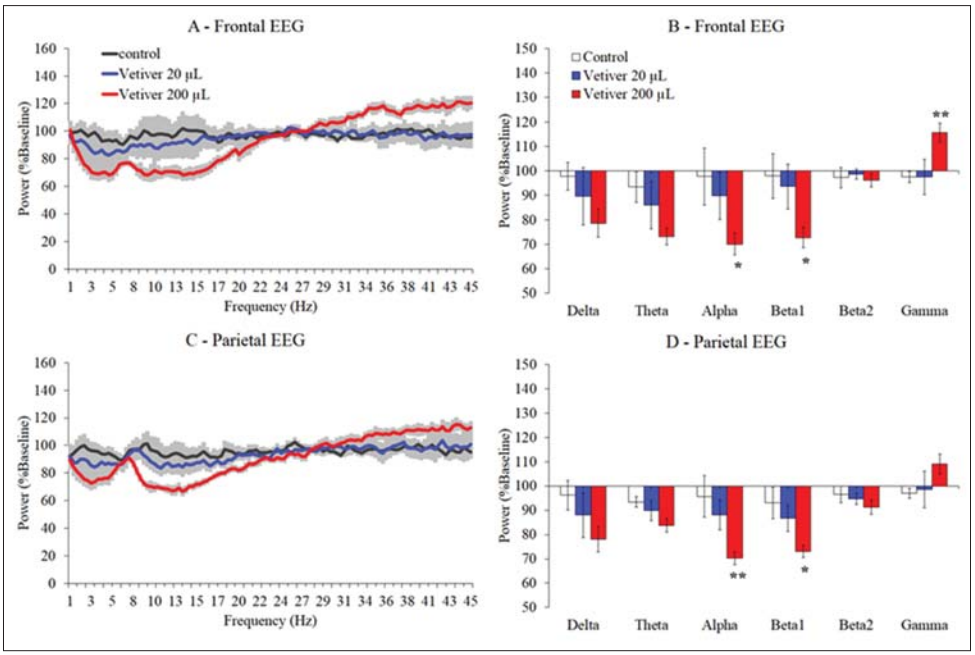

Figure 4: Effects of vetiver essential oils inhalation on frontal EEG power spectrum and 6 discrete band powers in frontal cortex (a and b respectively) and parietal cortex (c and d respectively). *, ** = $p < 0.05$, $p < 0.01$, respectively

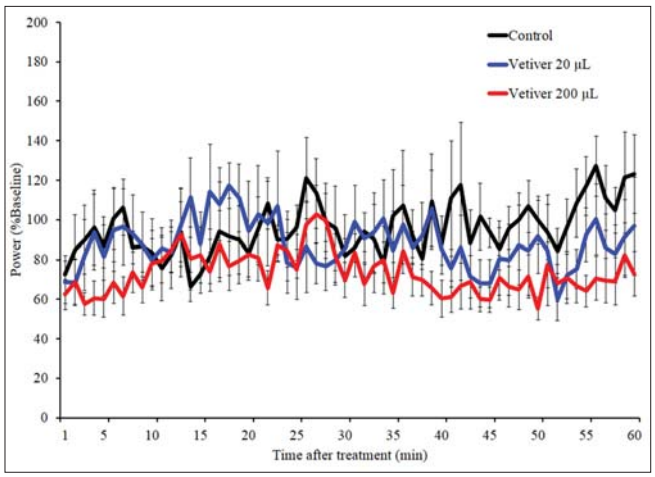

Figure 5: Effects of vetiver essential oils inhalation on frontal slow wave activity

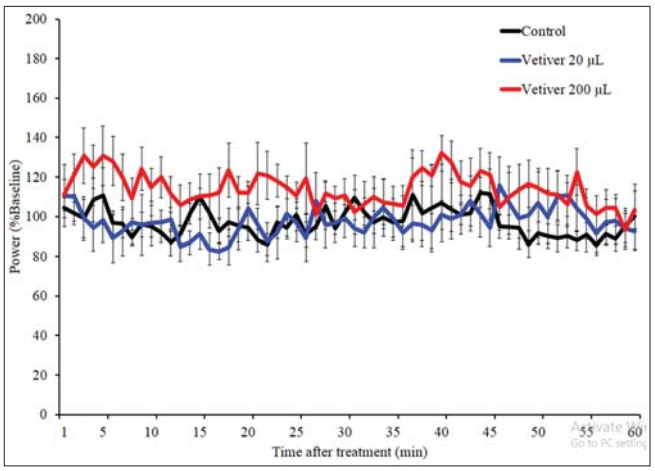

Figure 6: Effects of vetiver essential oils inhalation on frontal gamma activity

route for volatile substances. Therefore, it was an ideal purpose to examine the effects of inhaled vetiver EO on EEG pattern. As shown in the results, significant changes in EEG activity were seen following vetiver EO inhalation though the weight of the EO (in cotton wool) remained almost unchanged after the test. It means that the administration system ventilated extremely low amounts of the EO through recording chamber. This suggests that inhalation is probably the most effective route of EO administration. Previously, the inhalation has been consistently found to deliver EO to various organs including the brain and produce anxiolytic-like effect [28].

There are some advantages of EEG studies. With the use of FFT, it is possible to measure power in discrete frequency ranges of the EEG spectrum. Basically, slow frequency power increases during sleep, whereas fast frequency power is associated with wakefulness [29]. Mainly, overall brain wave activity can be overviewed in broad EEG power spectrum. Power alteration of some discrete frequency bands clearly reflects specific patterns of brain activity. In general, the decrease power in slow-wave activity and/or increase power in fast wave was positively correlated with increased alertness [30,31]. Moreover, EEG technique provides continuous data that reflect time course effect of EO treatment. In the present findings, refreshing action of vetiver EO was detected immediately after the inhalation started and persisted at least 1 h. This data would be beneficial for the odor management.

Most of previous reports of vetiver studies have focused on its anxiolytic or sedative effects proposed to be through GABAergic

activities [32,33]. Moreover, it was also demonstrated to produce significant anticonvulsant activity in animal model [34]. In general, anticonvulsant drugs such as benzodiazepines and valproic acid are used against seizures both in patients and in animals by enhancing GABAergic synaptic transmission [35]. These findings might suggest sedative and hypnotic properties. However, the results from the present study indicated stimulating effect of vetiver EO. Apart from its anxiolytic or sedative property, the plant has also been tested for other CNS effects. In a study using mice model of amnesia induced by scopolamine, different extracts of vetiver significantly inhibited acetylcholinesterase activity and reversed the amnesia [36]. Consistently, the ethanolic extracts of this plant were found to enhance learning and memory possibly through cholinergic transmission in the brain [32]. Altogether, vetiver appeared to be capable of inducing learning process in the brain and exploratory behavior that would probably results in increasing alertness and fast frequency oscillation.

In terms of application, alertness is one of the basic factors that would enhance physical and mental performances. Previously, treatment with EO from *Acori graminei* rhizome resulted in improvement of cognitive performances and increased levels of neurotransmitters especially monoamines that would enhance wakefulness [23]. The use of vetiver EO for refreshing might be an alternative choice in addition to the consumption of tea or coffee. In terminal task, human subjects who inhaled the volatile compounds of vetiver EO showed faster reaction times and stimulation of sympathetic nerve activity [37]. Its volatile compounds may help subjects to maintain performance in a visual discrimination task. Ultimately, the stimulating effects of vetiver EO might be beneficial for learning and memory processes. In conclusion, the present findings provide information that vetiver EO may be used as a stimulant to improve alertness and task performance.

ACKNOWLEDGMENTS

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Original Research

Biological synthesis of silver nanoparticles from *Adansonia digitata* L. fruit pulp extract, characterization, and its antimicrobial properties

Chennareddy Maruthi Kesava Kumar, Pulicherla Yugandhar, Nataru Savithramma

Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India

Address for correspondence:
Pulicherla Yugandhar, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India.
E-mail: yugandharbotany@gmail.com

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ABSTRACT

Aim: In the present study, we report a cost-effective, eco-friendly, and an efficient alternative method for large scale production of silver nanoparticles (AgNPs) from *Adansonia digitata* fruit pulp extract. The study mainly focused on the synthesis, characterization, and antimicrobial properties of AgNPs. **Materials and Methods:** Synthesis of AgNPs with the help of standard protocol and characterized by ultraviolet (UV)-vis spectrophotometry, Fourier transform infra-red (FTIR), X-ray diffractometer (XRD), atomic force microscopy (AFM), scanning electron microscopy (SEM) with EDAX, transmission electron microscopy (TEM) and explore their potential growth inhibitory effect on 07 bacterial and 05 fungal pathogens. **Results:** The synthesized AgNPs are characterized by UV-vis spectrophotometry shows a broad peak at 434 nm. The FTIR spectroscopic analysis clearly reveals phenols and proteins are main responsible for reduction and stabilization of nanoparticles. XRD studies show the nanoparticles are crystalline in nature owing 44 nm in size. EDAX spectrum shows a 33.28 weight percentage of Ag metal in the reaction medium confirms the purity of AgNPs. High resolution and magnification studies with AFM, SEM, and TEM reveal the nanoparticles are polydispersed, spherical in shape, having the size range from 3 to 57 nm without any agglomeration between the particles. Further, the antimicrobial studies reveal the potentiality of nanoparticles against different microbial pathogens. **Conclusion:** The present study is mainly focused on the synthesis of AgNPs from *A. digitata* fruit pulp extract. Here, we succeed to synthesize a narrow range of particles and validate its potential antimicrobial activity on different microorganisms. Based on this, we conclude that *A. digitata* pulp extract is a good source toward the reduction of AgNPs and acts as environment benign antimicrobial agents.

KEY WORDS: *Adansonia digitata*, antimicrobial activity, characterization, fruit pulp, silver nanoparticles

INTRODUCTION

Synthesis of metal nanoparticles with the help of plant extracts is an emerging field of nanotechnology, due to their novel properties, terrific applications in biomedicine and its eco-friendly nature [1]. In recent past great efforts were made for synthesis of environment benign and eco-friendly nanoparticle from plants such as iron oxide nanoparticles from *Medicago sativa* [2], copper nanoparticles from *Magnolia kobus* [3], calcium nanoparticles from *Boswellia ovalifoliolata* [4], gold nanoparticles from *Avena sativa* [5], zinc oxide nanoparticles from *Catharanthus roseus* [6], and silver nanoparticles (AgNPs) from *Syzygium alternifolium* [7]. Silver is one among the metal nanoparticles focused much interest due to its wide variety of applications [8]. It has different biological activities such as antimicrobial [9], anthelmintic [10], antilarvicidic [11], antioxidant [12], anticancer [13], anti-inflammatory [14], hepatoprotective [15], and wound healing activity [10]. Conventional methods for synthesizing AgNPs are mainly by chemical, physical, and microbe-mediated synthesis. In these chemical and physical methods, usage of hazardous chemicals, high energy requirements, difficult and wasteful materials generate potential and biological hazards to the environment [16]. Whereas in the case of microbe-mediated synthesis is not feasible industrially due its lab maintenance. Therefore biological synthesis of AgNPs by using plant materials is easy, efficient, and eco-friendliness in comparison to chemical mediated or microbe-mediated synthesis [17] and they possess secondary metabolites having the redox capacity to reduce metal nanoparticles in an easiest way [18].

Adansonia digitata L. belongs to the family Malvaceae is a large tree indigenous to Africa and found in many countries also. Traditionally, the fruit pulp dissolved in water or milk is used as a drink or sauce for food in Africa. Fruit pulp and powdered seeds are used for the treatment of diarrhea and dysentery in India [19]. The high content of Vitamin C in fruit pulp is recommended to pregnant women's for daily intake [20]. In recent times, different scientists prove different activities of pulp such as hepatoprotective [21], antimicrobial [22], antiviral [23], antioxidant [24], antidiarrheal [25], hypoglycemic [26], anti-inflammatory, and antioxidant [27] activities. Due to high medicinal values and mythological significance of this plant is known as "Kalpavriksha" (a tree which fulfill all desires) in India [28]. In our previous studies, synthesis of AgNPs from stem bark and leaf extract of *A. digitata* acts as excellent reducing agents and show potential antimicrobial activity against different microorganisms [29,30]. However, the potentiality of fruit pulp mediated synthesis of AgNPs is not carried out so far. Hence, the present study is aimed to synthesize AgNPs from *A. digitata* fruit pulp extract, characterize and to know the potentiality of AgNPs against different microbial pathogens.

MATERIALS AND METHODS

Collection of Plant Material

2 to 4 kg weight mature fruits possess a great amount of pulp is collected from Acharya Ranga Agricultural University, Tirupati and cross checked by herbarium (Voucher no: SVU362) deposited in Department of Botany, Sri Venkateswara University, Tirupati. Cut open the fruit and separate the seeds adhesive to pulp. Grounded the collected pulp with the help of mortar and pestle, sieved the content for the synthesis of nanoparticles.

Extract Preparation and Synthesis of AgNPs

For synthesis, 25 g of fruit pulp is extracted with 100 ml of distilled water on boiling water bath for 30 min, filter the content with Whatman No. 1 filter paper and stored at room temperature. From this, 5 ml of the extract is taken into 250 ml of Erlenmeyer conical flask and titrated against with the solution of 1 mM $AgNO_3$ at a 60-80°C temperature. The contents are cooled and centrifuged at 10,000 rpm for 20 min to avoid the presence of any biological impurities, and it is used for further characterization and antimicrobial studies.

Characterization of AgNPs

Confirmation of synthesized nanoparticles is AgNPs by ultraviolet (UV)-vis spectrophotometry absorption spectra using a spectro UV 2080 double beam, between the wavelength scan range of 190-700 nm, 1200 L/mm spectrophotometer, Analytical technologies, India. To know the possible bio-molecules responsible for reduction and stabilization of AgNPs by Fourier transform infra-red (FTIR) spectra in the scan range of 4,000 to 500 cm^{-1} transmittance with an ALPHA interferometer, Bruker, Ettlingen, Karlsruhe, Germany by KBr pellet method. X-ray diffraction of synthesized nanoparticles is examined by an X-ray diffractometer (XRD) (Shimadzu, XRD-6000) equipped with Cu Kα radiation source using Ni as a filter at a setting of 30 kV/30 mA to know the crystalline nature of AgNPs. Purity of AgNPs was analyzed by FEI Quanta 200 FEG high resolution (HR)-scanning electron microscopy (SEM) machine equipped with EDAX instrument. To know the size, shape, agglomeration pattern, and dispersed nature of the nanoparticles are done by atomic force microscopy (AFM) by NOVA NT-MDT SOLVER NEXT, Russia. SEM by FEI Quanta 200 FEG HR-SEM machine. Transmission electron microscopy (TEM) using HF-3300 advanced 300 kV TEM/STEM from Hitachi.

