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Full Length Research Paper

An evaluation of the anticancer activity in *Hopea odorata* extracts

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Possible anticancer characteristics of *Hopea Odorata* extracts were investigated by cell proliferation and viability studies of cells in culture. The mechanism of action was studied by determining the rate of apoptosis and expression of protein involved in signal transduction. The result indicated that the butanol extract of *H. Odorata* had selective inhibition to both Hep G2 and Chang cells with IC_{50} of 20.14 and 377 µg/ml, respectively. Growth inhibition by the extract showed an increased of apoptosis at concentration of 25 µg/ml. Cell study demonstrated morphologic changes characteristic of apoptosis such as chromatin condensation and fragmentation, as well as formation of apoptotic bodies. However, MAPK kinase signal transduction pathway indicated no difference in ERK1 and ERK2 expression level after exposure at varying time. P53 protein level also showed no changes in expression compared to control. In conclusion, the increase in apoptosis observed was not due to changes in MAPK pathways involving ERK1, ERK 2 and p53 but may involve other pathway, which require further investigation.

Key words: Antiproliferation, *Hopea Odorata*, apoptosis.

INTRODUCTION

Hopea Odorata belongs to the Dipterocarpaceae family, locally known as Merawan siput jantan. It can grow up to 120 feet to produce good quality timber. The wood of *H. Odorata* varies in colour from a very pale yellow, or white to brown when first cut and characteristically darkens to a brownish or yellowish-brown colour after more or less prolonged exposure to the air. The dammar of this tree is said to have medicinal property used in treating sores and wounds (Burkill, 1935). Phytochemistry studies reported that the heartwood of *H. Odorata* contain certain types of phenolic compounds (Coggon et al., 1964). These polyphenols are reported useful as antioxidants, antimutagens, scavengers of free radicals and therefore have implications in the prevention of pathologies such as cancer and cardiovascular disease (Scalbert et al., 2005). In the quest for anticancer agents from natural sources, thousands of natural compounds were screened using both *in vitro* and *in vivo* methods (Balis, 2002). Due to the potential ability of these polyphenols as probable antitumour/antimutagen factor, the study was conducted to examine the cytotoxic and apoptotic effect of crude extract (CE) of *H. Odorata* using *in vitro* methods involving liver cancer cell lines (Hep G2) and a normal liver cell line (Chang cell).

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MATERIALS AND METHODS

Chemicals and reagents

Tissue culture disposables and growth media for cell culturing were purchased from Nalgene and Flow Lab, respectively. Tetrazolium compound (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methoxysulfate; PMS) and 5-Bromo-2-deoxy-Uridine (BrdU) were purchased from Promega (USA) and Boehringer Mannheinn (Germany), respectively. Three *H. Odorata* extracts tested were prepared according to the Figure 1. All the plant extracts were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The final concentration of DMSO used was adjusted to 1% (v/v), the concentration used in control cell. All other chemicals were purchased from Sigma Chemical Co.

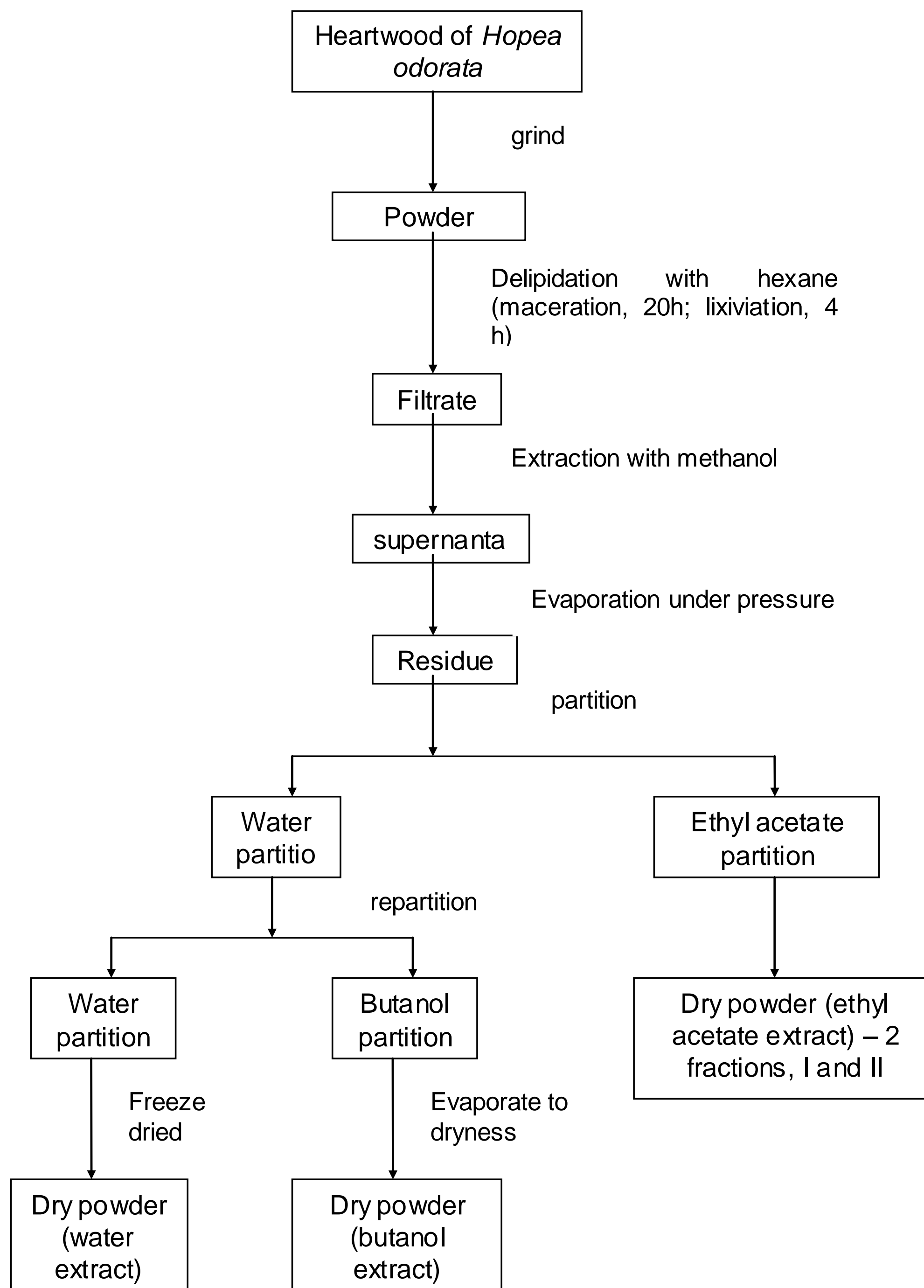

Figure 1. Preparation of *Hopea Odorata* extracts.

Cell culture

The hepatoma cell line, Hep G2 and Chang normal liver cell line were obtained from American type cell collection (ATCC), Rockville, MD and was cultured in Earle's minimum essential medium (MEM) containing L-Glutamine and sodium bicarbonate and supplemented with 10% fetal bovine serum in 5% CO_2 incubator at 37°C. Stock was allowed to grow to 80 to 90% confluence in T-75 flask. Culture medium was changed every two to three days. Cell viability was monitored using trypan blue exclusion test.

In vitro cytotoxicity assay

The proliferation assay was performed in 96 well flat bottom micro-titer plate at a plating density of 2×10^4 cell/well in a total volume of

100 ml of medium. Both Hep G2 cell and Chang cell were incubated at 37°C for 48 h with and without the test compounds at the following final concentration; 0, 10, 25, 50, 70, 100, 250, 500, 750 and 1000 μg/ml. Cells grown in a micro titer plate (MTP) were incubated with BrdU (10 µmol/l) for 18 h. The cells were then fixed with ethanol/HCl followed by incubation with nucleases to partially digest the DNA. The cell proliferation was then measured by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Gratzner, 1982). Incorporated BrdU was detected with the monoclonal anti BrdU-POD, Fab fragments and the bound conjugate was visualized with the soluble chromogenic substrate 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and measured using an ELISA reader at wavelength of approximately 490 nm.

Cellular growth in the presence and absence of plants extract was also determined by using the MTS/PMS assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, USA), according to the manufacturer's protocol. This assay is a colorimetric method, in which MTS is bioreduced to a formazan product that is soluble in tissue culture medium by dehydrogenase enzyme that is found in metabolically active living cells (Barltrop et al., 1991). The intensity of formazan product as measured at absorbance wavelength of 490 nm absorbency, is directly proportional to the number of living cells in cultured and is the measure of cell viability (Mossman, 1993). IC_{50} values were expressed as microgram of compound concentration per mililitre that caused a 50% growth inhibition as compared to controls (cell growth in the absence of extract).

Apoptosis assay

Apoptosis is characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintergration. In this study, Cellular DNA fragmentation ELISA purchased from Boehringer Mannheim was used to determine the apoptotic cell death. 2×10^4 BrdU labelled cells in the presence and without *H. odorata* extract, incubated for 24 h. At the end of the incubation, cells were centrifuged and cellular lysate was analysed for apoptosis.