Antimicrobial Studies of AgNPs

The antimicrobial activity of biologically synthesized nanoparticles are analyzed on seven pathogenic bacteria such as *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538 (Gram-positive), *Escherichia coli* ATCC 25922, *Klebsiella pneumonia* ATCC 43816, *Proteus vulgaris* ATCC 13315, *Pseudomonas aeruginosa* ATCC 15442, *Salmonella typhimurium* ATCC 14028 (Gram-negative), and five fungal pathogens such as *Alternaria solani* ATCC 32904, *Aspergillus flavus* ATCC 9643, *Aspergillus niger* ATCC 16404, *Penicillium chrysogenum* ATCC 11709, and *Trichoderma harzianum* ATCC 20476 procured from Department of Microbiology, Sri Venkateswara University, Tirupati. Disc diffusion method [31] was followed for testing antimicrobial activity against biologically synthesized AgNPs, and comparative studies were made with plant pulp extract, 1 mM $AgNO_3$ as negative controls and streptomycin or fluconazole as a standard for bacteria and fungi, respectively. 7 mm sterile discs are prepared from Whatman no. 1 filter paper and 20 μl of plant extract, 1 mM $AgNO_3$ solutions, and 10 μg/disc streptomycin/fluconazole are loaded on separate discs and allowed to air dry for 1 h at sterile conditions. Apart from these, a 5, 10, 20, 40, 60, 80 μg/ml concentrations of synthesized AgNPs are tested separately to know the minimum inhibitory concentration. 20 and 40 μg/ml concentration of AgNPs show minimum inhibitory effect and 80 μg/ml concentrations of AgNPs show maximum inhibitory concentration. Due to this, we prefer 80 μg/ml concentrations of AgNPs to check the antimicrobial activity on different microbial organisms. Freshly prepared nutrient agar media for bacteria and potato dextrose agar media for fungi are poured into sterile petri plates, allowed to 30 min for solidification. The plates are swabbed with 100 μl of microbial cultures and placed the previously prepared discs; the experiment is carried out in triplicates. The plates are incubated at 37°C for 24-48 h, and then the zone of inhibition is measured with the help of a scale and tabulated the results.

RESULTS

Reduction of Ag^+ into Ag^0 nanoparticles was observed visually by means of a color change pattern of the reaction medium. *A. digitata* fruit pulp having thick cream color, upon synthesis the color change from creamy to light yellow indicates the formation of nanoparticles. This color changed nanoparticles solution was analyzed by UV-vis spectrophotometry shows a broad absorption peak at 434 nm further confirms the formation of nanoparticles

are AgNPs [Figure 1]. FTIR spectroscopic studies of these synthesized nanoparticles show broad transmittance peaks at 3322 cm^{-1} assigned for an O-H bond of phenols and 1636 cm^{-1} assigned for an N-H bond of primary amines [Figure 2]. XRD spectrum of synthesized AgNPs shows the crystalline nature of AgNPs and gives four intensive peaks at 38.1°, 46.2°, 64.5°, and 77.3° of 2θ degrees of X-axis corresponds to 111, 200, 220, and 311 Bragg reflections of Y-axis [Figure 3]. These peaks are indexing the face-centered cubic nature of AgNPs. The mean particle diameter of synthesized AgNPs is 44 nm, calculated according to Debye-Scherrer equation ($D = k\lambda/\beta \cos \theta$) and it coincides with powder diffraction file of Joint Committee on Powder Diffraction Standards file No. 04-0783 [32]. The full width at half maximum values, i.e. k = 0.44 was derived from 38, 46, 64, and 77 Bragg reflections of the X-axis. 2 μm resolution studies of biologically synthesized AgNPs with AFM reveal the particles are polydispersed, spherical in shape, having the size range from 25 to 57 nm, and there is no agglomeration observed between the particles [Figure 4a]. Raw data obtained from this AFM microscope is treated with a specially designed image processing software (NOVA-TX) to further exploit the three-dimensional (3D) image of nanoparticles [Figure 4b]. 500 nm resolution studies with 20 kV electron energy passing through the nanoparticles coated thin films on a clean glass slide reveals the nanoparticles are spherical in shape, polydispersed and having the size range from 18 to 32 nm [Figure 5a]. The same sample was analyzed with the help of EDAX instrument shows 33.28% presence of Ag^0 in the sample [Figure 5b] along with 05.29% of carbon, 03.28% of nitrogen, 31.80% of oxygen, 08.37% of sodium, 02.57% of magnesium, 01.01% of aluminum, 00.78% of silicon, 07.87% of aurum, and 05.74% of calcium [Table 1]. 33.28% of Ag metal in the sample indicates the high purity of

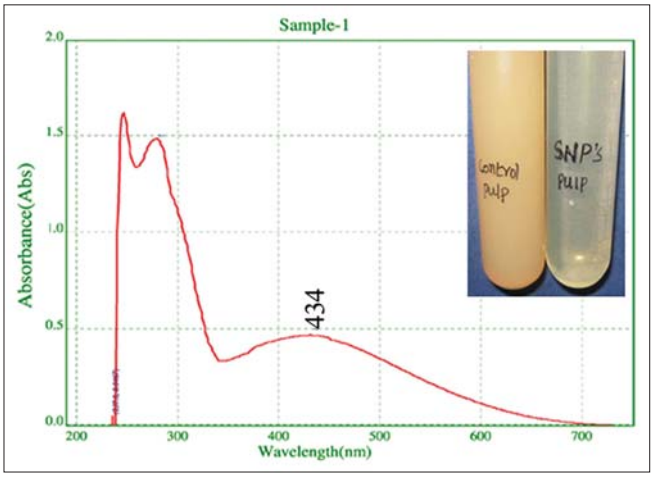

Figure 1: Ultraviolet-vis spectrum of synthesized silver nanoparticles shows broad peak at 434, 280, and 247 nm of narrow peaks is due interference of phytoconstituents in the medium. Inset figure shows color change pattern from cream to light yellow

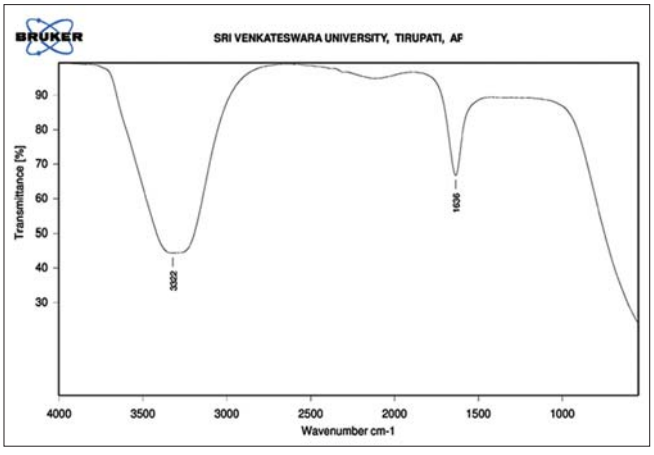

Figure 2: Fourier transform infra-red spectrum of synthesized silver nanoparticles shows broad peaks at 3322 cm^{-1} and 1636 cm^{-1}

Table 1: Metal analysis by EDAX spectrum shows different weight percentages of sample

Element	Weight (%)
C	05.29
N	03.28
O	31.80
Na	08.37
Mg	02.57
Al	01.01
Si	00.78
Au	07.87
Ag	33.28
Ca	05.74

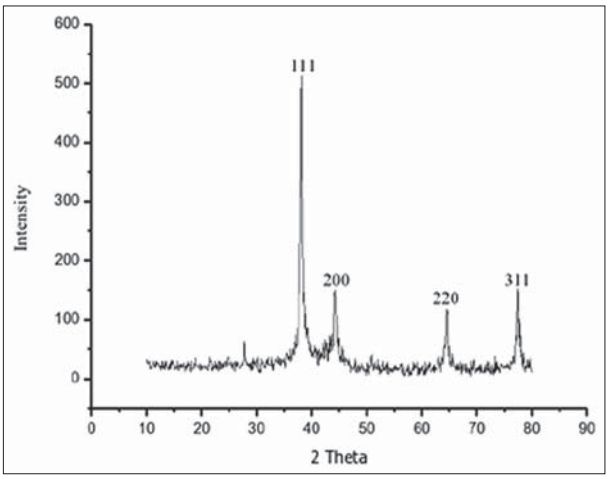

Figure 3: X-ray diffractometer pattern of synthesized silver nanoparticles shows four intensive peaks

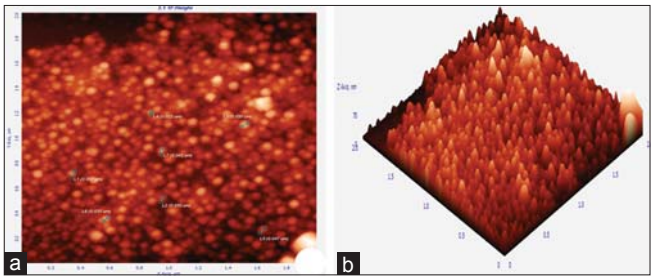

Figure 4: Atomic force microscopy micrograph of synthesized silver nanoparticles (AgNPs), (a) 2 μm resolution studies 25-57 nm size, spherical shaped, polydispersed particles, (b) three-dimensional image of AgNPs analyzed by NOVA-TX software

synthesized nanoparticles. Crystalline nature of synthesized AgNPs was analyzed using selected area electron diffraction (SAED) by directing the electron beam perpendicular to nanoparticles. The SAED pattern of synthesized AgNPs shows the spots had been corresponding to 111, 200, 220, and 311 of the crystallographic nature of face-centered cubic structures [Figure 6a]. 20 nm resolution studies with 300 kV electron energy passing through nanoparticles coated thin films on copper grid show the nanoparticles are polydispersed, spherical in shape and size range from 3 to 7 nm [Figure 6b]. To know the antimicrobial potency of biologically synthesized AgNPs from fruit pulp of *A. digitata* was analyzed against two Gram-positive, five Gram-negative, and five fungal pathogens by disc diffusion method. Among the bacterial pathogens, the highest inhibition zones were observed in *P. vulgaris* followed by *K. pneumoniae*, *P. aeruginosa*, *S. typhimurium*, *E. coli*, *B. subtilis*, and *S. aureus*. Whereas in the case of fungi, the highest inhibition zones were observed in *T. harzianum*, followed by *A. niger*, *A. flavus*, *P. chrysogenum*, and *A. solani*. [Figure 7 and Table 2].

DISCUSSION

When the addition of $AgNO_3$ solution to the plant extract the color of the plant extract is changed gradually according to the quantitative addition of $AgNO_3$ solution. This color change is due to the reduction of nanoparticles with the help of electrons present in the fruit pulp extract coming from NAD and ascorbic acid as electron donors present in the plant extracts [33]. Significantly higher amount of ascorbic acid, i.e., >300 mg/100 g was reported in fruit pulp of *A. digitata* [34]. This solution was subjected to UV-vis spectrophotometry between the scan ranges of 190-750 nm, a broad peak is obtained at 434 nm. The same type of results was observed in leaf extract mediated synthesis of AgNPs from *Couroupita guianensis* [35]. The color change pattern and broad peak obtained in UV-vis spectrophotometry are due to Surface Plasmon Resonance nature of AgNPs present in the medium. These nanoparticles absorb light at different wavelengths and excited due to charge density at the interface between conductor and insulator to give a respective peak on UV-vis spectrophotometry. FT-IR is a sensitive tool to analyze functional groups present in the biological samples. It relies on the light absorbance between 4000 cm^{-1} to 500 cm^{-1} of the electromagnetic, infrared region. In our case, the synthesized nanoparticles show broad peaks at 3322 cm^{-1} and 1636 cm^{-1}. The same type of results was observed in *Myristica fragrans* seed extract mediated synthesis of AgNPs [36]. From this FTIR study, clearly reveals hydroxyl groups of phenols and amide groups of proteins from plant extract forming a layer to the nanoparticles and acting as a capping agent to prevent agglomeration and providing stability in the medium. Based on these FTIR studies, we suggest that the bio-molecules present in plant extracts play dual role in formation and stabilization to AgNPs.

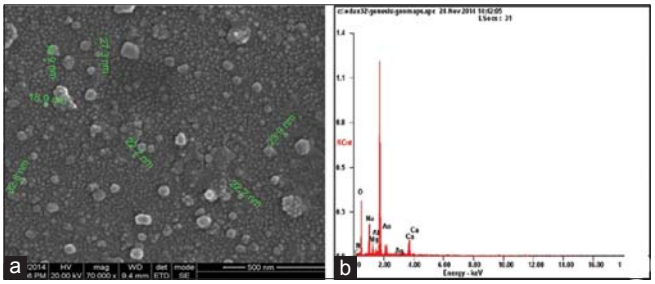

Figure 5: Scanning electron microscopy micrograph of synthesized silver nanoparticles (AgNPs) (a) 500 nm resolution studies shows 18-32 nm size, spherical shaped particles, (b) EDAX spectrum of synthesized AgNPs shows 33.28 weight percent of Ag metal in the sample

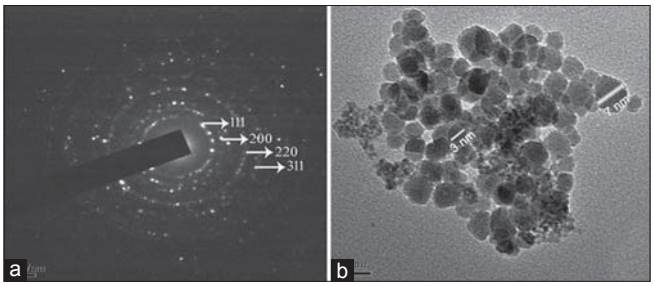

Figure 6: Transmission electron microscopy micrograph of synthesized silver nanoparticles (AgNPs), (a) selected area electron diffraction pattern shows characteristic crystal spots of elemental silver, (b) 20 nm resolution studies of AgNPs shows 3-7 nm size, spherical shaped nanoparticles

Table 2: Zone of inhibition (mm) of AgNPs on microbial pathogens and comparative studies with different controls

Name of the organism	Plant extract (mm)	1 mM $AgNO_3$ solution (mm)	AgNPs		Streptomycin/ fluconazole (mm)
			Disk diffusion assay (mm)	Minimum inhibitory concentration (μg/ml)	
B. subtilis	8.9±0.25	9.3±0.69	19.3±0.96	20±0.00	26.6±1.06
S. aureus	7.3±0.73	9.9±0.86	15.9±1.09	40±0.00	23.2±0.85
E. coli	7.2±0.48	8.1±0.38	22.0±0.38	20±0.00	35.9±0.69
K. pneumoniae	8.9±0.38	14.1±0.53	25.8±1.22	20±0.00	36.3±0.78
P. vulgaris	10.3±0.18	11.3±0.81	27.3±0.72	20±0.00	37.1±0.67
P. aeruginosa	7.5±0.38	9.3±0.20	24.5±0.91	20±0.00	33.2±0.70
S. typhimurium	9.1±0.43	9.4±0.25	24.4±0.60	20±0.00	34.6±0.32
A. solani	0	8.2±0.43	10.1±0.39	40±0.00	17.5±0.62
A. flavus	8.1±0.39	8.0±0.10	12.2±0.44	40±0.00	18.1±0.42
A. niger	0	0	12.4±0.21	40±0.00	20.3±0.32
P. chrysogenum	0	8.2±0.25	11.4±0.17	40±0.00	17.3±0.36
T. harzianum	0	9.0±0.40	14.4±0.17	40±0.00	24.4±0.32

AgNPs: Silver nanoparticles, *B. subtilis: Bacillus subtilis, S. aureus: Staphylococcus aureus, E. coli: Escherichia coli, K. pneumonia: Klebsiella pneumonia, P. vulgaris: Proteus vulgaris, P. aeruginosa: Pseudomonas aeruginosa, S. typhimurium: Salmonella typhimurium, A. solani: Alternaria solani, A. flavus: Aspergillus flavus, A. niger: Aspergillus niger, P. chrysogenum: Penicillium chrysogenum, T. harzianum: Trichoderma harzianum*

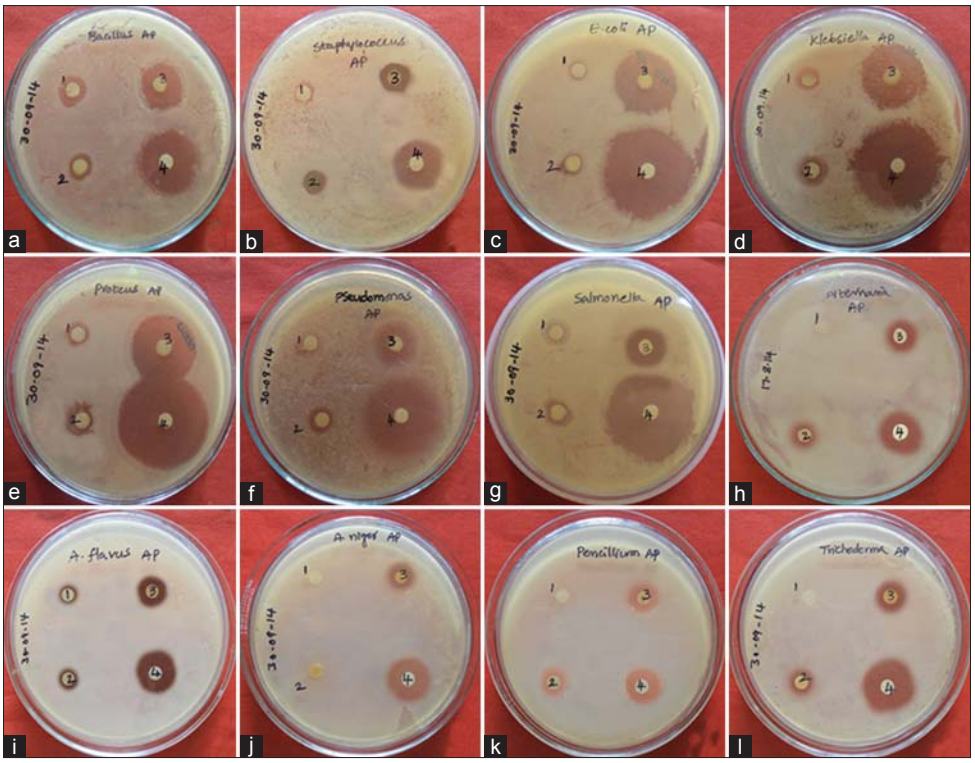

Figure 7: Antimicrobial studies of biologically synthesized silver nanoparticles (AgNPs) from *Adansonia digitata* pulp extract, (a) *Bacillus subtilis*, (b) *Staphylococcus aureus*, (c) *Escherichia coli*, (d) *Klebsiella pneumoniae*, (e) *Proteus vulgaris*, (f) *Pseudomonas aeruginosa*, (g) *Salmonella typhimurium*, (h) *Alternaria solani*, (i) *Aspergillus flavus*, (j) *Aspergillus niger*, (k) *Penicillium chrysogenum*, (l) *Trichoderma harzianum*, (1) Plant extract, (2) $AgNO_3$, (3) AgNPs, (4) streptomycin/fluconazole

AFM is a primary tool for analyzing size, shape, agglomeration pattern and also offers visualizations of 3D views of the nanoparticles, unlike the electron microscopes. It has an advantage over combination of HR, samples do not have to be conductive and does not require the high-pressure vacuum conditions. Better resolution and percentage presence of nanoparticles were demonstrated by SEM with EDAX instrument provides reliable characterization and morphology of the particles when compare to AFM. The four intensive Bragg reflections are appear in XRD pattern [Figure 3] is correlated with SAED pattern [Figure 6a] of AgNPs, clearly indicates the nanoparticles are crystalline in nature. Higher resolution studies with TEM analysis shows size, shape, and agglomeration pattern of nanoparticles. It achieves better resolution than SEM due to electron energies higher than 20 kV used in SEM. Finally, all the microscopic studies reveal the biologically synthesized AgNPs are spherical in shape, having the size range from 3 to 57 nm polydispersed and no agglomerations were found between the particles.

To test the inhibitory effect of biologically synthesized nanoparticles on different microorganisms shows potential antimicrobial activity. Bacterial species show the highest inhibitory activity when compare to fungi because the cell walls of fungi are made up of chitin is more rigid when compare to bacterial cell walls containing peptidoglycans. Among the bacteria, Gram-negative species show the highest inhibitory zones when to compare to Gram-positive species, because the Gram-negative species containing a less amount of peptidoglycans. Silver is a precious metal used as an effective antimicrobial agent before the advent of AgNPs. Due to overuse of silver products, decreased the efficiency of silver agents as antibiotic. In recent times with the advancement of nanotechnology, the interest in the use of AgNPs as antibacterial agents had been rekindled [37]. Antimicrobial activities of AgNPs are dependent on the size and shape of the particles. Small sized nanoparticles have higher antimicrobial activity than larger particles because they have large surface area to interact bacteria efficiently [38]. In recent times, the scientists produce 30 to 40 nm size, spherical shaped AgNPs from fruit extract of *Vitis vinifera* shows excellent antimicrobial activity against *B. Subtilis* and *K. planticola* [39]. 10 to 70 nm size, spherical shaped AgNPs synthesized from *Emblica officinalis* fruit extract shows potential antimicrobial activity against *S. aureus*, *B. subtilis*, *E. coli*, and *K. Pneumoniae* [40]. *Euphorbia hirta* leaf mediated synthesis of AgNPs having a spherical shape with 40-45 nm size shows good antifungal efficacy [41]. In our studies also 3-57 nm size, spherical shaped AgNPs produced from *A. digitata* pulp extract confirmed by HR microscopic studies with AFM, SEM, and TEM proved these AgNPs are eco-friendly antimicrobial agents against different microorganisms.

CONCLUSION

In the present study, we report an eco-friendly, non-toxic, cost-effective method for synthesis of AgNPs from *A. digitata* pulp extract as reducing agent. In this method, naturally

occurring materials are acts as reducing agents such as biomolecules such as phenols and proteins present in plant extract as a simple and alternative to complex physical or chemical synthetic procedures. 3 to 57 nm size, spherical shaped, polydispersed nanoparticles are produced from A. *digitata* pulp extract confirmed by AFM, SEM, and TEM prove this plant extract as an effective reducing agent for the production of narrow range of nanoparticles by industrial scale. Further antimicrobial studies reveal that small size, spherical shaped particles have immense activity against different microbial pathogens and acts as eco-friendly antimicrobial agents.

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Review Article

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Ayurvedic management of pulmonary tuberculosis: A systematic review

Janmejaya Samal

International Union against Tuberculosis and Lung Disease (The Union), Pune, Maharashtra, India

Address for correspondence:
Janmejaya Samal, International Union against Tuberculosis and Lung Disease (The Union), Pune, Maharashtra, India.
E-mail: janmejaya_samal@yahoo.com

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ABSTRACT

Background: Tuberculosis (TB) is a global public health crisis. 25% of world's TB cases are found in India. Ayurveda, an ancient medical science may offer some solution to this problem. Hence, a systematic review was carried out to assess the role of Ayurveda for the management of TB. **Methodology:** A systematic review was carried out using published literature obtained through "PubMed" until April 2015. The key words used for literature search include "Ayurveda, role and TB." **Results and Discussion:** It was observed that a couple of single and compound drugs have been used for the management of TB. However, none of the studies could reflect the true anti-TB activities of any drug, both single and compound. Two of the studies revealed *in vitro* anti-TB properties of some herbs which can potentially be brought into the realm of a clinical trial to test their efficacy in a human subject. Most of these Ayurvedic therapeutic preparations studied in different clinical settings primarily reflected their adjunct properties for the management of TB. These studies revealed that Ayurvedic therapeutics was able to reduce associated symptoms and the adverse drug effects of ATDs (anti-TB drugs). Furthermore, some of the preparations showed potential hepato-protective properties that can be simultaneously administered with ATDs. **Conclusion:** Distressingly research on the role of Ayurveda in the management of TB is very scanty and mostly limited to adjunct or supportive therapy. Being a global public health crisis, it is highly recommended to carry out clinical trials on TB patients using Ayurvedic drugs and therapeutic regimens.

KEY WORDS: Adjunct therapy, Ayurveda, Herbal Drugs, *Rajayakshma*, Tuberculosis

INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis or the other members of Mycobacterium* complex such as *Mycobacterium africanum* and *Mycobacterium bovis*, known to affect the humans. Since the times immemorial, it has affected many civilizations around the world and has been a major reason for many death tolls during the earlier days. It remains as the number one killer infectious disease among the adult population in developing countries even today. The WHO's 1990 global disease burden report ranked TB in the seventh position and expected to continue the same until 2020 in terms of morbidity [1]. It is a global public health crisis. In India, TB continues to be a devastating health crisis with more than 3, 00,000 deaths, 2.2 million new cases with an economic loss of $23bn (£14.9bn; €20.3bn) each year [2]. In spite of noticeable progress achieved TB always offers newer challenges such multidrug-resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) along with debilitating side effects of anti-TB drugs. In India, TB in the community is managed by a centrally sponsored TB control program known as Revised National TB Control Programme (RNTCP). The main target of this program is 85% cure rate and 70% case detection rate. Since 2007, India has achieved this global target of cure rate and case detection rate. Furthermore, RNTCP has 100% coverage rate under DOTS. Although India has achieved the global targets but the problem does not end there owing to a multiplicity of problems [3]. Despite being a centrally sponsored program and free treatment and diagnostic services people still visit private practitioners at their first point of contact when a symptom arises. Studies report that around 50-80% of the patients visit private practitioners for TB treatment [4]. Currently, Ayurvedic drugs are not a part of RNTCP. However, private practitioners use various types of Ayurvedic medicines, both general practitioners and chest physicians, to support TB management [5]. This ancient medical science can offer some solutions to these problems hence a systematic review was carried out to assess the role of Ayurveda for the management of TB.

Ayurvedic Concept of Pulmonary TB (PTB)

PTB is aptly compared with Rajayakshma in Ayurveda. *Rajayakshma* is primarily attributable to *Dhatukshaya* (tissue emaciation or loss). This process universally initiates the process of pathogenesis in *Rajayakshma* patients. In addition, there is inevitable metabolic dysfunction (Dhatwagninasana), in which rasa (tissue fluid), rakta (blood), mamsa (muscle), meda (adipose tissue), and *sukra* (generative tissue) are lost. This leads to ultimate deterioration of immunity or *ojokshaya*. As per Ayurvedic concepts, an unusual metabolic change occur leading to loss of various *dhatus* (tissue) such as *Ojokshaya*,

sukra, *meda dhatus* to *rasa dhatu* preceding each other, which is known as *Pratilomakshaya* [6].

METHODOLOGY

A systematic search strategy was adopted using "PubMed (US National Library of Medicine, USA)" for the main search process. The key words used for the purpose of PUBMED search include "Ayurveda, role and TB." No limits were adopted such as; journals, years of publication, language, types of articles, or authors, but the articles published in English language only were included for the purpose of review. This was done purposefully to obtain a comprehensive list of articles published until April 2015 without the above-mentioned limits. In the second stage, the total articles obtained from searching the database using the above search criteria was pooled together, and articles were initially screened by reading the "title" and thereafter the "abstracts." Studies not satisfying the inclusion criteria were excluded at these stages. The remaining articles were screened in the final stage by reading the full-text and those not meeting inclusion criteria were excluded. The Figure 1 gives an idea about the flow of information through different phases of this systematic review as per Preferred Reporting Items for Systematic review and Mata Analysis (PRISMA) guidelines (http://www.prisma-statement.org/).

RESULTS

In this section, the results are mentioned in two phases. In the first phase studies related to the Ayurvedic management of TB as an adjunct therapy are delineated and in the next part the studies related to Ayurvedic drugs showing *in-vitro* anti-TB properties are described.