Flourescence microscopic analysis

Hep G2 cell (7×10^5 cell) was cultured in EMEM medium suplemented with 10% fetal bovine serum in petri dish. After 24 hours of incubation, the medium was replaced with EMEM containing fetal bovine serum with 25 µg/ml *H. odorata* butanol extract. The cells were incubated for 4 h and the fixed with 1% cold formadelhyde in PBS for 30 min. The cell was then washed with PBS and stained with 1 ml 10 µg/ml Propium iodide. The specimens were analysed by flourescence microscopy.

Differential staining of cells

Cell cultured on cover slips and exposed to *H. Odorata* butanol extract for 24 h were fixed in 100% methanol. The nuclei were then stained with acid dye and finally, the cytoplasm was stained using basic dye. The morphological appearance of the cell was viewed under a light microscope.

Protein extraction and western blot analysis.

8.5×10^6 cells were cultured and after 24 h incubation, the mediun was replaced with a fresh medium containing 25 µg/ml butanol extract of *H. odorata*. Cells were incubated at different time duration of 0, 2 h, 6 h, 12 , 18 and 24 h. Cells were then trypsinized and lysated, supernatant was kept at 80°C until used. 60 µl protein were boiled at 95°C for 5 min and were resolved on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose and immunoreacted with primary antibodies, followed by a 1 h incubation with secondary antibodies conjugated with alkaline phosphatase. The primary antibodies were mouse monoclonal anti-human p53, ERK 1 and ERK 2 (Pharmagen). Visualization was performed using chemiluminescence detection (Pierce).

RESULTS AND DISCUSSION

The viability assay results are concurrent with the proliferation assay in which only butanol extract of *H. Odorata* showed anti proliferation effect. Figure 2 represents the results of anti proliferation for 48 h as measured by BrdU assay in an *in vitro* system. Results are expressed as the percentage of absorbency of cells incubated with different doses of extracts against control. As shown in Figures 2 and 3, only butanol extract showed significant growth inhibition, IC_{50} (Hep G2) is equal to 20.14 µg/ml and at the same time need a higher concentration (IC_{50} = 377.60 µg/ml) to produce the same effect in normal Hepar cell, Chang. Those data suggested that the Butanol extract have selective toxicity towards liver tumour cells. Two other extracts did not show any significant inhibition. Hence, only butanol extract was used for further studies.

Cellular DNA fragmentation ELISA revealed that anti proliferation demonstrated by butanol extract via apoptosis pathway. Figure 4 shows detection of nucleosomes in the cytoplasmic fractions at different extract concentration after 24 h incubation. Fluorescence microscopy analysis revealed that the Butanol extract of *H. Odorata* induced apoptosis and caused morphological changes of cells undergo apoptosis. Cell undergoing apoptosis process showed sequence of morphological modification that include nucleoplasm and cytoplasm condensation with a pronouned decrease in cell volume, chromatin condensation and fragmentation, plasma membrane blebbing and degeneration of the nucleus into membrane bound apoptotic bodies (Steller, 1995). For differential staining, the result demonstrated cells undergo nuclear shrinkage, cytoplasmic constriction and formation of apoptotic bodies as shown in Figure 5. Signal transduction has been the target for anti cancer by inhibiting phosphorylation process (Seymour, 1999). One of the pathways is MAP kinase which is involved in extracellular signal regulation and ERK 1 and ERK2 are among the unique component in this transduction pathway (Willbacher et al., 1999). While the p53 is the nuclear phosphoprotein involved in stimulating apoptosis directly by binding and act on several protein in variety pathway including C-Abl, basal transcription factor and so on (Oliner, 1992). Western blot Analysis demonstrated (Figure 6) that after treatment with 25 g/ml *H. Odorata*, the level of p53 protein was apparently unchanged with

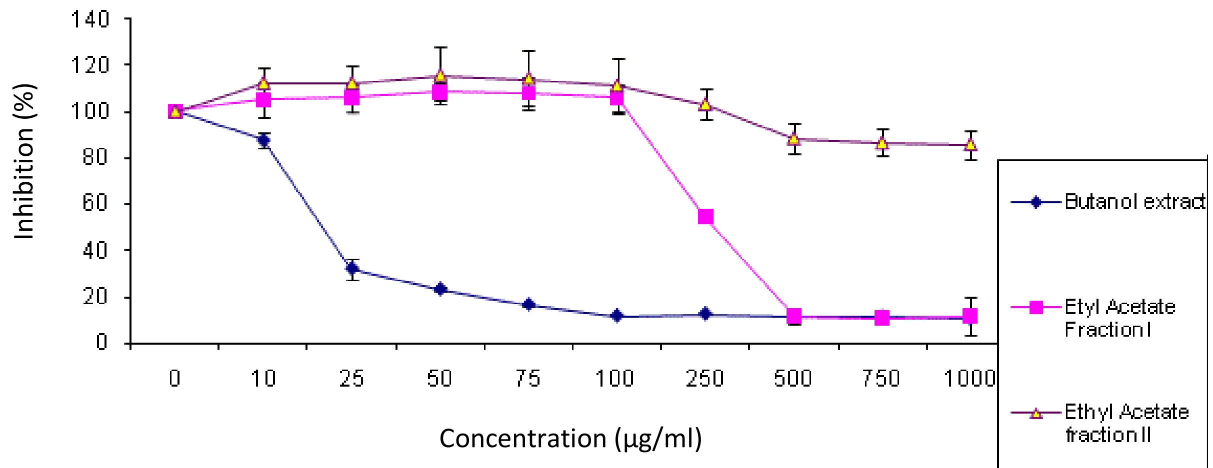

Figure 2. Percentage of cell proliferation, measuring with BrdU labelling assay in the presence of plant extracts at different concentration after 48 h incubation.

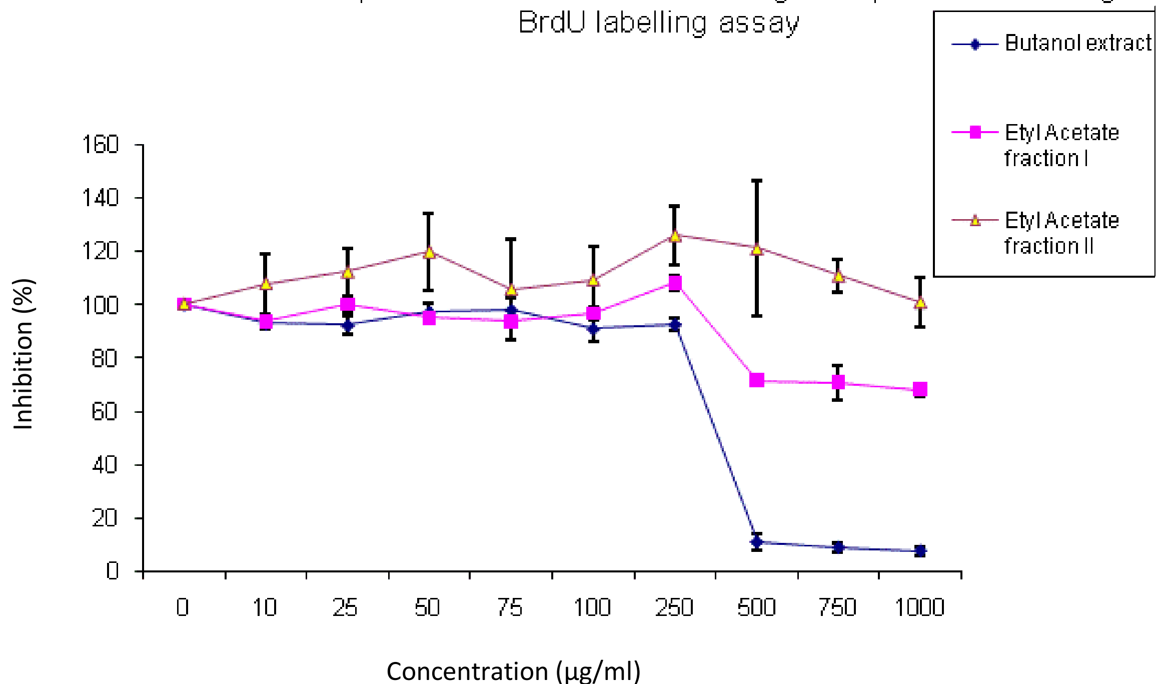

Figure 3. Percentage Chang cell inhibition after 48 h incubation in the presence of *Hopea odorata* extracts at different concentration.

respect to the level found in the untreated cells. ERK 1 and ERK 2 expression also remained unchanged after having treatment with *H. Odorata* from 0 hour up to 24 hours. The apoptosis induced by *H. Odorata* butanol