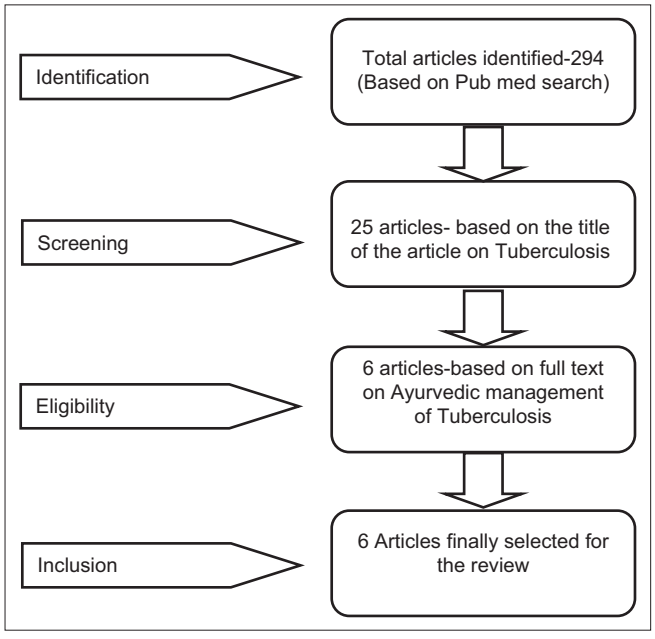

Figure 1: Flow of information through different phases of this systematic review (As per PRISMA guidelines)

Ayurvedic Management of TB (Adjunct and Supportive)

Four different studies conducted in different clinical settings in India evaluated the properties of some of the Ayurvedic therapeutics as adjunct to anti-TB treatment. Table 1 summarizes the properties of some of the Ayurvedic therapeutics as adjunct to anti-TB drugs. Vyas *et al*. conducted a single blind controlled trial to evaluate the adjunct properties of *Rasayana* compound among 133 TB patients receiving "Cat-1" therapy under RNTCP. The *Rasayana* used in this study is composed of *Amalaki* (*Emblica officinalis* Gaertn.)-Pericarp, 1 part, *Guduchi* (*Tinospora cordifolia* willd.)-Stem, 1 part, *Ashwagandha* (*Withania somnifera* L.)-Root, 1 part, *Yashtimadhu* (*Glycyrrhiza glabra* Linn.)-Root, 1 part, *Pippali* (*Piper longum* Linn.)-Fruit, ½ parts, *Sariva* (*Hemidesmus indicus* R.Br.)-Root, ½ Parts, *Kustha* (*Saussurea lappa* Falc.)-Root, ½ parts, *Haridra* (*Curcuma longa* Linn.)-Rhizome, ½ parts and *Kulinjan* (*Alpinia galangal* Linn.)-Rhizome, ¼ parts and administered in capsule form. They conducted the study at OPD level at three different hospitals; (1) Institute of Post Graduate Training and Research in Ayurveda (IPGT and RA), Hospital, Gujarat Ayurveda University, Jamnagar, (2) District TB Centre and Hospital, Jamnagar, and (3) Guru Govind Singh Hospital, P. N. Marg, Jamnagar. The study was carried out for 60 days, and the capsule was administered at a dose of 450 mg. Only sputum smear-positive patients from Category I of PTB or extra PTB and age group of >13 years were selected for the study, rest excluded from the study. The study found that the compound is helpful in alleviating the associated symptoms of PTB in the treatment group (TG) compared to the control group (CG). The results were statistically significant ($P < 0.001$). The *Rasayana compound was found to decrease cough (83%)*, fever (93%), dyspnea (71.3%), hemoptysis (87%), and increase body weight (7.7%) [7].

Dornala and Dornala conducted a study to evaluate the clinical efficacy of *Bhringarajasava* as *Naimittika Rasayana* in PTB. The study was conducted at the Out Patient Department (OPD) of the State TB Training and Demonstration Centre, S.R. Nagar, Hyderabad, Andhra Pradesh, India among 60 PTB patients who were already under directly observed treatment short course (DOTS). Each patient was administered 30 ml of *Bhringarajasava* with an equal quantity of water, ½ an hour after food, thrice a day during the intensive phase of treatment, and followed up to 6-8 months based on treatment category. *Bhringarajasava* is a liquid formulation composed of *Bhringaraja* (Eclipta prostrate Linn.), as active ingredient along with Haritaki (*Terminalia chebula* Retz.), Pippali (*Piper longum* Linn.), Jatiphala (*Myristica fragrance* Houtt.), Lavanga (*Sygizium aromaticum* Linn.), Twak (*Cinnamomum zeylanicum*), Ela (*Elatteria cardamomum*), Tamalapatra (*Cinnamomum tamala*), Nagakesara (*Messua ferrea*), and Gudam (old cane jaggery). The investigators evaluated the role of *Bhringarajasava* in 15 subjective parameters and 3 objective parameters. The role of this formulation on these subjective parameters is as follows; the improvement is mentioned in both TG and in CG; *Amsaparsabhitapah* (pain in costal and scapular region) - 35% in CG and 100% in TG, *Samtapakarapadayoh* (burning sensation in palms and

Table 1: Ayurvedic management of TB as an adjunct therapy

Author	Journal and YOP	Study type	Therapeutic regimen/drugs	Sample size and inclusion criteria	Place of study	Major outcomes
Vyas *et. al.*	AYU Journal/ 2012	Single blind controlled trial	*Rasayana* drug in a capsule form	133 TB patients with Cat-I, type as per RNTCP classification with age >13 years	The study was conducted at OPD level in three hospitals (1 Ayurveda and 2 Modern Hospital)	The compound was found to decrease Cough (83%), fever (93%), Dyspnea (71.3%), Hemoptysis (87%), and increase body weight (7.7%) with high statistical significance ($P<0.001$)
Dornala and Dornala	AYU Journal/ 2012	Clinical Trial	*Bhringarajasava* (A liquid Ayurvedic preparation)	60 patients with Sputum+ve cases (Cat-I), Sputum –ve cases (Cat-III), Relapse, Failure, and Default cases (Cat-II) as per RNTCP categorization at the time of the study	The study was conducted at OPD level in the State TB Training and Demonstration Centre, S.R. Nagar, Hyderabad, Andhra Pradesh, India	The response to the drug is marked ($P<0.05$), moderate ($P>0.05$) and marked ($P<0.01$) in Cat-I, Cat-II, and Cat-III patient, respectively
Debnath *et al.*	JAIM/2012	Clinical Trial	Ashwagandha (*Withania somnifera*) AND Chyawanprash	99 patients undergoing anti-TB treatment	Patipukur TB Hospital annexed to J B Roy State Ayurvedic Medical College and Hospital, Kolkata, India	The symptoms subsided, body weight showed improvement, ESR values were normal, there was an appreciable change in IgA and IgM patterns and significantly increased the bioavailability of isoniazid and pyrazinamide were recorded
Sharma *et al.*	IJTK/2004	Clinical Trial	Group-1, Liv-600 Capsule. Group-2 Decoction of *Bhumyamalaki*	10 patients with Liv-600+10 patients with standardized decoction of *Bhumyamalaki* patients+10 patients with placebo	PG department of Kayachikitsa, Rajiv Gandhi PG Ayurveda College and Hospital, Paprola, HP, India. Patients were selected from OPD and IPD	Both of these preparations exhibited hepato-protective properties compared to the placebo after 12 weeks of treatment on periodic liver functions evaluations

OPD: Outpatient department, IPD: Inpatient department, TB: Tuberculosis

Soles) - 50% in CG and 75% in TG and complete relief in Cat-III, patients, *Jwara* (Pyrexia) - <75% in CG and 100% in TG, *Bhaktadwesha* (Anorexia)-mild in CG and complete in TG, *Swasa* (Dyspnea) - <65% in CG and >90% in TG, *Kasa* (Cough) - moderate relief in CG and progressive relief, later occasional-nonproductive and easy expectoration in TG after 15 days of treatment, *Shonita darshanam* (Hemoptysis) - the relief is not statistically significant among CG and were given another drug but encouraged results were observed in TG, *Swarabheda* (Hoarseness of voice) - the relief is not statistically significant among CG but encouraged results were observed in TG, *Anilath shula* (pain in visceral organs) - complete relief in TG, *Sankochamsaparshyoh* (shoulder and scapular emaciation) - very encouraging in TG patients over the CG, Daha (burning sensation) - complete relief in TG, *Atisara* (Diarrhea) - no manifestation among TG, *Pittaraktasyachagama* (Hematemesis) - manifested in 2 patients and controlled immediately, *Sirasaha paripoonata* (Heaviness in the head) - relieved in 10 days among TG and in CG after 3-4 months, *Kantadwamsa* (Tracheal shift) - observed only in one case with fibrosed and consolidated lung. The role in objective parameters is as follows; Wight loss-in CG there was further weight loss but in TG there was no weight loss and is statistically significant, Sputum Conversion-sputum conversion was found among TG after intensive phase but in CG 10 patients out of 30 again put on intensive phase, Skiagram (chest X-ray [CXR]) - Density of the opacity in the CXR was less than on previous studies, with lessening of the cavities and resolution of fibrotic changes noted in the TG patients [8].

Debnath *et al.* conducted a study to assess the use of adjunct therapy of Ayurvedic medicine with anti-tubercular drugs in the therapeutic management of PTB. It primarily aimed to evaluate the toxicity reduction and early restoration by adjunct therapy of Ayurvedic drugs by increasing the bioavailability of ATDs. The study was conducted among 99 newly diagnosed PTB patients from both the sexes aged between 10 and 65 years. The subjects were administered 500 mg of *Ashwagandha* -2 caps, twice daily and *Chyawanprash* (as per Indian Pharmacopeia)-10 g, thrice daily for a period of 28 days. The study reported amelioration of symptoms, improvement of body weight, normalization of erythrocyte sedimentation rate, appreciable change in IgA and IgM patterns, and significant increase in bioavailability of isoniazid and pyrazinamide. Bioavailability of isoniazid and pyrazinamide showed 7-10% increased value after 28 days of treatment [9].

Sharma *et al.* conducted a study to assess the hepato-protective properties of Ayurvedic herbs among patients receiving anti-TB treatment. The study was conducted among three groups of subjects, each with 10 subjects aged 15-70 years, for a period of 90 days with normal liver Anatomy and Physiology confirmed by ultrasonography and biochemical analysis at the time of

the investigation. Group-1 received capsule Liv-600, thrice a day composed of 200 mg of hydro alcoholic extract of *Berberis aristata*, *Solanum nigrum*, and *Aloe vera*, Group-2 received freshly prepared decoction of *Bhumyamalaki* (*Phyllanthus fraternus*) prepared from 10 g of aerial plant, and the Group-3 received 600 mg of starch powder as placebo. To evaluate, the hepato-protective efficacies of the above drugs the investigators used subjective and objective parameters such as liver function test including serum bilirubin, alanine transaminase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase. No significant elevation in AST and ALT were observed in TG compared to CG, whereas no significant elevation was observed for serum total bilirubin and alkaline phosphatase in both TG and CG [10].

In vitro Drug Trials for Anti-TB Activities

Two studies reported *in vitro* anti-TB properties of some Ayurvedic herbs. Table 2 summarizes the *in vitro* anti-TB properties of some of the Ayurvedic Herbs. Komal Kumar *et al.* conducted a study to evaluate the *in vitro* anti-TB properties of the leaves of five legumes. These include *Kingiodendron pinnatum* Rox. Hams., *Humboldtia brunonis* Wall., *Indigofera cassioides* Rottl. ex DC, *Derris scandens* Benth., and *Ceasalpinia mimosoides* Lamk. Non-polar and polar solvent extracts of leaves of these medicinal legumes were tested against *M.* TB H37RV, and minimum inhibitory concentrations (MICs) were determined by the agar-based proportion assay. Isoniazid was used as positive control to evaluate the anti-TB activity of the crude extracts of medicinal legumes. Except *I. cassioides* methanol extracts of the leaves of all the five legumes completely inhibited the growth of *M.* TB at the concentration of 50 μg/ml. Ethyl acetate extract of two legume leaves, *K. pinnatum* and *D. scandens* and chloroform extract of three legume leaves of *H. brunonis*, *C. mimosoides*, and *D. scandens* showed anti-TB activity. However, petroleum ether extract of only *I. cassioides* showed the activity, similarly, the methanol extract of only *H. brunonis* leaves showed the activity [11]. Gupta *et al.* conducted a study to evaluate *in vitro* anti-TB activity of five medicinal plants *viz*., *Acalypha indica*, *Adhatoda vasica*, *Allium cepa*, *Allium sativum*, and *Aloe vera*. Aqueous extracts of leaves of *A. indica*, *A. vasica*, bulbs of *A. cepa*, cloves of *A. sativum*, and pure gel of *A. vera* leaves, were tested *in vitro*. Percentage inhibition was used to evaluate the activity in L-J (Lowenstein-Jensen) medium which was calculated by mean reduction in a number of colonies on extract containing as compared to extract free controls. Extracts of all the five plants *A. vasica*, *A. indica*, *A. cepa*, *A. vera*, and *A. sativum* exhibited anti-TB activity in L-J medium, the proportion of inhibition of these plants extract in respect to that mentioned above is 95, 32, 37, 72, 32%, respectively, for MDR isolate DKU-156 and 68, 86, 79, 72, 85%, respectively, for another MDR isolate JAL-1236, while for sensitive *M.* TB H37Rv, inhibition was found to be 68, 70, 35, 63, and 41%, at 4% v/v concentration in L-J medium. There was no inhibition against rapid grower *Mycobacterium fortuitum* (TMC-1529). In BacT/ALERT also extracts of these plants showed significant inhibition against *M.* TB [12].