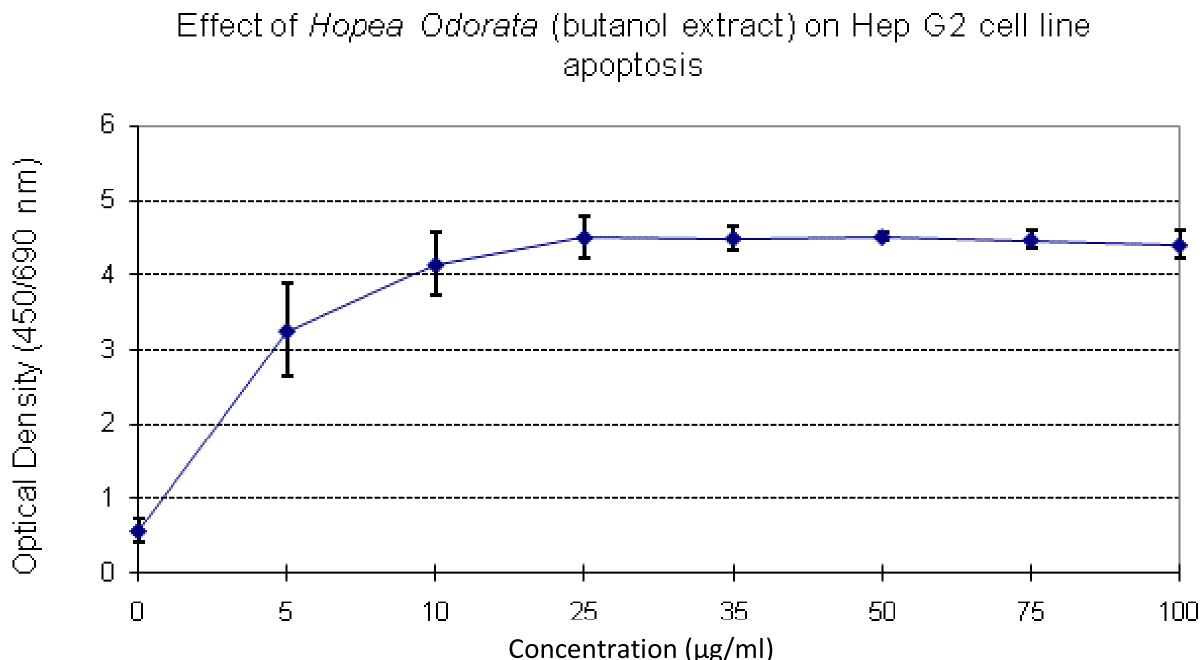

Figure 4. Measuring apoptosis with the cellular DNA fragmentation ELISA BrdU-labelled Hep G2 cells were cultured in the presence of *Hopea Odorata* Butanol extract for 24 h. After incubation, the lysates were tested by ELISA.

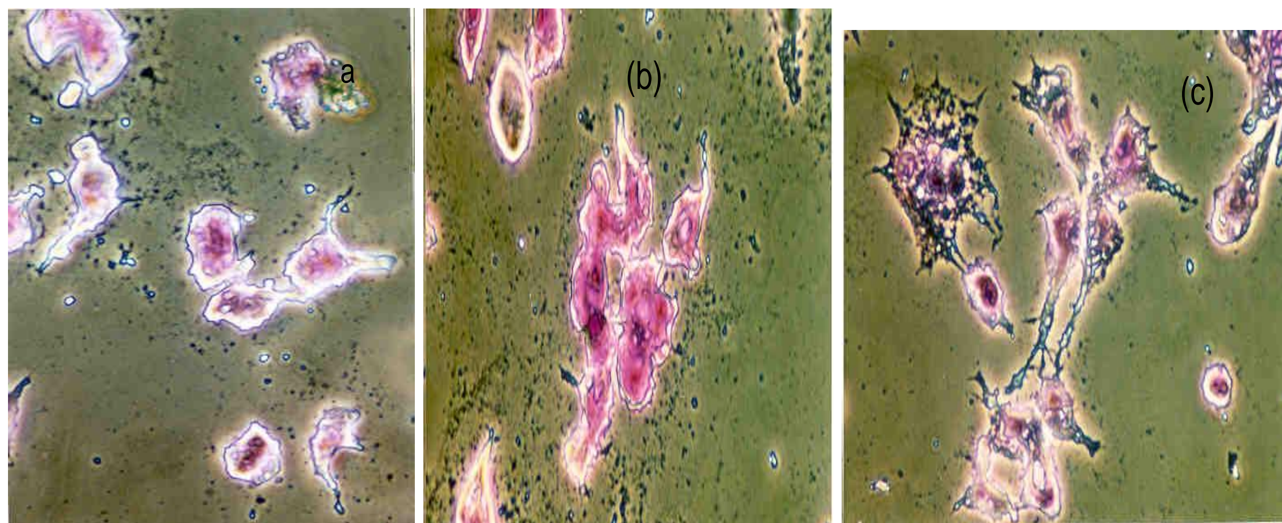

Figure 5. Morphological features of *Hopea Odorata* butanol extract treated Hep G2 cells (25 µg/ml) after 24 h incubation examined by differential staining. (a) and (b) showed nuclear shrinkage and cytoplasmic constriction, (c) showed formation of apoptotic bodies.

extract in Hep G2 seem to be determined by other mean of mechanism. In this case, apoptosis is not accompanied by the increments in p53. Thus, the probability of *H. Odorata* being involved in yet other signal transduction pathway leading the proliferation pathway of cell growth and further studies will be needed.

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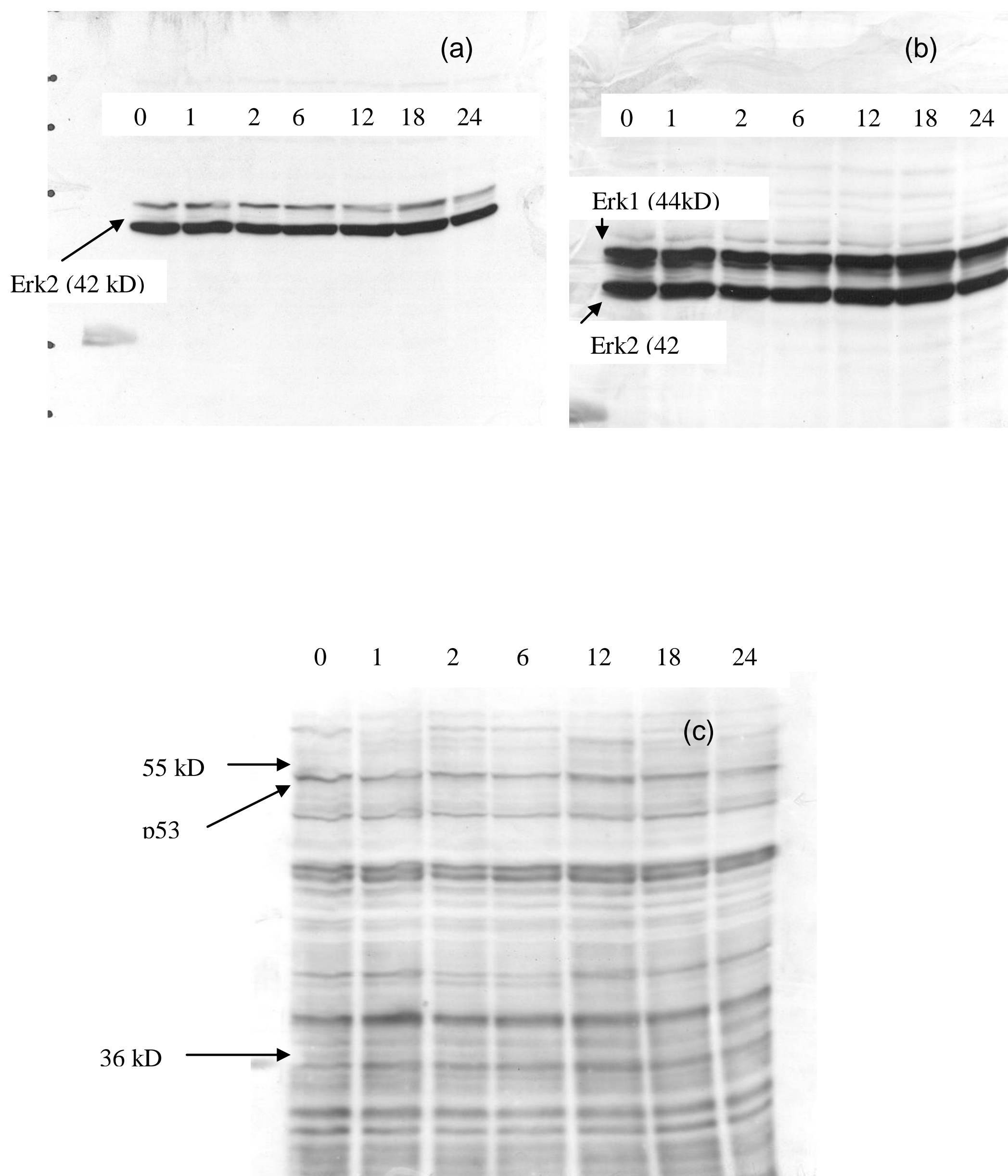

Figure 6. Western blot Analysis of (a) ERK 2, (b) ERK 1 & ERK 2, and (c) p53. Extracts were prepared from cells as described in methods. Cells were exposed to 25 µg/ml plant extract at different time duration (0, 1, 2, 6, 12, 18, and 24 h. 60 µg of protein were submitted to western blot analysis and assayed for particular proteins expression.

REFERENCES

Balis FM (2002). Evolution of anticancer drug discovery and the role of cell based screening. J. Natl. Cancer Inst., 94(2): 78-79.

Barltrop JA, Owen TC, Cory AH (1991). 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazoly)-2,5-diphenyl tetrazolium bromide (MTT) reducing to purple water soluble formazans as cell viability indicators. Bioorg. Med. Chem. Lett., 1: 611.