DISCUSSION

It was observed that a couple of single drugs and compound drugs are useful for the management of TB. However, none of the studies could reflect the true anti-TB activities of any drug, both single and compound. Two studies revealed *in vitro* anti-TB properties of some herbs which can potentially be brought into the realm of a clinical trial to test their efficacy in a human subject. Most of these therapeutic preparations studied at different clinical set ups reflected their adjunct properties for the management of TB. These drugs were able to possibly reduce associated symptoms and the adverse drug effects of ATDs. Some of the preparations showed potential hepato-protective properties that can be used as adjunct to ATDs. The first study primarily focused on the use of a *Rasayana* drug as an Adjunct with the simultaneous administration of

Table 2: Ayurvedic drugs showing *in-vitro* anti-TB properties

Author	Journal and YOP	Study type	Therapeutic regimen/ drugs	Methodology	Place of study	Important findings
Kumar *et al.*	AYU Journal/ 2014	*In-vitro* drug trial	Non-polar and polar solvent extracts of leaves of *K. pinnatum* Rox. Hams., *H. brunonis* Wall., *I. cassioides* Rottl.ex DC., *D. scandens* Benth., and *C. mimosoides* Lamk	MICs were determined by the agar-based proportion assay	Biodiversity Conservation Laboratory, the Department of Environmental Science, University of Mysore, India	Crude leaf extracts of these plants completely inhibited the growth *M.* TB at the concentration of 50 μg/ml
Gupta *et al.*	Indian Journal of Medical Research/2010	*In-vitro* drug trial	Aqueous extracts of leaves of *A. indica, A. vasica, A. cepa, A. sativum*, and *A. vera*	Aqueous extracts of leaves of these plants were tested *in vitro* for their activity against two MDR isolates (DKU-156 and JAL-1236)	Department of Botany, School of Life Sciences, Khandari Campus, Dr. B.R. Ambedkar University, India	All these plants exhibited activity against MDR isolates of *M.* TB. While the anti-TB activity of *A. vera, A. vasica*, and *A. sativum* against MDR isolates confirm earlier results, the activity of the extracts of *A. indica* and *A. cepa* is reported for the first time

TB: Tuberculosis, MIC: Minimum inhibitory concentrations, MDR: Multidrug-resistant, *A. indica: Acalypha indica, A. vasica: Adhatoda vasica, A. cepa: Allium cepa, A. sativum: Allium sativum, A. vera: Aloe vera, K. pinnatum: Kingiodendron pinnatum, H. brunonis: Humboldtia brunonis, I. cassioides: Indigofera cassioides, D. scandens: Derris scandens, C. mimosoides: Ceasalpinia mimosoides, M.* TB: *Mycobacterium tuberculosis*

ATDs. It provided better physical and mental well-being to the patients by potentiating therapeutic efficacy of ATDs and also counteracting the unwanted effects caused by ATDs. *Rasayana* therapy has an anti-oxidant effect along with nutritive value, immune-modulator, immune-protective properties, and free radical scavenging property [7]. In the second study, the authors focused on the role of a *Naimittika Rasayana*, a unique concept used in chronic diseases described in Ayurveda. It promotes vigor and vitality and instills the ability to hold out disastrous outcomes of diseases as in the case of PTB which has similar untoward effects due to the disease itself and the effects of ATDs as well [13]. Most importantly, the second study delineated an objective parameter, "sputum conversion," which creates some degree of skepticism. As the authors have mentioned that the TG got sputum conversion after the intensive phase which was not seen among some patients in CG poses a question, whether it is mere coincidence or the *Rasayana* has really some effect on *M.* TB. Similarly, the study conducted by Debnath *et al.* also reflected on the reduction of the bacterial load with the patients who were on adjunct therapy along with ATDs. This requires further investigation to establish and validate the fact. The Debnath *et al.* study reported a very significant finding of increased bioavailability of isoniazid and pyrazinamide, which is potentially a great hope in today's situation of drug resistance. Furthermore, TB is associated with social determinants such as poor living condition, overcrowding, poor nutrition, and above all poverty. Again poverty leads to under nutrition, which itself is affected by both scarcity of food and intra-household distribution. A poor nutritional status also affects drug absorption, resulting in sub-therapeutic serum drug levels, which may lead to non-response to drug therapy [14]. Hence, supplementation of these drugs (*Ashwagandha* and *Chyawanprash)* along with first line ATDs could improve bioavailability and help in combating the problem of drug resistance and improve curability. Currently at the national level such initiatives are not a part of TB control program which can seriously be thought of looking at the current scenario of widespread drug resistance. Most importantly, in all the studies, the commonest improvement as a result of Ayurvedic intervention is gain in body weight, which is undoubtedly a cardinal improvement in the part of a PTB patient.

Furthermore, the hepatotoxic properties of ATDs are proved. Isoniazid causes liver damage owing to its reactive metabolites generated from Acetyle hydrazine. Rifampicin is an enzyme inducer and promotes the formation of reactive metabolites and thus hepatotoxic in the form of impairment of uptake of bilirubin and acute cellular necrosis [15]. Pyrazinamide has also potential to induce hepatocellular damage [16]. Hence, the adjunct intake of Liv-600 capsule and decoction of *Bhumyamalaki* could be extremely beneficial in combating hepatotoxicity as a consequence of administration of ATDs. In addition, in the post-trial analysis, it was observed that the effect of freshly prepared decoction of *Bhumyamalaki* is more effective than Liv-600 composed of three drugs. The activity is attributable to their anti-cholestatic action, reduction in free radical damage and cell protein necrosis as well [10]. Hence, freshly prepared decoction of *Bhumyamalaki* can be promoted, as a home remedy, at the community level as a hepato-protective agent with the co-administration of ATDS, which appears to be a cost-effective measure to combat hepatotoxicity of ATDs.

In addition to clinical trials, few herbal drugs showed potential anti-TB activities *in vitro*. The study conducted by Kumar *et al.* showed anti-TB properties of five different legumes. The leaf extracts of the legumes completely inhibited the growth of *M.* TB at MIC level of 50 μg/ml. This is acceptable as activity is considered significant if MIC values are below 100 μg/ml for crude extract and moderate when MIC is between 100 to 625 μg/ml [17,18]. The frontline anti-TB drugs have MICs in the range of 0.025-2.0 μg/ml and if the activity of any new entity is to be compared (compound/extract), its activity should also be in the same range. However, since extracts are crude preparations they may be considered active at higher concentrations also [11]. Gupta *et al.* conducted a study to evaluate the anti-TB properties of some herbs on two MDR *M.* TB isolates. In addition to some other first line and second line drugs, these MDR isolates were earlier found to be resistant to at least rifampicin and isoniazid. Hence, further studies should be carried out using various fractions of crude extracts of these plants as well as their semi-purified/purified principles responsible for anti-TB activity (specially for MDR and XDR isolates of *M.* TB) to find out the MIC in suitable broth based media as MICs defined in broth are more accurate [12]. Most importantly *in vitro* studies could be brought into the arena of the clinical trial to test their efficacy in human subjects in today's situation of MDR and XDR TB.

Studies reveal that chest physicians are treating TB patients using few Ayurvedic preparations along with ATDs. The chest physicians are of the opinion that these medications act as immune modulators if given along with second line anti-TB drugs [5]. Furthermore, there is a growing interest in identifying the compounds responsible for the anti-mycobacterial activity of traditional medicine and developing them as potential TB drugs [19]. Many drugs of the Indian System of Medicine have come to the realm of national health programs for the management of common ailments, as in national health mission. Similarly, few drugs could also be brought into the same platform and be implemented in the national TB control program [20,21].

CONCLUSION

TB has been a major public health crisis for the developing world including India. Due to increase in MDR and XDR strains of *M.* TB, there is an urgent need to find newer solutions to combat this problem. Distressingly research on the role of Ayurveda in the management of TB is very scanty and mostly limited to adjunct or supportive therapy. However, the adjunct role of Ayurveda drugs cannot be simply neglected for not qualifying as agents to combat the *M.* TB rather their role as agents of increasing bioavailability of ATDs and counteracting the adverse drug interactions should be properly utilized. Being a global public health crisis and having the state of current drug resistance, it is highly recommended to carry out clinical trials on TB patients using Ayurvedic drugs and therapeutic regimens. The drugs those proved

potent in combating the adverse drug reactions of both the first line and second line anti-TB drugs could be successfully added to the drug regimens of TB for better curability and to reduce drug resistance. Similarly, the drugs which showed potential anti-TB properties *in vitro* could also be useful in today's world of drug resistance.

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Herbs as an antioxidant arsenal for periodontal diseases

Asha Ramesh, Sheeja Saji Varghese, Jayakumar Nadathur Doraiswamy, Sankari Malaiappan

Department of Periodontics, Saveetha Dental College and Hospital, Chennai, India

Address for correspondence:
Asha Ramesh, Department of Periodontics, Saveetha Dental College and Hospital, Chennai, India.
E-mail: ash.periopg@gmail.com

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ABSTRACT

Herbal medicines have long been used as a traditional mode of therapy for various ailments in India. They are being used increasingly as dietary supplements to ward off common diseases. Periodontal diseases are highly prevalent and can affect up to 90% of the world population. Gingivitis is the mild form whereas periodontitis results in an irreversible loss of supporting structures of the teeth. Even though periodontal pathogens form a crucial component in the etiopathogenesis of periodontitis, there is a growing body of evidence suggesting oxidative stress playing a pivotal role in the disease initiation and progression. Studies have shown a direct correlation between increased levels of biomarkers for tissue damage induced by reactive oxygen species (ROS) to the severity of periodontal disease. Thus, the focus of attention has revolved back to herbal medicines due to their wide spectrum of biological and medicinal activities, lower costs, and higher safety margin. Internet databases Pubmed and Google Scholar were searched, and the most relevant articles were considered for review. This review briefly describes the various herbs with antioxidant capacity and their potency in the treating periodontal disease.

KEY WORDS: Antioxidant capacity, herbal medicine, periodontitis

INTRODUCTION

Herbal medicines, including herbs, herbal preparations and finished herbal products, contain as active ingredients parts of plants or other plant materials perceived to have therapeutic benefits [1]. About 80% of the worldwide population use herbal products for their basic health care (primary care) such as extracts, teas and other active principles, a market estimated at US$ 50 billion per year [2]. Herbal products are preferred over conventional drugs due to wide biological activity, higher safety margin, and lower costs. Furthermore, the conventional drugs are known to cause various side effects, and continuous intake has resulted in antibiotic resistance. Thus, herbal medicines are being used increasingly as dietary supplements to fight or prevent common diseases [3].

Periodontitis is a chronic inflammatory disease which results in the destruction of supporting structures of the teeth. The etiology is multifactorial with periodontopathogens forming a major crux in the initiation and progression of the disease. Plaque build-up allows the growth of anaerobic bacteria [4], which eventually leads to the recruitment and activation of neutrophils. This further results in the upregulation of pro-inflammatory cytokines and also leads to the release of neutrophilic enzymes and ROS. Prolonged exposure of the connective tissue to these insults results in the degradation and subsequent loss of ligamentous support and alveolar bone, eventually leading to tooth loss [5].

Periodontal therapy includes both surgical and nonsurgical management of the disease process. Various antimicrobials and chemotherapeutic agents, such as chlorhexidine, triclosan, cetylpyridinium chloride, have been tried and tested in the management of periodontal diseases. Due to its multifactorial etiology and complex disease process, the treatment of periodontitis is still a formidable task to dentists. Therefore, herbal remedies have been sought to achieve antimicrobial, antioxidant, antiseptic, anti-inflammatory, and anti-collagenase effects. The following review briefly describes the role of oxidative stress in periodontitis and the various herbs with antioxidant potential used in its management.

OXIDATIVE STRESS IN PERIODONTITIS

It is well-established that oxidative stress is an important cause of cell damage associated with the initiation and progression of many chronic diseases [6-8]. A recent review by Bullon describes the mounting evidence that the basis for the interrelationships between chronic periodontitis and atheromatous disease and diabetes lies at the fundamental intracellular level, namely oxidative stress and mitochondrial dysfunction [9]. Oxidative stress is the disturbance in the pro-oxidant and antioxidant balance, in favor of the former, resulting in potential tissue damage. Consecutive oxidation-reduction reactions of molecular oxygen by various enzymes results in the production of molecules such as superoxide anion, hydrogen peroxide, hydroxyl radical, singlet oxygen,

nitric oxide, hypochlorous acid which together constitute the term "ROS" [10]. All the cells in the body are capable of generating ROS, of which polymorphonuclear neutrophils are of prime importance with respect to periodontitis. Neutrophils are a part of innate immunity which comprise the first line of host defense and are located at sites of microbial invasion. They are activated by inflammatory mediators and can generate increased levels of ROS, which not only attack the periodontopathogens but also the surrounding tissues [11]. The ROS is generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) system present in the neutrophils and it catalyzes the reduction of molecular oxygen to superoxide anion. Subsequent reductions result in the production of hydrogen peroxide and hydroxyl radical. Similarly, superoxide dismutase enzyme present in all the cells catalyzes the dismutation of superoxide radical to hydrogen peroxide. Many studies have shown that ROS regulate the formation and function of osteoclasts, i.e., the activation and bone resorption ability [12-14]. Bone resorption which results in alveolar bone loss and ultimately tooth loss is the hallmark feature of the periodontal disease. NADPH oxidase system plays a role in periodontal pathologies and its involvement is the strongest in aggressive periodontitis [15].

To combat the oxidative stress, all the cells in the body are equipped with an intrinsic store of molecules known as "antioxidants." Antioxidants may be regarded as "those substances which when present at low concentrations, compared to those of an oxidizable substrate, will significantly delay, or inhibit oxidation of that substrate" [16]. They function by scavenging free radicals as and when they form and thereby preventing oxidative stress. They can also sequester transition metal ions and prevent Fenton's reaction or catalyze the oxidation of other molecules. Various antioxidant molecules include vitamins C, E, coenzyme Q, carotenoids, enzymes such as glutathione reductase, glutathione transferase, superoxide dismutase, and peroxiredoxin.

Numerous studies have shown that the total antioxidant capacity in periodontitis patients is significantly lower when compared to healthy controls [17-19] or in subjects who have received periodontal therapy [20]. These findings have triggered the use of exogenous supplements for the treatment of periodontal disease. Herbal antioxidant remedies have been the focus of research in recent times. A literature search was performed using keywords such as "plant extracts," "herbs," "herbal medicine," "antioxidants," "oxidative stress" "periodontal disease" in PubMed, and Google Scholar. The most relevant articles were included in this review and it gives an overview about the potential of herbal medicine in the management of periodontitis.

GREEN TEA

Components

Green tea is made from the leaves of *Camellia sinensis* that have undergone minimal oxidation during processing. It contains the highest concentrations of antioxidants called polyphenols [21]. Polyphenols contained in teas are classified as catechins. There are six primary catechin compounds in green tea: Catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate (ECg), and epigallocatechin gallate (EGCg). EGCg has been the focus of extensive research among all the other compounds and it is a very potent antioxidant. Green tea also contains carotenoids, tocopherols, ascorbic acid, minerals such as zinc, selenium, chromium, and certain phytochemical compounds.