Burkill IH (1935). A Dictionary of the Economic Products of the Malay Peninsula. The Crown Agents for the Colonies, London, 1(A-H): 1192-1193.

Coggon P, Janes NF, King FE, Molyneux RJ, Morgan JWW, Sellars K (1964). Hopeaphenol, an extractive of the Heartwood of *Hopea Odorata* and *Balacarpus Heimii*. University of Nottingham.

Gratzner HG (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. Science, 218(4571): 474-475.

Mossman T (1993). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods, 65: 5-63.

Oliner JD, Kinzler GP, Olson DC, George DL (1992). Amplication of gene encoding a p-53-associated protein in human sarcomas. Nature, 358: 80-83.

Scalbert A, Johnson IT, Saltmarsh M (2005). Polyphenols: antioxidants

and beyond. Am. J. Clin. Nutr., 81(suppl): 215S-217S.
Seymour L (1999). Novel anticancer agents in development: exciting prospects and new challenges. Cancer Treat. Rev., 25: 301-312
Steller H (1995). Mechanisms and genes of cellular suicide. Science, 267: 1445-1449.
Willbacher JL, Goldsmith EJ, Cobb MH (1999). Phophorylation of MAP Kinase by MAP/ERK involved multiple regions of MAP Kinases. J. Biol. Chem., 274(24): 16988-16994.

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Full Length Research Paper

Anti-*Helicobacter pylori* and anti-internalization activities of Thai folk remedies used to treat gastric ailments

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Invasion of gastric epithelium cells by *Helicobacter pylori* is associated with antibiotic resistance and persistence infection, which contribute to eradication failure in many circumstances. The aims of this study were to investigate the anti-*H. pylori* and anti-internalization activities of thirteen Thai plant extracts used for gastric ailments in traditional medicine including *Curcuma longa* (L.), *Kaempferia parviflora* Wall. Ex Baker, *Abelmoschus esculentus* (L.) Moench, *Musa sapientum* (L.), *Aloe vera*, *Centella asiatica* (L.), *Allium sativum* (L.), *Zingiber officinale* Roscoe, *Alpinia galanga* (L.) Swartz, *Andrographis paniculata*, *Ocimum basilicum* (L.), *Ocimum sanctum* (L.) and *Cymbopogon citratus*. The minimum inhibitory concentrations against 11 clinical isolates and 2 reference strains of *H. pylori* were examined using an agar dilution method. The level of internalization against HEp-2 cells by *H. pylori* was compared among 3 extracts of *C. longa*, *K. parviflora* and *M. sapientum* by a conventional gentamicin internalization assay. From thirteen methanolic extracts, the extracts of *C. longa* and *K. parviflora* exhibited significant anti- *H. pylori* activities at minimum inhibitory concentration (MIC) of 32 and 64 µg/ml, respectively. The extracts from *C. longa* and *M. sapientum* showed inhibitory effects on the internalization of *H. pylori* to HEp-2 cells at 3 h of treatment. However, the adverse effect of *C. longa* was found at 6 and 12 h by enhancing the internalization activities. Thus, appropriate use of medicinal plants may be valuable for curing and preventing *H. pylori* infection.

Key words: *Helicobacter pylori*, medicinal plants, minimum inhibitory concentration (MIC), anti-internalization.

INTRODUCTION

Helicobacter pylori infection represents the major cause of peptic ulcer disease and gastric cancer (National Institutes of Health, 1994). It was classified by the World Health Organization as a Group I, or definite, human carcinogen. Approximately 50% of the world's population is estimated to be infected with *H. pylori*. Transmission occurs via human-to-human contacts, fecal-oral route and intrafamilial transmission from mother to her offspring (Magalhaes and Luzza, 2006). The recommended eradication therapy continues to be triple therapy with a proton pump inhibitor (PPI) and two antibiotics of clarithromycin, amoxicillin, or metronidazole for 7 days (Cavallaro et al., 2006). However, the eradication rate achieves only 60 to 80% in clinical practice. The treatment failure resulted from an increasing of antibiotic resistance (Vilaichone et al., 2006) and reducing treatment efficacy due to intracellular survival (Andersen and Holck, 1990; Evans et al., 1992). A minor *H. pylori* population which localizes inside gastric mucosal cells could not be entirely killed even with a long-time administration of pertinent antibiotics (Korman et al., 1997). This leads to the relapses of gastric ulcers. Some other disadvantages of long antibiotic treatment attribute

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to drug resistance development and drug side-effects. Therefore, the alternative approaches of herbal medicines have become more prominent and been extensively studied worldwide in order to find new anti-*H.pylori*. (Stamatis et al., 2003; Ndip et al., 2007; Shih et al., 2007). A number of natural products have been shown to have anti-*H. pylori* activity such as garlic extracts, cinnamon extracts, tea catechins, wasabi and honey (Mabe et al., 1999; Ohta et al., 1999; Osato et al., 1999; Tabak et al., 1999; Shin et al., 2004). Some spice and food plants used in Thai traditional medicine for the treatment of gastrointestinal diseases had been proven to exhibited growth-inhibitory effects against *H. pylori* including *Myristica fragrans* (aril), *Barringtonia acutangula* (leaf), *Kaempferia galanga* (rhizome), *Cassia grandis* (leaf), *Cleome viscosa* (leaf), *Myristica fragrans* (leaf), *Syzygium aromaticum* (leaf), *Pouzolzia pentandra* (leaf), *Cycas siamensis* (leaf), *Litsea elliptica* (leaf) and *Melaleuca quinquenervia* (leaf) (Bhamarapravati et al., 2003). This study aimed to screen the anti-*H. pylori* and anti-internalization of thirteen Thai folk remedies which have been frequently prescribed for treating gastric ailments.

MATERIALS AND METHODS

Plant materials and extraction

Plant materials were purchased from supermarket and Thai traditional drug store at Pak Klong market in Bangkok, Thailand. 500 g of roughly ground air-dried plant materials were extracted twice with methanol. The extracts were filtered and concentrated by evaporation at 40°C until dryness. Each sample was dissolved at 0.25 to 1 g/ml with dimethyl sulfoxide (DMSO). These stock solutions were filtrated through 0.2 µm millipore and stored at -20°C before use.

Bacterial strains and culture condition

The *H. pylori* strains used in this study were two standard strains (ATCC 43504 and ATCC 43526) and eleven clinical isolates obtained from Division of Gastroenterology, Department of Medicine, Thammasat University Hospital, Pathumthani, Thailand and from Department of medicinal sciences, Ministry of Public Health, Nonthaburi, Thialand. The bacteria were grown on brain heart infusion agar containing 7% (v/v) sheep blood and were incubated at 37°C for 3 to 5 days under microaerophilic conditions using gas generating kit (Mitsubishi, Japan).

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined, in duplicate, by an agar dilution method following the National Committee for Clinical Laboratory Standards Institute guidelines. Briefly, 1 ml of each diluted plant extract was mixed with 24 ml of unsolidified Mueller-Hinton agar supplemented with 5% sheep blood and poured into sterile Petri dishes. Final concentrations of each plant extract range from 16 to 512 µg/ml except *Musa sapientum* were ended at 25,000 µg/ml. A 72-h *H. pylori* strains were suspended in 0.85% NaCl to a turbidity of McFarland standard no. 2 (10^7 to 10^8 CFU/ml). Subsequently, three microlitres of each inoculum was spotted on each plate and incubated as previously described for 3 days. The MIC was defined as the lowest concentration of each extract at which no visible growth was observed.

Culture of HEp-2 cells

HEp-2 cells from a patient with human larynx carcinoma were kindly provided by Dr.Pornthep Tiensiwakul, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand and were cultured in a medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL Laboratories, USA), 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution (Gibco BRL Laboratories, USA) at 37°C in 5% CO_2 with 80% humidity. The cells were washed two times with Hank's balanced salt solution (HBSS), treated with trypsin, and seeded on six wells tissue culture plates at a density of 1×10^6 cells/ml per well. After seeding, the cells were allowed to attach overnight in the same atmosphere as described previously to obtain 80% confluence.

Gentamicin internalization assay

Six wells with confluent layers of HEp-2 cells were used for examining the internalization ability of *H. pylori* ATCC 43504. After washing twice with HBSS, 900 µl of antibiotic-free DMEM and 100 µl of *H. pylori* suspension (about 10^8 CFU/ml) were added to each well. The treatment times were set at 3, 6, 12 and 24 h separately in each well in the absence or presence of the extracts. The upmost dilution of DMSO used at 5% was incorporated as control. Then, the cells were gently washed two times with HBSS, and treated with HBSS containing 100 µg/ml gentamicin and incubated for one hour to kill extracellular bacteria. HEp-2 monolayer were then washed three times with HBSS and lysed in distilled water for 10 min to release intracellular bacteria. The cell lysate was diluted in HBSS in 10-fold dilutions and 100 µl were plated onto brain heart infusion agar supplemented with 7% (v/v) sheep blood, incubated as previously described for 3 to 5 days to determine the number of viable intracellular bacteria. Colony-forming units of *H. pylori* were counted and were calculated as the percentage of bacteria surviving gentamicin treatment in proportion to the total number of bacteria added to the well.

Statistical analysis

All experiments were carried out independently in duplicate experiments. The inhibitions of internalization by various extracts at various time intervals were evaluated by one-way analysis of variance (ANOVA) and Kruskal-Wallis Test. Statistical significance was accepted at the $P = 0.05$ level.

RESULTS

Effects on growth of *H. pylori*

Methanol extracts of 13 herbal medicines used traditionally for gastric ailments and also as food ingredients were screened *in vitro* for the anti-*H. pylori* activities. The minimum inhibitory concentrations (MICs) against all the13 tested strains of *H. pylori* were shown in Table 1. *Curcuma longa* exhibited the strongest growth

Table 1. Minimum inhibitory concentrations (MICs: µg/ml) of extracts against 13 *H. pylori* strains.

Extract	Common name	Parts	MIC (µg/ml)
C. longa (L.)	Kra-min-chan (Thai)	Rhizome	32
K. parviflora Wall. Ex Baker	Kra-chai-dam (Thai)	Rhizome	64
Abelmoschus esculentus (L.) Moench	Kra-jeab-mon (Thai)	Fruit	>512
M. sapientum (L.)	Banana	Fruit	25,000
Aloe vera	Aloe vera	Gel in leaf	>512
C. asiatica (L.)	Bai Bua Bok (Thai)	Leaf	>512
A. sativum (L.)	Garlic	Capita	>512
Z. officinale Roscoe	Ginger	Rhizome	>512
Alpinia galanga (L.) Swartz	Galanga	Rhizome	>512
Andrographis paniculata	Fa-Talai-Jorn (Thai)	Whole	>512
O. basilicum (L.)	Basil	Stem	>512
O. sanctum (L.)	Thai Basil	Leaf	>512
C. citrates	Lemon Grass	Stem	>512

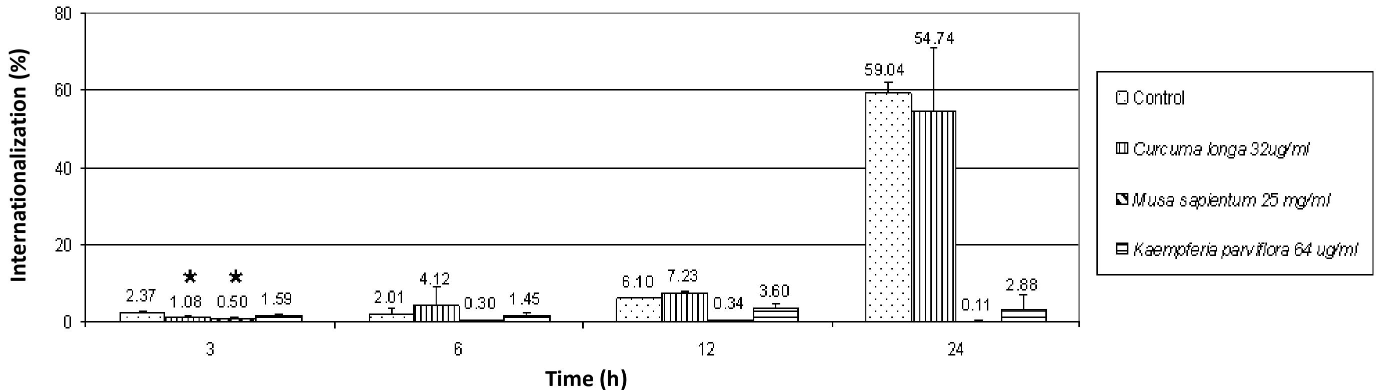

Figure 1. Internalization assay of *H. pylori*-infected HEp-2 cells in the absence or presence of plant extracts at 3, 6, 12 and 24 h of incubation. The extracts were added at the concentration of the MICs. Data are presented as mean ±SD of separate duplicate experiments with two wells per experiment.

inhibition with MIC of 32 µg/ml. Also significant anti-*H. pylori* actions with MIC of 64 µg/ml belonged to *Kaempferia parviflora*. While, no growth inhibiton against *H. pylori* was found in *Abelmoschus esculentus*, *M. sapientum*, *Aloe vera*, *Centella asiatica*, *Allium sativum*, *Zingiber officinale*, *Alpinia galanga*, *Andrographis paniculata*, *Ocimum basilicum*, *Ocimum sanctum* and *Cymbopogon citrates* (MIC > 512 µg/ml).

Effects on anti-internalization of *H. pylori* against HEp-2 cells

Two medicinal plants with superior anti-*H. pylori* activities (*C. longa* and *K. parviflora*) and one common medicinal plant with anti-gastric cancer (*M. sapientum*) (Mukhopadhyaya et al., 1987; Goel et al., 2001; Mohan Kumar et al., 2006) were tested for their anti-internalization activities against *H. pylori* ATCC 43504 into HEp-2 cells at 3, 6, 12 and 24 h of incubation using the concentration equal to their MICs. The MIC of *M. sapientum* was equal to 25 mg/ml. The results were summarized in Figure 1. It was found that the internalization capability of *H. pylori* was increased at the extended incubation time. Only, the extracts from *C. longa* and *M. sapientum* showed significant inhibition of internalization at 3 h of treatment compared to the untreated control (Figure 1; $P < 0.05$). If the internalization activities of control at each time was equal to 100%, the percent internalization of overall tests were reduced ranging from 21 to 67, 15 to 72, 6 to 59 and 0.2 to 5% at 3, 6, 12 and 24 h of incubation time, respectively. Surprisingly, *C. longa* enhanced the invasion during 6 and 12 h of treatment. The percent internalization of *C. longa* treated was 4.12 and 7.23%, while he untreated controls were 2.01 and 6.1%, at 6 and 12 h, respectively.

DISCUSSION

Adhesion and invasion to the gastric epithelial take the crucial role in the pathogenesis of *H. pylori* infection. The intracellular habitat of *H. pylori* established the persistent infection in the human gastric cells and caused the major problem of eradication failure (Bjorkholm et al., 2000). The intracellular *H. pylori* provided the repopulation in the extracellular environment (Amieva et al., 2002). Accompany with the increase of microbial resistant strains that reduced the effectiveness of antibiotic treatments (Levy, 2002). *In vitro* studies have shown that the intracellular bacteria are resistant to extracellular antibiotics, such as amoxicillin and gentamicin (Molyneux and Harris, 1993). Although, the intracellular antibiotic such as clarithromycin, significantly increased the efficacy of therapy, the prevalence of resistant strains to such agent still appeared with the rate of about 20%. The prevalence of clarithromycin and metronidazole resistances vary considerably between each geographic regions from 1.0 to 26.7% and 25 to 39.2%, respectively (Storskrubb et al., 2006; Gu et al., 2006; Janssen et al., 2006). At present, clarithromycin-containing triple therapy is no longer acceptable as empiric therapy (Graham and Shiotani, 2008). Plant extracts are the alternative source of antimicrobial agents with multiple organic components including phenols, quinines, flavones, tannins, terpenoids, and alkaloids (Cowan, 1999). For centuries, numerous folk medicines have been used for curing the gastrointestinal disorders in many countries all over the world. Most studies have been focused to their anti-*H. pylori* activities (Bae et al., 1998; Yesilada et al., 1999; Stamatis et al., 2003; Ndip et al., 2007; Zaidi et al., 2009). In Thailand, some spice and food plants had been shown to exhibit the growth inhibition against *H. pylori* (Bhamarapravati et al., 2003). In this study, *C. longa* and *K. parviflora* were shown to have significant anti-*H. pylori* activities. The MIC of *C. longa* was related to the previous data (Mahady et al., 2002). *Curcuma longa* conferred the inhibitory action against *H. pylori* invasion to HEp-2 cells in the beginning period (3 h) but it enhanced the bacterial internalization ability in the extended times (6 and 12 h). The inducement of cell invasion should be seriously concerned apart from its benefit such as pharmacological effects, anti-inflammatory, antimutagen, antibacterial, antiviral and antioxidant effects (Araújo and Leon, 2001). Curcumin (diferuloylmethane), the major constituent derived from the rhizomes of *C. longa* (Turmeric), has been shown to prevent gastric and colon cancers in rats (Ikezaki et al., 2001). Moreover, it was able to block the NF-κB activation and cellular motogenic response of epithelial cells induced by *H. pylori* infection (Foryst-Ludwig et al., 2004). While, *K. parviflora* was also the attractive plants, which has been reported to possess anti-allergic, antibacterial (Tewtrakul and Subhadhirasakul, 2007), antiplasmodial activities (Yenjai et al., 2004), as well as anti-peptic ulcer effects (Rujjanawate et al., 2005). Its anti-gastric ulcer activity was suggested to be related partly to preservation of gastric mucus secretion (Rujjanawate et al., 2005). Although, *M. sapientum* was weakly active against the growth of *H. pylori*, its effect against gastric cancer was interesting. *M. sapientum* had been indicated for gastric ulcer protection and healing activities through its prominent effects on mucosal glycoprotein, cell proliferation, free radicals and antioxidant systems (Mukhopadhyaya et al., 1987; Goel et al., 2001; Mohan Kumar et al., 2006). In our studies, *M. sapientum* exhibited the strongest inhibition against *H. pylori* invasion to HEp-2 cells. However, the adverse effects due to the high quantity of the extract have to be further elucidated. In conclusion, we reported the *in vitro* potential anti-*H. pylori* activity and anti-internalization activity of some Thai medicinal plants used in traditional treatment of gastrointestinal disorders. Further studies are needed to be carried out to investigate their mechanisms and effects *in vivo*. This finding may be useful in the future drug development in combination with antibiotics for effective treatment and prevention of *H. pylori*-associated gastric cancer.