Role in Periodontitis

Green tea catechins have been observed to have profound effects on periodontal pathogens. Anaerobic bacteria like *Porphyromonas gingivalis* and *Prevotella* spp. are the main etiological agents in periodontitis. *In vitro* studies have shown that these compounds inhibit the growth of *P. gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens* [22-24]. It also prevents the adherence of *P. gingivalis* onto human buccal epithelial cells [25]. A study also showed that both ECg and EGCg inhibited *P. gingivalis* derived collagenase activity [26].

Bone resorption which occurs in periodontitis is due to the interplay between osteoblasts and osteoclasts. In an animal study, it was shown that EGCg reduced lipopolysaccharide-mediated bone resorption in both *in-vivo* and *in-vitro* conditions. It also showed that EGCg suppressed LPS mediated gene expression such as RANKL, cyclooxygenase-1 and the cytokine PGE2 in mouse osteoblasts. This clearly suggests that the catechin present in green tea is highly potent in suppressing the bone resorption mediated by an inflammatory response as seen in periodontal disease [27].

There are various reports about the use of green tea in various forms in the management of periodontitis. Pilot studies on the usage of green tea as a dentifrice and a local drug delivery system have observed an improvement in the periodontal status of the patients suffering from chronic periodontitis [28,29]. A clinical trial indicated that green tea mouthwash had a comparable antiplaque efficacy to chlorhexidine gluconate (gold standard) when used for a period of 1 week [30]. These preliminary results show that further research is needed to explore and tap the benefits of green tea, and utilize it in the management of periodontal diseases.

TRIPHALA

Components

Triphala is a well-known powdered preparation in ayurvedic medicine used since ancient time. It consists of equal parts of Amalaki (*Emblica officinalis*), Haritaki (*Terminalia chebula*) and Bahera (*Terminalia belerica*). Amalaki is an excellent source of vitamin C and also contains carotene, nicotinic acid, D-glucose, D-fructose, riboflavin, empicol, and mucic and phyllemblic acids. Haritaki contains anthraquinone glycoside, chebulinic acid, tannic acid, terchebin, vitamin C, and arachidonic, linoleic, oleic, palmitic, and stearic acids. Bahera contains chebulagic acid, ellagic acid and its ethyl ester, gallic

acid, fructose, galactose, glucose, mannitol, and rhamnose. All the three components present in triphala have a wide range of pharmacological activity and are potent antioxidants.

Role in Periodontitis

Triphala has a strong antimicrobial, antioxidant and anti-collagenase properties. The antioxidants present in Triphala reduce the oxidative burden and protect cells from the damage caused by free radicals [31,32]. Bahera is the most active antioxidant followed by Amalaki and Haritaki. A clinical trial has shown that Triphala mouthwash is as efficacious as 0.2% chlorhexidine in antiplaque and anti-inflammatory activities [33].

The antibacterial effect has been assessed in an *in-vitro* study where Triphala concentrations of 50 μg/ml inhibited *Streptococcus mutans* species. This antiplaque effect may be attributable to tannic acid present in Triphala, which is well adsorbed on the surface of bacterial cells resulting in protein denaturation and ultimately to bacterial cell death [34].

Triphala has also been known to inhibit the collagenases derived from polymorphonuclear leukocytes which are responsible for connective tissue destruction in periodontal disease. This has been corroborated by an *ex-vivo* study where tissue samples were treated with triphala, kamillosan extracts, and doxycycline, and gelatin zymography was done. Triphala showed 76.6% reduction of matrix metalloproteinase-9 (MMP-9) activity, whereas kamillosan and doxycycline showed 46.36% and 58.7%, respectively, at concentrations of 1500 μg/ml [35].

RUBIA CORDIFOLIA

The roots of this plant have been used in ayurvedic medicine. It also contains an organic compound known as Alizarin, which gives the red color to textile dyes. Mollugin, a major component of *R. cordifolia* has been shown to possess anti-inflammatory property [36]. A recent study showed that mollugin inhibited RANKL-induced osteoclast differentiation and bone resorbing activity of mature osteoclasts. Mollugin reduced the phosphorylation of signaling pathways activated in the early stages of osteoclast differentiation, including the MAPK, Apt, and GSK3β and inhibited the different genes associated with osteoclastogenesis such as *Osteoclast-associated* receptor, tartrate resistant acid phosphatase, ICAM-1, cathepsin K, DC-STAMP and OC-STAMP. Furthermore mice treated with mollugin showed significant restoration of lipopolysaccharide-induced bone loss as indicated by micro-computed tomography and histological analysis of femurs [37]. However, further studies are required to use this herbal product as a novel therapeutic approach to treat bone degenerative disorders such as periodontitis, rheumatoid arthritis, and osteoporosis.

PIPERINE

It is an alkaloid which is present in plants such as *Piper nigrum* and *Piper longum*. It is shown to have antioxidant and anti-inflammatory properties. In an animal model, LPS stimulated mice when treated with piperine showed reductions in the nitrite level and lowered the TNF-α level. This study corroborates the free radical scavenging activity of piperine [38]. Another study on rat periodontitis model revealed that piperine significantly down-regulated the production of interleukin-1β, MMP-8, and MMP-13. Piperine clearly inhibited alveolar bone loss and reformed trabecular microstructures in a dose-dependent manner. Histological staining showed that piperine significantly reduced the infiltration of inflammation in soft tissues [39].

SUMAC

Sumac (*Rhus coriaria*) is a well-known spice used widely as an herbal medicine for its anti-inflammatory, antimicrobial, and antioxidant properties [40]. The existing literature on sumac fruit extracts show that they has marked antioxidant activity against lipid peroxidation and free radicals *in vitro* [41,42]. In an experimental animal study, Wistar rats with ligature-induced periodontitis were administered with sumac extracts orogastrically at a dosage of 20 mg/kg/day. Serum total oxidant status (TOS) and oxidative stress index (OSI) were significantly reduced in the sumac extract treated rats. Furthermore, the serum total antioxidant status was similar to the non-ligated rats. Sumac extracts have the potential to reduce alveolar bone loss by affecting TOS and OSI levels in periodontal disease in rats [43].

GINKBO BILOBA

G. biloba (EGb) leaf extract is among the widely used herbal dietary supplement in the US [44]. It is composed of ginkgo flavone glycosides (24%), terpenoids (6%) and less than 5ppm of ginkgolic acid. Its purported biological effects include: Scavenging free radicals [45], lowering oxidative stress [46], and anti-inflammation [47]. In ligature-induced periodontitis rat model, systemic administration of EGb (28-56 mg/kg/day) resulted in reduced osteoclastic counts, decreased inflammation and induced osteoblastic activity [48].

PSIDIUM GUAJAVA

Guava has an excellent antioxidant property because it is primarily rich in Vitamin C (Ascorbic acid). It also has quercetin, carotenoids, and polyphenols which augment its antioxidant action [49,50]. Guava leaf extracts and essential oil from the stem have the ability to scavenge hydrogen peroxide, superoxide anion and inhibit the formation of hydroxyl radical [51,52]. The decoction of the root bark is recommended as a mouthwash and decoction of leaves as an effective gargle for bleeding gums [53].

CURRENT DEVELOPMENTS

Recent *in vitro* studies have shown that herbs such as *Lythrum salicaria* and *Ascophyllum nodosum* have shown to possess potent antioxidant properties. *L. salicaria* aqueous extracts inhibited ROS production from stimulated neutrophils which were isolated and cultured from humans [54]. A polyphenol rich

extract from *A. nodosum* demonstrated significant anti-lipid peroxidation activity and antioxidant activity by scavenging superoxide anion, hydroxyl, and peroxyl radicals [55]. Preliminary studies on a mouthwash containing microencapsulated natural extracts such as avocado oil, manuka oil, propolis oil, grapeseed extract, *Aloe vera*, green tea, coenzyme Q10 (6% GingiNat) have shown significant efficiency on plaque, gingivitis and halitosis due to its antioxidant and immunoregulatory properties [56,57]. Pradeep *et al*. showed that a gel and powder formulation derived from *Acacia arabica* demonstrated a significant improvement in plaque and gingival scores when compared to 1% chlorhexidine in gingivitis patients [58].

CONCLUSION

The herbal medicines have shown to possess a wide array of biological properties such as antimicrobial, antioxidant, and anti-inflammatory effects. The natural phytochemicals present in these herbs aid in suppressing the alveolar bone loss, which is the striking feature in periodontitis. Furthermore, the oxidative burden established due to the chronicity of the disease can be alleviated with the antioxidant property of these herbs. Although many studies, have shown the potency of herbal medicines as an alternative to conventional therapy, there still lies a void in research with respect to the clinical application of these agents in periodontics. Future targeted trials in learning the mechanism of action of these herbal remedies are warranted.

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Review Article

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Natural polyphenols: Influence on membrane transporters

Saad Abdulrahman Hussain[1], Amal Ajaweed Sulaiman[1], Hasan Alhaddad[1], Qasim Alhadidi[2]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq, [2]Department of Medicinal and Biological Chemistry, College of Pharmacy and Pharmaceutical Sciences, University of Toledo, 3000 Arlington Avenue, Toledo, OH, 43614, USA

Address for correspondence:
Saad Abdulrahman Hussain, Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq.
E-mail: saad_alzaidi@yahoo.com

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ABSTRACT

Accumulated evidence has focused on the use of natural polyphenolic compounds as nutraceuticals since they showed a wide range of bioactivities and exhibited protection against variety of age-related disorders. Polyphenols have variable potencies to interact, and hence alter the activities of various transporter proteins, many of them classified as anion transporting polypeptide-binding cassette transporters like multidrug resistance protein and p-glycoprotein. Some of the efflux transporters are, generally, linked with anticancer and antiviral drug resistance; in this context, polyphenols may be beneficial in modulating drug resistance by increasing the efficacy of anticancer and antiviral drugs. In addition, these effects were implicated to explain the influence of dietary polyphenols on drug efficacy as result of food-drug interactions. However, limited data are available about the influence of these components on uptake transporters. Therefore, the objective of this article is to review the potential efficacies of polyphenols in modulating the functional integrity of uptake transporter proteins, including those terminated the effect of neurotransmitters, and their possible influence in neuropharmacology.

KEY WORDS: Herb-drug interactions, membrane transporters, polyphenols, therapeutic application

INTRODUCTION

Polyphenols encompass several classes of compounds that produced in plant as secondary metabolites, and they were routinely consumed with human diet. Until now, about 7000 different chemical molecules together with their metabolites were identified in many types of fruits and vegetables [1]. They received increasing attention due to their well-documented therapeutic significance in many diseases and disorders [2]. Chemically, all polyphenols have one or more hydroxylated aromatic rings that account for both structural and physicochemical properties, and allow their classification into several chemical classes including lignans, flavonoids, stilbene, isoflavones and phenolic acid derivatives [1]. All polyphenols have reducing properties; they can donate hydrogen to oxidized cellular constituent, and play a significant role against oxidative stress-related pathologies, like cardiovascular diseases, cancer and variety of neurodegenerative disorders [3]. Moreover, many *in vitro* and *in vivo* studies suggested that the beneficial effects of polyphenols extended to involve cell signaling, since they act as regulatory factors of gene transcription that affect many important processes like cell growth and apoptosis [4-7]. Flavonoids are the most abundant polyphenols that widely consumed by peoples worldwide; therefore, many researchers focused on the effects of many flavonoids like resveratrol [8], quercetin, epigallocathechin-3-gallate (EGCG), rutin and curcumin, as a health promoting compounds in treatments of several diseases [9-11]. However, low bioavailability of most polyphenols represents the main hurdle in their use as dietary supplements. Bioavailability of polyphenols is closely related to the biotransformation process, which mainly based on Phase II conjugation reactions of the free hydroxyl groups with methyl group, sulfate or glucuronic acid [12,13]. The diversity in polyphenols structure increases the possibility of different interaction patterns with membrane transporters at different anatomical sites [14,15]. The balance between absorption and excretion of dietary polyphenols can be achieved by modulation of the tissue uptake system, suggesting that certain cells may readily incorporate them by specific mechanisms; for instance, morin can cross the vascular endothelium by a rapid, energy-dependent transport system that can also transport other hydroxylated compounds [16,17]. Recently, kinetic studies showed that the functional integrity of both uptake and efflux transporters represent the basis behind different tissue

distribution of orally administered drugs in certain organs such as liver, kidney, and brain in animals that chronically challenged with polyphenolic compound, and reflects the importance of these transporters as a site for food-drug interactions [18,19]. On the other hand, such interaction can be utilized for treatment of central nervous system (CNS) related disorders like depression, anxiety, and other psychoneuronal diseases associated with functional abnormalities of monoamine transporters (MATs) [20]. Accordingly, polyphenols may be considered as a potential modulators that can maintain homeostasis within brain tissues, and provide adaptation against neuronal stress [21,22].