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REFERENCES

Amieva MR, Salama NR, Tompkins LS, Falkow S (2002). *Helicobacter pylori* enter and survive within multivesicular vacuoles of epithelial cells. Cell. Microbiol., 4: 677-690.

Andersen LP, Holck S (1990). Possible evidence of invasiveness of *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis., 9: 135-138.

Araújo CC, Leon LL (2001). Biological activities of *Curcuma longa* L. Mem. Inst. Oswaldo. Cruz, 96: 723-728.

Bae EA, Han MJ, Kim NJ, Kim DH (1998). Anti-*Helicobacter pylori* activity of herbal medicines. Biol. Pharm. Bull., 21: 990-992.

Bhamarapravati S, Pendland SL, Mahady GB (2003). Extracts of spice and food plants from Thai traditional medicine inhibit the growth of the human carcinogen *Helicobacter pylori*. *In Vivo,* 17: 541-544.

Bjorkholm B, Zhukhovitsky V, Löfman C, Hultén K, Enroth H, Block M, Rigo R, Falk P, Engstrand L (2000). *Helicobacter pylori* entry into human gastric epithelial cells: A potential determinant of virulence, persistence, and treatment failures. Helicobacter, 5: 148-154.

Cavallaro LG, Egan B, O'Morain C, Di Mario F (2006). Treatment of *Helicobacter pylori* infection. Helicobacter, 11(Suppl 1): 36-39.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev., 12: 564-582.

Evans DG, Evans DJ Jr, Graham DY (1992). Adherence and internalization of *Helicobacter pylori* by HEp-2 cells. Gastroenterology, 102: 1557-1567.

Foryst-Ludwig A, Neumann M, Schneider-Brachert W, Naumann M (2004). Curcumin blocks NF-kappaB and the motogenic response in *Helicobacter pylori*-infected epithelial cells. Biochem. Biophys. Res. Commun., 316: 1065-1072.

Goel RK, Sairam K, Rao CV (2001). Role of gastric antioxidant and anti-*Helicobactor pylori* activities in antiulcerogenic activity of plantain banana (*Musa sapientum* var. *paradisiaca*). Indian J. Exp. Biol., 39: 719-722.

Gu Q, Xia HH, Wang JD, Wong WM, Chan AO, Lai KC, Chan CK, Yuen MF, Fung FM, Wong KW, Lam SK, Wong BC (2006). Update on clarithromycin resistance in *Helicobacter pylori* in Hong Kong and its effect on clarithromycin-based triple therapy. Digestion, 73: 101-106.

Janssen MJ, Hendrikse L, de Boer SY, Bosboom R, de Boer WA, Laheij RJ, Jansen JB (2006). *Helicobacter pylori* antibiotic resistance in a Dutch region: trends over time. Neth. J. Med., 64*:* 191-195.

Graham DY, Shiotani A (2008). New concepts of resistance in the treatment of *Helicobacter pylori* infections. Nat. Clin. Pract. Gastroenterol. Hepatol., 5: 321-331.

Ikezaki S, Nishikawa A, Furukawa F, Kudo K, Nakamura H, Tamura K, Mori H (2001). Chemopreventive effects of curcumin on glandular stomach carcinogenesis induced by N-methyl-N'-nitro-N-nitrosoguanidine and sodium chloride in rats. Anticancer Res., 21: 3407-3411.

Korman MG, Bolin TD, Engelman JL, Pianko S (1997). Sucralfate as an alternative to bismuth in quadruple therapy for *Helicobacter pylori* eradication. Helicobacter, 2: 140-143.

Levy SB (2002). The 2000 Garrod lecture. Factors impacting on the problem of antibiotic resistance. J. Antimicrob. Chemother., 49: 25-30.

Magalhaes QDM, Luzza F (2006). Epidemiology of *Helicobacter pylori* infection. Helicobacter, 11(Suppl1): 1-5.

Mabe K, Yamada M, Oguni I, Takahashi T (1999). *In vitro* and *in vivo* activities of tea catechins against *Helicobacter pylori.* Antimicrob. Agents Chemother., 43: 1788-1791.

Mahady GB, Pendland SL, Yun G, Lu ZZ (2002). Turmeric (*Curcuma longa*) and curcumin inhibit the growth of *Helicobacter pylori*, a group 1 carcinogen. Anticancer Res., 22: 4179-4181.

Mohan KM, Joshi MC, Prabha T, Dorababu M, Goel RK (2006). Effect of plantain banana on gastric ulceration in NIDDM rats: role of gastric mucosal glycoproteins, cell proliferation, antioxidants and free radicals. Indian J. Exp. Biol., 44: 292-299.

Mukhopadhyaya K, Bhattacharya D, Chakraborty A, Goel RK, Sanyal AK (1987). Effect of banana powder (*Musa sapientum* var. *paradisiaca*) on gastric mucosal shedding. J. Ethnopharmacol., 21: 11-19.

Molyneux AJ, Harris MD (1993). *Helicobacter pylori* in gastric biopsies--should you trust the pathology report? J. R. Coll. Physicians, 7: 119-120.

National Institutes of Health (1994). NIH Consensus Conference. *Helicobacter pylori* in peptic ulcer disease. NIH Consensus Development Panel on *Helicobacter pylori* in Peptic Ulcer Disease. J.A.M.A., 272: 65-69.

Ndip RN, Malange TAE, Mbullah SM, Luma HN, Malongue A, Ndip LM, Nyongbela K, Wirmum C, Efange SM (2007). *In vitro* anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. J. Ethnopharmacol., 114: 452-457.

Ohta R, Yamada N, Kaneko H, Ishikawa K, Fukuda H, Fujino T, Suzuki A (1999). *In vitro* inhibition of the growth of *Helicobacter pylori* by oil-macerated garlic constituents. Antimicrob. Agents Chemother., 43: 1811-1812.

Osato MS, Reddy SG, Graham DY (1999). Osmotic effect of honey on growth and viability of *Helicobacter pylori.* Dig. Dis. Sci., 44: 462-464.

Rujjanawate C, Kanjanapothi D, Amornlerdpison D, Pojanagaroon S (2005). Anti-gastric ulcer effect of *Kaempferia parviflora*. J. Ethnopharmacol., 102: 120-122.

Shih YT, Wu DC, Liu CM, Yang YC, Chen IJ, Lo YC (2007). San-Huang-Xie-Xin-Tang inhibits *Helicobacter pylori*-induced inflammation in human gastric epithelial AGS cells. J. Ethnopharmacol., 112: 537-544.

Shin IS, Masuda H, Naohide K (2004). Bactericidal activity of wasabi (Wasabia japonica) against *Helicobacter pylori*. Int. J. Food. Microbiol., 94: 255-261.

Stamatis G, Kyriazopoulos P, Golegou S, Basayiannis A, Skaltsas S, Skaltsa H (2003). *In vitro* anti-*Helicobacter pylori* activity of Greek herbal medicines. J. Ethnopharmacol., 88: 175-179.

Storskrubb T, Aro P, Ronkainen J, Wreiber K, Nyhlin H, Bolling-Sternevald E, Talley NJ, Engstrand L, Agréus L (2006). Antimicrobial susceptibility of *Helicobacter pylori* strains in a random adult Swedish population. Helicobacter, 11*:* 224-230.

Tabak M, Armon R, Neeman I (1999). Cinnamon extracts' inhibitory effect on *Helicobacter pylori*. J. Ethnopharmacol., 67: 269-277.

Tewtrakul S, Subhadhirasakul S (2007). Anti-allergic activity of some selected plants in the *Zingiberaceae* family. J. Ethnopharmacol., 109: 535-538.

Vilaichone RK, Mahachai V, Graham DY (2006). *Helicobacter pylori* diagnosis and management. Gastroenterol. Clin. North Am., 35: 229-247.

Yenjai C, Prasanphen K, Daodee S, Wongpanich V, Kittakoop P (2004). Bioactive flavonoids from *Kaempferia parviflora*. Fitoterapia, 75: 89-92.

Yeşilada E, Gürbüz I, Shibata H (1999). Screening of Turkish anti-ulcerogenic folk remedies for anti-*Helicobacter pylori* activity. J. Ethnopharmacol., 66: 289-293.

Zaidi SF, Yamada K, Kadowaki M, Usmanghani K, Sugiyama T (2009). Bactericidal activity of medicinal plants, employed for the treatment of gastrointestinal ailments, against *Helicobacter pylori*. J. Ethnopharmacol., 121: 286-291.

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Full Length Research Paper

Establishment of plant regeneration of *Michelia champaca* L. through cell suspension culture technique

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***Michelia champaca* L. is a woody tree species, which has high economical value as a basic material for medicinal and fragrance products. However, there are several problems encountered in conventional propagation of *M. champaca*, which are generally low through seed germination, while vegetative layering technique produces limited planting materials. This experiment was conducted to establish high frequency *M. champaca* regeneration through cell suspension culture technique. Liquid Murashige and Skoog medium containing 2 mgL^{-1} α-naphthaleneacetic acids induced high frequency of somatic embryos formation (47.67 ± 4.53 per ml). Meanwhile for germination of somatic embryos, the highest total number of somatic embryos germinated into plantlets obtained was 34% in hormone free Murashige and Skoog medium. Plant regeneration of *M. champaca* via cell suspension culture technique which was successfully established in this study could be used for mass production of planting materials of this tree.**

Key words: *Michelia champaca*, somatic embryogenesis, cell suspension culture technique.

INTRODUCTION

Michelia champaca L. belongs to Magnoliaceae family. It is native to tropical and subtropical South and Southeast Asia. *M. champaca* has commercial value especially the flower whereby the essential oil could be extracted from the flowers and use for production of perfumes, cosmetics and hair oil (Barlow et al., 1991). There is a high demand for plant materials of *M. champaca* but their production is limited.Conventionally, *M. champaca* can be propagated via seed and/or layering technique. However, there are several problems associated to propagation of *M. champaca* such as low percentage of seeds germination caused by alkaloids content in seeds that inhibit seed germination (Zabala, 1990; Wan et al., 1990).

The other problem related to vegetative propagation via layering is that the method is too slow to meet the need of elite varieties in time. To overcome this problem, tissue culture technique through cell suspension culture has a potential for rapid mass production of plantlets.

The cell suspension culture technique offers advantages when rapid cell division or many cell generations are desired, or when a more uniform treatment application is required (Phillips and Hansen, 1995). The new cells are formed when they are dispersed into the liquid medium and become clusters and aggregates. Cells in suspension can exhibit much higher rates of cell division than do cells in callus culture (Gurel et al., 2002). Through this technique, somatic embryogenesis, the process of embryo formation from somatic cells (Stasolla and Yeung, 2003) could produce high multiplication rates of cells, possibility of cryopreservation of embryogenic callus, and potential for scalling-up the process in bioreactor systems (Karami and Kordestani, 2007). Plant regeneration through

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Abbreviations: NAA, α-Naphthaleneacetic acid; **GA$_3$**, gibberellic acid; **DMRT,** Duncan´s multiple range tests; **Sd**, standard deviation.

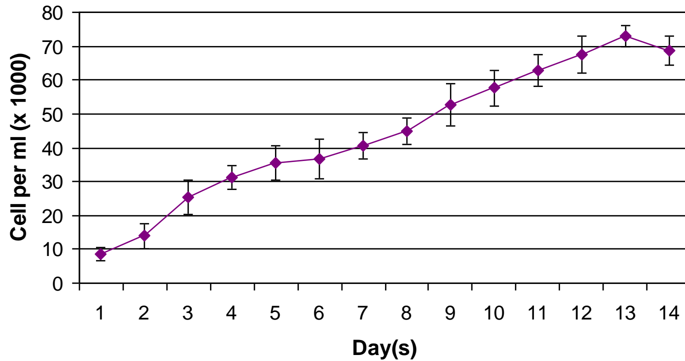

Figure 1. Cell growths in liquid MS medium containing 2 mgL-1 NAA. Vertical bars represent ± SD (n=10).

somatic embryogenesis via cell suspension culture technique could also be used for mass production of planting materials and as a potential pathway for production of secondary metabolite.

This paper reports on the establishment of plant regeneration of *M. champaca* from immature seed derived embryogenic callus through cell suspension culture technique. It is the first report on cell suspension culture of *M. champaca*.

MATERIALS AND METHODS

Explants and surface sterilization procedure

The immature seeds were separated from the fruit aggregates and disinfested using 0.2% (w/v) Benlate solution for 15 min followed by 70% (v/v) of ethanol for 2 min and finally 20% (v/v) Sodium hyphochlorite (Clorox) solution for 15 min. The explants were then rinsed with sterile distilled water for three times. After surface sterilization, the immature seeds were isolated from the fruit by removing the fruit pericarp. The seeds taken from seed carpel were then cut at the corner side of the seed by using scalpel blade. The seed explants were cultured on a basal Murashige and Skoog (MS) medium supplemented with 2 mgL^{-1} NAA for induction of embryogenic callus. The embryogenic callus produce was then transferred into liquid MS medium containing 2 mgL^{-1}NAA for enhancing high frequency somatic embryo formation with ten replications and each replication data were recorded ten times reading using haemocytometer.

Initiation and establishment of cell suspension culture

The embryogenic calli at four months of culture in embryogenic callus induction medium were used as explants. Any unwanted parts were removed from the embryogenic callus, and approximately 1 g was placed in each of 100 mL flask containing 10 mL of liquid medium. The medium used was full MS (Murashige and Skoog, 1962) medium supplemented with 30 gL^{-1} sucrose and 2 mgL^{-1} (w/v) of NAA at the medium pH of 5.8. The cell suspension cultures were placed on an incubation shaker at 100 rpm. Subculture into fresh medium was performed at the end of the exponential phase determined from the plotted cell growth curve. During subculture the suspensions were filtered through polypropylene meshes with pore size of 250 µm. The cultures were kept at 25 ± 2°C in the growth room with a daily fluorescence light of 16 h. The light intensity was 13.5 to 18 $\mu molm^{-2}s^{-1}$.

Somatic Embryo germination and development

The cotyledonary somatic embryos at the sixth subculture (3 months) were used as explants. The cotyledonary were transferred on solid MS hormone free medium containing 30 gL^{-1} of sucrose and solidified with 3.9 gL^{-1} gelrite agar for maturation and to remove the residual effect of plant growth regulators from earlier experiments. After two weeks on MS free hormone medium, the somatic embryos were then transferred on germination medium containing different concentrations of GA_3 (0, 0.5, 1 and 1.5 mgL^{-1}) with ten replications and each replication consisted approximately of ten to fifteen somatic embryos.

Experimental design and statistical analysis

On initiation and establishment of cell suspension culture the experiment was arranged in a Completely Randomized Design (CRD) and data was performed by using standard deviation. Meanwhile for embryo germination and development, data were analyzed using the analysis of variance (ANOVA) and Duncan New Multiple Range Test (DNMRT) at α = 5% for comparison between treatment means. Statistical calculation on standard deviation was estimated by using MS excel program and Duncan's new multiple range test was estimated using the SAS program version 9.1 (SAS institute, Cary NC, USA).

RESULTS AND DISCUSSION

Initiation and establishment of cell suspension culture

Embryogenic callus produced from immature seed of *M. champaca* at the fourth month of culture were selected for the establishment of cell suspension culture. The embryogenic callus was identified by their sharp yellowish color, friable and when checked under the inverted microscope (Axiovert 135 model ZEISS, Germany) showed cells with highly dense cytoplasm. The embryogenic calli were then placed in a 100 ml flask containing 10 ml of full MS liquid medium containing 2 mgL^{-1}NAA. The number of cells produced per ml of cell suspension was counted daily using a haemocytometer and the cell growth curve was plotted to determine the appropriate time for subculture of the cell suspension. The number of cells increased from day to day due to cell division and cell enlargement. The cell population entered a stationary phase after 13 days of culture with a cell count of seventy three thousand cells per ml (Figure 1). Subculture was performed at the end of the exponential phase which was on the thirteenth day of culture. Subculture was necessary as the cells need optimum nutrients for growth and development into somatic embryos and the presence of NAA was essential to stimulate the somatic embryo formation.

According to Gray (1996) the auxins induced cells to

Table 1. The estimated yield of somatic embryos in cell suspension cultures established from immature seed of Champaca explants in full MS medium containing 2 mgL^{-1} NAA.

Sub culture	Pro embryo masses (cell clump) per ml ± Sd	Globular-Heart-Torpedo shaped somatic embryos per ml ± Sd	No. of flasks attained
2nd	8330 ± 4.61	-	1
3rd	9800 ± 9.47	-	2
4th	15750 ± 6.21	10800 ± 6.85	4
5th	13850 ± 8.78	11800 ± 7.26	6
		Cotyledonary ± SD	
5th	47.67 ± 4.53	6	5th

Sd= Standard deviation.

become embryogenic and subsequently to promote repetitive cell division of embryogenic cell population and high concentration of auxin prevented cell differentiation and embryo growth. The agitation of medium in cell suspension culture also plays a role in evenly exposing the cells to nutrients and hormone inside the medium. Through cell suspension culture, the suitability of a medium for somatic embryo development can be assessed since the components of the medium can be controlled precisely to induce somatic embryo development in a uniform manner. The agitation of liquid culture medium enhances aeration inside the culture vessel and provides a uniform distribution of nutrient and contact to the whole surface of cells in culture.

The cells in the suspension culture became embryogenic and developed into somatic embryos at the fourth to the fifth subculture (Table 1). Turbidity of the cell suspension indicated more cells were becoming embryogenic. These cells were small, rounded and with distinct nucleolus which then divided into two, four and eight celled structure (Figure 2A, B, C and D) and became proembryogenic masses (Figure 3A and B). The proembryogenic masses then formed globular stage (Figure 4), heart stage (Figure 5A and B), torpedo stage (Figure 5C) and finally the formation of cotyledonary stage (Figure 5D).All subcultures cycles, showed high frequency on proembryogenic masses production per ml, globular-heart-torpedo stage somatic embryos production per ml, and mean number of cotyledonary somatic embryos per ml (Table 1). The results showed that cell suspension culture technique could produce high frequency of somatic embryo for *M. champaca* plant.