PLASMA MEMBRANE TRANSPORTERS

To maintain the normal cell functions, transport of organic and inorganic molecules across the lipid bilayer of the plasma membrane is vital for life and maintenance of homeostatic mechanisms [23]. It represents a tough barrier for most polar molecules, while enables passage of hydrophobic molecules only through passive diffusion [24]. Transport of ions, polar organic compounds, in addition, to the transport against concentration gradient of many chemicals (e.g., nutrients and metabolites) requires a special transport system that rely on a source energy utilized either through the existed potential of chemical gradients or coupled with enzymatic reactions that consume anion transporting polypeptide (ATP) [25]. With few exceptions, transport substrates of the rotary motor and P-type ATPases are limited to metal ions or protons; however, ATP-binding cassette (ABC) type transports a broad range of substrates, including amino acids, sugars, nucleosides, vitamins, peptides, lipid molecules, oligonucleotides and polysaccharides [26,27]. There are more than 1300 membrane transporter proteins that are broadly classified into efflux and influx types relative to the direction of substrate flow. Moreover, utilization of energy for transportation allow further sub-classification into active and passive transporters [28]. The group of active transporters included three subclasses, primary, secondary and tertiary depending on their driving force, which could be either ATP or the chemiosmotic gradient [29]. These transport systems are mostly substrate-specific and saturable, and can be competitively or noncompetitively blocked, and genetically regulated. In addition, active membrane transport is vectorial, where the substrate is either transported into or out of the cell; however, the same transporter does not perform both actions [30,31]. In general, the term active transporters cover the following:

i. ATP-binding cassette protein (ABC): Members are mainly expressed on the membrane of excretory organs, where they regulate extrusion of wide range of chemically different substrates against electrochemical gradient to the extracellular region. This group includes seven subfamilies represented by ABCA, ABCB which also known as p-glycoprotein (P-gp) or multidrug resistance (MDR), ABCC or MDR proteins, ABCD, ABCF and ABCG which include breast cancer resistance protein (BCRP or ABCG2) [32,33].
ii. The solute carrier (SLCs) transporters or uptake transporters: Members are mainly located in cell membrane of organs having tubular lumen structures like liver, kidney and intestine; they were organized into more than 50 families and have chemically related substrate specificity. They transported diverse substrates including charged and uncharged organic molecules, in addition to inorganic ions. The most characterized families of this class are the organic ATPs (OATPs), which include OATP1, OATP2, OATP3, OATP4, OATP5, and OATP6 [34,35]. In addition, the organic anion transporters (OATs), which include seven members represented by OAT1, OAT2, OAT3, OAT4, OAT6, OAT7 and OAT10, perform the transport of substrates with molecular weights of 400-500 Da. Meanwhile, the organic cations transporters (OCTs) include twenty members, some are well identified like OCT1, OCT2, OCT3, OC12 and the organic creatinine transporters, where the latter is recently included and involved in bidirectional transport of creatinine to maintain its physiological level [36]. In aeneral, OCTs catalyzed transport of monoamines neurotransmitters, Zwitter ions, and some anion substrates with different tissue distribution [37-39]. Since SLTs have regulatory role in absorption, uptake and elimination of different drug molecules, trace elements and dietary nutrients, they are considered as important site of kinetic drug-drug and/or drug-nutrient interactions, where modulation of their function can influence the availability and therapeutic efficacy of many ligands including drugs and other health promoting supplements [40].
iii. MATs: This class included three members of membrane transporters (NET, SET and DAT), which catalyze transport of norepinephrine, serotonin, and dopamine. They are expressed at the presynaptic area within both CNS and the periphery [41], and involved in regulating neurotransmission, utilizing Na/Cl gradient as driving energy that induce conformational changes to enhance uptake and release of monoamines [42,43]. In spite of functional importance of peripheral MATs, no sufficient information are available compared to those present in CNS, which have been widely studied [44]. Impairment of neurotransmitter homeostasis is correlated with many CNS disorders such as depression, anxiety, attention deficit hyperactivity disorder, schizophrenia, and Parkinson's disease [45], and suggests MATs as important therapeutic targets. In this regard, treatment of depression has been focused on modulating monoamine neurotransmission, mainly achieved through blockade of MATs [46]. Meanwhile, development of non-selective tricyclic antidepressants, selective serotonin reuptake inhibitors and serotonin and norepinephrine reuptake inhibitors is the most valuable outcome of this concept [47]. Moreover, drugs of abuse including cocaine and amphetamines interfere with MATs; accordingly, these transporters are considered as potential targets for treatment of drug addiction [48]. Dysfunction or complete deletion of DAT showed to decrease the clearance of dopamine that associated with spontaneous hyperactivity, sleep disturbances, motor deficiency, and cocaine abuse behavior [49]. Meanwhile, deletion of NET in animal models showed resistant depressive-like effects of stressors with anti-nociceptive activity, and such models were less vulnerable to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-

induced neurotoxicity [50]. The contribution of central serotonin in controlling mood and behavior was reported using SET-deficient rats, and anxiety could be ameliorated by a 5-HT1A antagonist [51]. Another study revealed that depletion of serotonin level in gastrointestinal tract (GIT) was observed in patients with irritable bowel syndrome that often associated with increased expression of SET in the colon, suggesting the role of SET inhibitors in its treatment [52,53].

INTERACTIONS OF NATURAL POLYPHENOLS WITH MEMBRANE TRANSPORTERS

Numerous data have summarized the broad biological properties and the diversity of polyphenols targets in the biological systems that mediate their activities. Both chemical structure and the nature of interaction of polyphenols with biomembranes are critical for their beneficial effects, and such interaction represents the underlying mechanism through which they affect the functional properties of membrane bound enzymes and transporter proteins, which alter transmembrane potential for endogenous and exogenous molecules [54]. Dietary polyphenols became increasingly popular, and some of them have a potential role in initiating adverse drug interactions that may be established through alterations in efflux and uptake transporters [55]. Similar to conventional drugs, many natural ingredients, including polyphenols, interact with various classes of drug uptake transporters. For example, the flavonoids apigenin, quercetin, and kaempferol block the transporter functions of OATP1A2 and OATP2B1, which are localized in the apical membrane of the intestinal lumen [56]. Furthermore, green tea catechins, herbal extracts, and citrus and grapefruit juice affect the OATP-mediated transport of many ligands [57-59].

INTERACTIONS OF NATURAL POLYPHENOLS WITH MDR TRANSPORTERS

MDR transporters are family of proteins that include P-gp as an important member, which was primarily characterized in MDR Chinese hamster ovary (CHO) cells. P-gp transports xenobiotics outward in a unidirectional pattern utilizing ATP as energy source [60]. Other MDR-related transporters were discovered within different types of cells such as MDR related proteins (MRP's) [61] and BCRP-1 [62]. Ligands that inhibit these efflux transporters were expected to elevate the intracellular concentrations of many therapeutic agents in similar fashion to P-gp blockade [63]. In addition to the potential role of P-gp in normal physiological processes, its overexpression on cancer cells decreases significantly the intracellular concentrations of a many chemotherapeutic agents [64]. It has been reported that natural polyphenols or their synthetic analogs can modulate the MDR transporters responsible for chemotherapy resistance, including P-gp, MRP1, and BCRP [65]. Flavonoids and stilbenes are known as the third generation of P-gp blockers and produce comparable effects to those of the already known P-gp inhibitors like verapamil and cyclosporine [66]. Many flavonoids have the capacity to inhibit BCRP; thus, consumption of flavonoids with high blocking activity can modify pharmacokinetics and levels of drugs that are extruded by BCRP [67]. In addition, many polyphenols are well-known as P-gp blockers including EGCG that down-regulates P-gp and BCRP but did not inhibit MRP1 in a tamoxifen resistant breast cancer cell line [68]. Other polyphenols with hydrophobic groups like prenyl substituents may be the future promising candidates for MDR reversal agents.

The activity of P-gp can be modulated by altering the physical state of the surrounding lipids and/or lipid composition. Polyphenols, including biochanin A, morin, phloretin, and silymarin, have been reported to influence the transport activity of ABCB1 protein. These effects resulted in a significant increase of daunomycin accumulation in P-gp expressing cells in a concentration-dependent manner, and explained by the competitive binding of the polyphenols to the ligand binding site on the P-gp molecule that end up with drug accumulation [69]. Others reported that quercetin inhibits the ATPase activity of P-gp, a mechanism that based on structural properties of this polyphenol [70]; however, the mechanism through which genistein modulates drug transport across plasma membrane seems different, where its interaction with MRP1 increased daunorubicin accumulation in cell lines overexpressed MRP1 without expression of P-gp [71]. In other studies that utilize membrane vesicle preparations to evaluate the direct inhibition of MRP1, polyphenols interact with various sites on the MRP1 molecule. In addition, they showed an increase in the activity of cystic fibrosis transmembrane conductance regulator (CFTR; ABCC7) chloride channel [65,72,73]. Catechins were also found to interact with P-gp and can modulate its transport activity relative to the type of tested compound, where some of them affect the fluorescent markers transported by P-gp, while others increase the transport of these markers [74]. Polyphenols like mangiferin and the mangiferin aglycone derivative norathyriol, as well as catechin, gallic acid and quercetin were investigated for their potential ability to influence ABCB1 gene and P-gp expression in HK-2 cells. Western blot analysis demonstrated a time and concentration-dependent modulation in P-gp activity that correlated to relative changes in the ABCB1 mRNA content [75]. Resveratrol, a well-known polyphenol, was found to improve the cytotoxic profile of docetaxel and doxorubicin in solid tumors through blockade of P-gp efflux and down-regulation of MDR1 gene [76]. More recently, silibinin dihemisuccinate improves the sensitivity of methotrexate-resistant human rhabdomyosarcoma cell lines to the cytotoxic activity of methotrexate in concentration-dependent pattern, most probably through modulation of methotrexate transport through the plasma membrane [77].

INTERACTION OF NATURAL POLYPHENOLS WITH UPTAKE TRANSPORTERS

Oral drug delivery is the most acceptable way of administration, mainly because of patient compliance and ease of administration. Many studies have suggested the role of specific transporters in GIT absorption of weak acids. In clinical practice, patients usually administer various types of drugs at the same time.

Thus, drug-drug interactions that involve membrane transporters may directly affect safety and efficacy of many drugs and food components. It has been reported that ferulic acid, widely used as a functional food, modulates the transport of many clinically effective agents, and baicalin inhibits the specific transport system that mediate transport of the active metabolite of irinotecan through the intestinal epithelium and ameliorate severe diarrhea associated with high doses of this compound [78,79]. Inhibition of OATP2B1 by polyphenols in apple juice may also contribute to limit drug-induced GIT toxicity, and may be of value in prophylaxis of late-onset diarrhea reported during CPT-11 therapy [80]. Herbal extracts and dietary polyphenols were investigated for their broad bioactivities including maintenance of glucose homeostasis, neuroprotection and regeneration, which often attributed to the functional control of uptake transport processes [81]. Tea polyphenols showed tendency to attenuate degeneration of dopamine neurons and toxicity induced by 6-OHDA and MPP by mechanisms not only related to anti-oxidant or metal-chelating properties, but may be directly mediated by modulation of transporters and intracellular signaling pathways [82]. Polyphenols are studied for their potential to alter the kinetic properties of other chemicals, mostly through their influence on metabolism and/or transportation at different tissue levels relative to their distribution. To date, few number of transporters were studied for their interaction with dietary polyphenols [56,83]. Although the precise molecular mechanisms by which polyphenols interact with uptake transporters are not well identified, several *in vitro* studies on cell lines expressing MATs revealed that polyphenols could interact with membrane functional proteins, like enzymes and/or transporters, as competitive or non-competitive ligands through direct interaction with the active or allosteric sites, altering their configuration and functional activity [84]. The tea polyphenols ECG and EGCG are good ligands for OATP1B3 and OATP1A2 that widely expressed in liver and intestinal epithelium [59], and they can inhibit the uptake of dopamine and MPP^+ by DAT, thus protected embryonic rat mesencephalic dopaminergic neurons against MPP^+-induced injury [85]. Moreover, an *in vitro* study showed that quercetin rather than naringin can specifically transported by intestinal OATP2B1 expressed in Caco2 cell line, and specific inhibition of these transporters can reduce uptake of quercetin. However, the conjugated polyphenols, as sulfated conjugates, were effectively taken up by OAT1 and OAT3 [86]. In neuropharmacology, the mechanisms by which polyphenols and their metabolites cross the blood-brain barrier (BBB) remain a hot spot for future research. Many *in vitro* studies evaluated the neuroprotective effects of polyphenols, and many evidence suggested that both aglycones and the conjugates behaves as ligands for OAT to cross the BBB. Such transport mediated by OATs and OATP1A2 may influence brain tissue levels of the administered flavonoids. In a kinetic study, galangin and apigenin competitively inhibit A and B isoforms of MAO, while other polyphenols showed antidepressant effect by altering transportation of NE and SE [87]. The medicinal herb hypricum, contained 6-15% of proanthocyanidin and 5% phenolic acid, was known to exert antidepressant effect by non-competitive reuptake inhibition of monoamines; however, its therapeutic potential remain questionable due to its poor bioavailability [88]. The sensitivity of membrane transporters to polyphenols was examined in comparison with bupropion, a reference inhibitor for both DAT/NET, using recombinant technique for CHO cell line expressed these transporters. The results showed that polyphenols have selectivity to inhibit MATs, with high potency and efficacy for DAT and NET more than SET, indicating competitive inhibition of DAT/NET [89]. Moreover, cells expressing OAT1 showed increasing tendency to the uptake of the aglycone silibinin three folds than control cells [90]. *Ginkgo biloba* flavonoids including kaempferol, quercetin and apigenin showed competitive inhibitory effect of OATp1B1 and OATp1B3 expressed on HEK293 cell line, with no significant effect on OCT [91]. In a study that investigates the role of hepatic MRP_2 in the disposition of polyphenols, both MRP_2 and BCRP exhibited an essential role in the efflux of glucuronidated quercetin and naringin [83]. Interestingly, hesperidin and EGCG enhance the activity of the clinically used benzodiazepine through a modulatory effect on GABA-A receptor where they bind two sites [92]. These modulatory effects of polyphenols may guide pharmacological research to develop natural products with therapeutic benefit for treatment of depression or other neurological disorders, besides their implication in drug-dietary supplement interactions [93]. On the other hand, polyphenols may act as modulators of transporter proteins directly by either increasing or decreasing the activity of membrane proteins, or indirectly by modifying the signaling pathways and expression of mRNA encoding these transporters. It has been shown that individual components of silymarin showed a significant but different inhibitory effect on OATp transporters in overexpressing cell lines in spite of their structural similarity and identical molecular weight; this finding revealed the influence of stereochemistry in modifying the interaction potential with OATP transport proteins, as observed from variable values of IC50 among these components [94]. Other study showed that both morin and silibinin competitively inhibit OAT1 with IC50 values of 0.5 and 25 μM, respectively, while ellagic acid was a potent inhibitor of OAT1 activity with IC50 of 207 nM [90,95]. Assessment of the modulatory effect of red wine polyphenols like resveratrol, quercetin and myricitin, using MPP^+ as a reference substrates for OCT1 and OCT3 in Caco2 cells expressing these transporters, indicated that these polyphenols increased the uptake of MPP^+ in a concentration-dependent manner, just like the effect of grape seed proanthocyanidins [96,97]. Moreover, other investigators showed that myricetin and catechin decrease MPP^+ uptake more than quercetin which displayed strong inhibitory effect [98]. In 2010, Zhao *et al* suggested that the cytoprotective effect of polyphenols could be mediated through modulation of the MATs, where both transgenic CHO and dopaminergic cell line (wild type) that specifically transported monoamines used to investigate the effect of the frutescens fruit polyphenols luteolin and apigenin. Both provided a significant increase in the uptake of DA and NE, with higher potency of luteolin over apigenin. These effects may be a consequence of conformational changes or translocation of transporter proteins induced by polyphenols. In addition, luteolin can counteract the inhibitory effect of cocaine, the competitive inhibitor of DAT. Thus, luteolin had

tendency to give antipsychotic and anti-addictive effects [99]. Inhibitors of SET were known to prolong neuronal signaling of serotonin to improve depression and control intestinal function. Investigation of polyphenols that founded in licorice like methyl glabridin, glabridin and glabrene using HEK-293 cell line expressing SET showed 53%, 60% and 47% inhibition of SET, respectively, suggesting the impact of dietary polyphenols on mood through modulation of SET [100]. To date, little information are available about expressional modulatory effect of polyphenols. It has been postulated that stress signals induced down-regulation of OAT1 and OAT3, while treatment of cell lines expressing these transporters with curcumin results in two folds up regulation of these transporters, and reduced the expression of OATP1B1 [101]. The isoflavon genisten was reported to decrease expression of NE uptake transporters in human neoplastoma cells, suggesting its potential pharmacological action of on sympathetic neurons [102]. The polyphenols of *Cynomorium songaricum* extract were investigated on CHO expressing MAT, and the results indicated that they inhibit DAT/NET transporters; however, more polar fractions possess dual glutamate/serotonin transporters inhibition suggested the multi-spectrum targeting effect of these polyphenols [103]. For instance, the multifunctional modulation of cell signaling pathways in neuroprotection activities of tea polyphenols was investigated using 6-OH-DA model of PD, where they decrease apoptosis through increasing the anti-apoptotic signaling proteins [104].

The classic concept of transporters in the CNS illustrated mechanisms that control many functions in the brain, including autonomic function, locomotion, hormones secretion, and behavioral and intellectual activities related to the emotion and reward [105]. Moreover, functional disturbances of these transporters predispose various CNS pathologies [43]. During neuronal transmission, the intensity and duration of synaptic signaling are determined, in part, through the reuptake of the signaling molecules through specific membrane-bound transporters, which are mostly belong to the SLC6 family [106]. In certain circumstances, these transporters can transport other substances including drug molecules and toxins, which may be associated with positive or negative modulation of their original function. In this regard, dopamine transporters enabled the transport of molecular toxins, such as MPP^+, 6-hydroxydopamine [107], and paraquat [108] into the dopaminergic neurons leading to selective dopaminergic neuron damage. Many researchers have reported the influence of polyphenols on DATs; pretreatment of mice with 7,8-dihydroflavone significantly attenuated the reduction of DATs in the striatum after repeated doses of methamphetamine [109], and consequently reduced the associated behavioral abnormalities and neurotoxicity. In certain occasions, DATs behave as molecular ports that accumulate neurotoxins and can be modulated by EGCG, and this effect is considered neuroprotective against MPP^+-induced neurotoxicity. Based on real time-PCR data, EGCG did not interfere with the transcription of DAT mRNA, suggesting direct inhibitory effect on DAT, probably through modulating its internalization to activate PKC [110].

In concentration dependent pattern, both cis and trans resveratrol interfere with the uptake of noradrenaline and 5-HT in rat brain, suggested this polyphenol as a potential source for many CNS acting drugs, including antidepressants [111]. The natural polyphenol hispidulin produced anticonvulsant activity through the inhibition of glutamate release from cortical synaptosomes, mostly through the blockade of presynaptic voltage-dependent Ca^{+2} channels [112]. Moreover, the neuroprotective effect of procyanidin against ischemic injury was attributed to attenuating the reduction in glutamate uptake mediated through interference with the ATP required for active reuptake at mitochondrial level [113]. Similar effect was observed for myricetin and quercetin in oxygen/glucose deprivation induced swelling in C6 glial cells [114]. In another model of injury of astroglial cells, EGCG significantly increases glutamate uptake in C6 glial cells, and this may contributes to the neuroprotective role of glial cells during excitotoxicity [115]. Other types of polyphenols, including luteolin and apigenin can act as activator of MATs, which might have positive impact on many hypermonoaminergic psychological disturbances, especially during cocaine addiction [99]. Currently available data clearly showed that natural products may be of therapeutic benefits in many experimentally-induced neurological disorders. Recently, many studies shed a light on natural polyphenols that may have therapeutic significance in the prevention and treatment of many specific neurological disorders. For example, berberine, curcumin, honokiol, and tanshinone IIA, were capable to cross the BBB and protect brain tissue against damage in various animal models of neurological disorders [116-118]. These plant-derived polyphenols also have been demonstrated to decrease glutamate release in rat brain tissues [119-122]. In summary, dietary polyphenols may influence a person's mood and improve quality of life. Several *in vitro* studies using cell line expressing uptake transporter proteins showed different potencies of polyphenols to modulate transporter proteins including MAT, especially NET, SET and DAT, either directly or indirectly. In this respect, the amount and duration of polyphenols consumed with diet may be the underlying factor involves modulation of both function and expression. This, in turn, could affect the bioavailability, distribution and transport of various substrates handled by these transporters. Understanding interactions of polyphenols with transport proteins seems to be a pre-request to predict the structures of potential flavonoid-based drugs and match with the desired biological effects. Moreover, this knowledge aid in prediction the possible side-effects associated with flavonoid usage to be minimized. Actually, challenge ahead and further research required to establish the exact mechanism and determine whether polyphenols and/or metabolites have an efficacy in treatment of disorders related to uptake transporters.

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Mini Review Article

Can propolis and caffeic acid phenethyl ester be promising agents against cyclophosphamide toxicity?

Sumeyya Akyol[1], Mehmet Akif Gulec[2], Haci Kemal Erdemli[3], Omer Akyol[4]

[1]Department of Medical Biology, Turgut Ozal University Medical Faculty, Ankara, Turkey, [2]Department of Medical Biochemistry, Faculty of Medicine, Turgut Ozal University, Ankara, Turkey, [3]Department of Biochemistry Laboratory, Corum Training and Research Hospital, Corum, Turkey, [4]Department of Medical Biochemistry, Hacettepe University Medical Faculty, Ankara, Turkey

Address for correspondence: Sümeyya Akyol, Department of Medical Biology, Turgut Ozal University Medical Faculty, Ankara, Turkey. E-mail: sumeyyaak@hotmail.com

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ABSTRACT

Propolis is a mixture having hundreds of polyphenols including caffeic acid phenethyl ester (CAPE). They have been using in several medical conditions/diseases in both *in vitro* and *in vivo* experimental setup. Cyclophosphamide (CP) has been used to treat a broad of malignancies including Hodgkin's and non-Hodgkin's lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, Ewing's sarcoma, breast cancer, testicular cancer, etc. It may cause several side effects after treatment. In this mini review, the protective effects of propolis and CAPE were compared each other in terms of effectiveness against CP-induced injuries.

KEY WORDS: Caffeic acid phenethyl ester, cyclophosphamide, propolis

INTRODUCTION

Propolis, having hundreds of polyphenols, is a mixture produced by the honeybee. This sticky, greenish-brown product has different compositions depending on the location of the bees and what trees and flowers they have access to. Propolis from Turkey or Egypt will not have the same chemical properties as propolis from Europe or Brazil. This is because it is very difficult for researchers to come to general conclusions about its health benefits. Caffeic acid phenethyl ester (CAPE) [Figure 1] is one of important compounds found in propolis that has antiviral [1], antioxidant, anti-inflammatory, antiproliferative, antitumor, and immunomodulatory effects [2]. This marvelous compound has been used to prevent oxidative stress-based deterioration in cells/tissues/organs in both cell culture and experimental animals. Lately, the protection of CAPE on central and peripheral nervous system as well as a reproductive system have been extensively reviewed [3-5]. Cyclophosphamide (CP) is an anticancer chemotherapeutic drug classified as an alkylating agent. It has extensively been used to treat a broad of malignancies including Hodgkin's and non-Hodgkin's lymphoma, Burkitt's lymphoma, chronic lymphocytic leukemia, Ewing's sarcoma, breast cancer, testicular cancer, etc. CP can cause several side effects on treatment. It has toxic effects in almost every system in the human body including the heart, liver, and kidney of which mostly due to its structural properties prone to induce oxidative stress *in vitro* and *in vivo*.

This study aimed to collect data and compare the protective effects of propolis and CAPE against CP-induced injury in animals. There are limited studies in this specific field. Lately,

ameliorative effect of propolis against CP-induced toxicity in mice was studied by El-Naggar *et al.* [6]. It throws light on the side effects of a common anticancer agent, CP, used in the treatment of various malignancies and possible remedies to prevent that type of side effects in vital organs such as liver and kidney. The proposed natural compound propolis has been found to be protective against CP toxicity. Uysal *et al.* [7] conducted an experimental animal study to determine protective role of CAPE on CP-induced hemorrhagic cystitis (HC). While CP-induced HC lead to increase in superoxide dismutase, catalase, and malondialdehyde activities/levels, CAPE significantly reduced these parameters showing the protective effects. In addition to this biochemical effects, CAPE also ameliorates edema, hemorrhage, inflammation, and mucosal ulceration of CP-induced HC.

We published a review article about toxicities of some therapeutic compounds and the protective effect of CAPE on chemotherapy- and radiotherapy-induced toxicity [8]. We have shown that CAPE has protective effects on oxidative stress-induced toxicities by doxorubicin (nephrotoxicity) [9], cisplatin (nephrotoxicity, ototoxicity, and hepatotoxicity) [10-13], and bleomycin (lung fibrosis) [14].

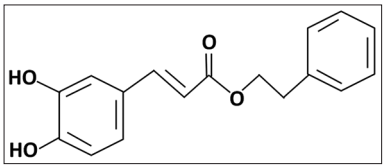

Figure 1: The chemical illustration of caffeic acid phenethyl ester

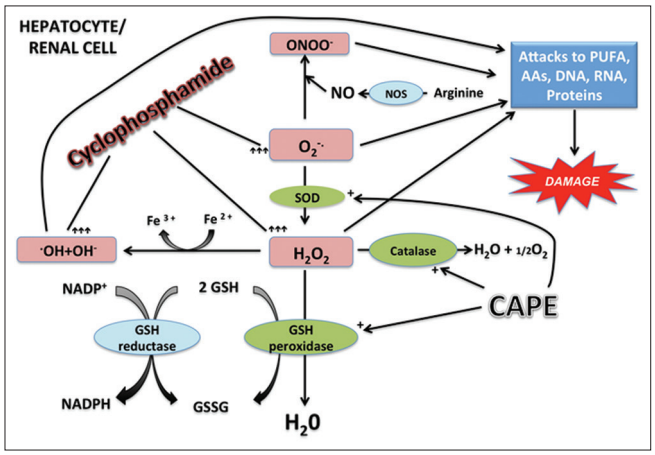

Figure 2: Proposed mechanism of how cyclophosphamide-induced oxidative stress is blocked by antioxidant enzymes in several parts of hepatocytes and renal cells and how CAPE shows its protective effects against oxidative stress. AAs: Amino acids, CAPE: Caffeic acid phenethyl ester, CAT: Catalase, Fe^{2+}: Ferrous iron, GPx: Glutathione peroxidase, GSH: Reduced glutathione, GR: Glutathione reductase, GSSG: Oxidized glutathione, H_2O: Water, $NADP^+$: Oxidized nicotinamide adenine dinucleotide phosphate, NADPH: Reduced nicotinamide adenine dinucleotide phosphate, O_2: Molecular oxygen, $O_2^{\cdot-}$: Superoxide anion radical, OH^-: Hydroxyl ion, OH: Hydroxyl radical, $ONOO^-$: Peroxynitrite, NO: Nitric oxide, NOS: Nitric oxide synthase, PUFA: Polyunsaturated fatty acid, SOD: Superoxide dismutase

Currently, there is no medically recommended dose for propolis, since the mixture of propolis is subjected to change depending on its source. The most successful medical application field of propolis is beauty and skin care, especially in acne vulgaris because of its antibacterial, antiviral, antifungal, and anti-inflammatory properties. Despite the fact that both water and ethanolic extractions of propolis have been used in the *in vivo* and *in vitro* experiments, water-soluble extracts of propolis exhibit higher antioxidant and inhibitory activities as compared ethanolic extract *in vitro* [15]. In this perspective, even though the extraction method selection is dependent on the authors' desire, it would be expected for authors to study propolis for their experiments comparatively by selecting propolis extracted by both extraction methods. CAPE is the most potent antioxidant agent of propolis mixture having free radical scavenging activity and potent inhibition of NF-κB. So, the protective antioxidant effect of ethanol extract of propolis on organs depends mostly on CAPE rather than other polyphenolic compounds such as flavonoids, phenolic acids, and their esters [Figure 2]. CAPE was shown to completely block the production of reactive oxygen species in human neutrophils and in the xanthine/xanthine oxidase systems at 10 μM concentration by its competent antioxidant capacity [16]. Indeed, CAPE has a regulatory effect on antioxidant enzyme activities such as catalase, superoxide dismutase, and glutathione peroxidase [7,17] [Figure 2].

It has been shown that CAPE application to the rats modifies the enzyme activity of cytochrome P450 (CYP) isoforms involved in the activation of diethylnitrosamine such as CYP1A1/2 and CYP2B12 [18]. Furthermore, treatment with CAPE of carbon tetrachloride-induced hepatotoxicity in mice blocks CYP2E1-mediated CCl_4 bioactivation and protects against fas/FasL-mediated apoptosis [19]. It will be very interesting to see the effect of CAPE on CYP2B6, which constitutes 3-6% of total hepatic CYP content and metabolizes several pharmaceuticals including CP [20]. To achieve this, further studies on the every single bioactive constituent of propolis such as CAPE and some other polyphenols are necessary to identify interactions mediating their biological effects on CYP2B6, since there are roughly 150 different polyphenolic compounds within propolis.

As a conclusion, studying propolis to prevent CP-induced oxidative stress in animals has several limitations since the proposed effect cannot be specified to one or several molecules within the mixture. In that case, every single bioactive constituent of propolis needs to be studied to show the source of real effects and the molecular mechanisms of this effects.

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