Martin (2003) stated that the development of higher number of somatic embryos of *Holostemma ada-kodien* was obtained through cell suspension cultures as compared with through solid medium cultures technique. Furthermore, Mauri and Manzanera (2003) reported that somatic embryo through liquid culture have some advantages such as high multiplication rate, embryos grow faster in size and fresh weight, due to the aeration and easier nutrient absorption for the cultures.

Somatic embryo germination and development

Somatic embryos at torpedo and cotyledonary stage from cell suspension culture after maturation were used as explants for germination somatic of embryo (Figure 6). Clumps of somatic embryos (approximately 10 to 15 somatic embryos) which were produced from cell suspension culture systems were cultured onto germination medium containing different concentrations of GA_3 (Figure 7). Somatic embryos produced from cell suspension culture germinated at the fourth to fifth week of culture (Figure 8).

Based on data obtained (Table 2), a significantly highest percentage of somatic embryos were produced from cell suspension culture system. A total of 34% germination was observed when somatic embryos germinated on hormone free MS medium. Through this treatment 29% of somatic embryos germinated into normal plantlets (Figure 9) and 5% abnormal plantlets (Figure 10). Treatment of hormone free MS medium was most suitable for germination of somatic embryos of *M. champaca* from cell suspension culture systems. Treatment with free hormone MS medium produced better result as compared with MS treatments containing different concentrations of GA_3 (0.5, 1 and 1.5 mgL^{-1}). Germination of somatic embryos through cell suspension culture was low (34%), due to browning problem when somatic embryos were transferred to germination media. The problem also happened on induction of embryogenic callus from immature seed of *M. champaca.* Tissue culture of *M. champaca* has problems due to presence of phenolic compound which causes browning. According to Lai and Lee (1994) the initiation of callus culture from the flower of *M. champaca* was slow and the calli turned brown quickly.

Based on the results in this study, MS (Murashige and Skoog, 1962) medium, which is rich with nutrients and

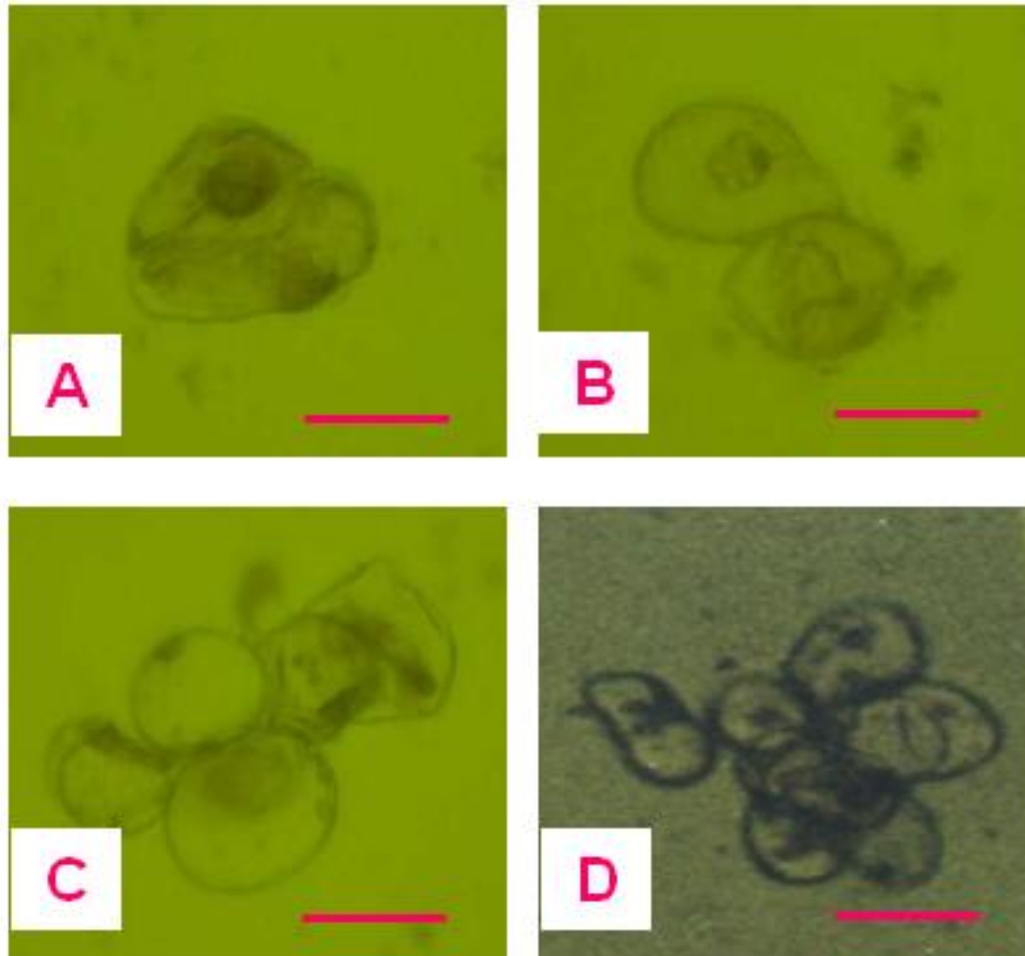

Figure 2. Single cell undergoing division into two (A) and (B), the cell divided into four (C), and the cell divided into eight (D), (Bar = 10 μm).

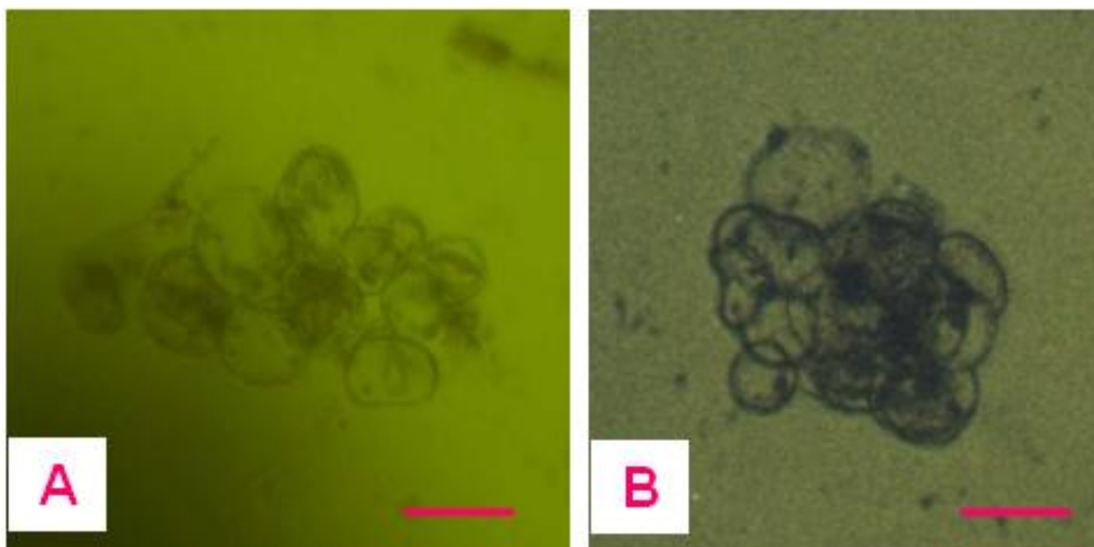

Figure 3. Pro-embryogenic masses in liquid cell suspension culture at the second subculture (A) and after the third subculture (B), (Bar = 10 μm).

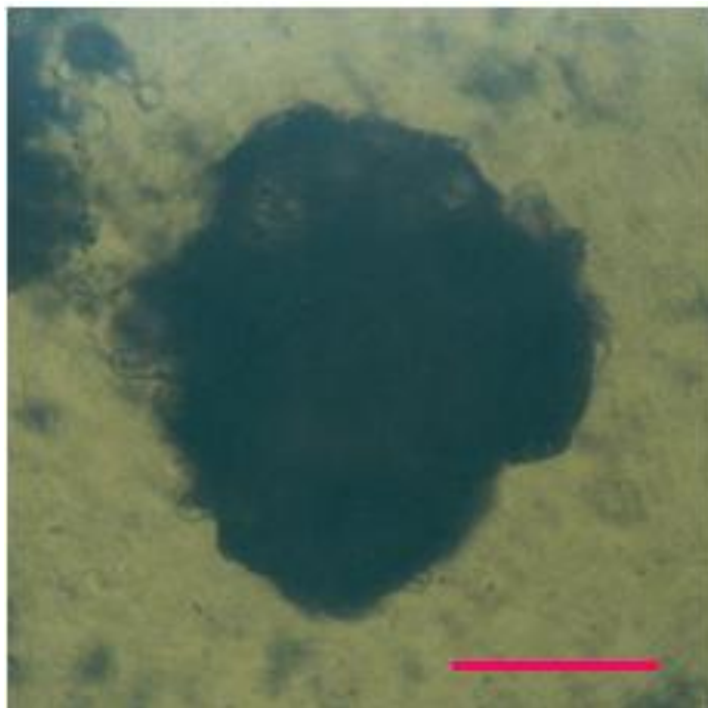

Figure 4. Globular stage of somatic embryos in liquid cell suspension culture under inverted microscope (Bar = 50 µm).

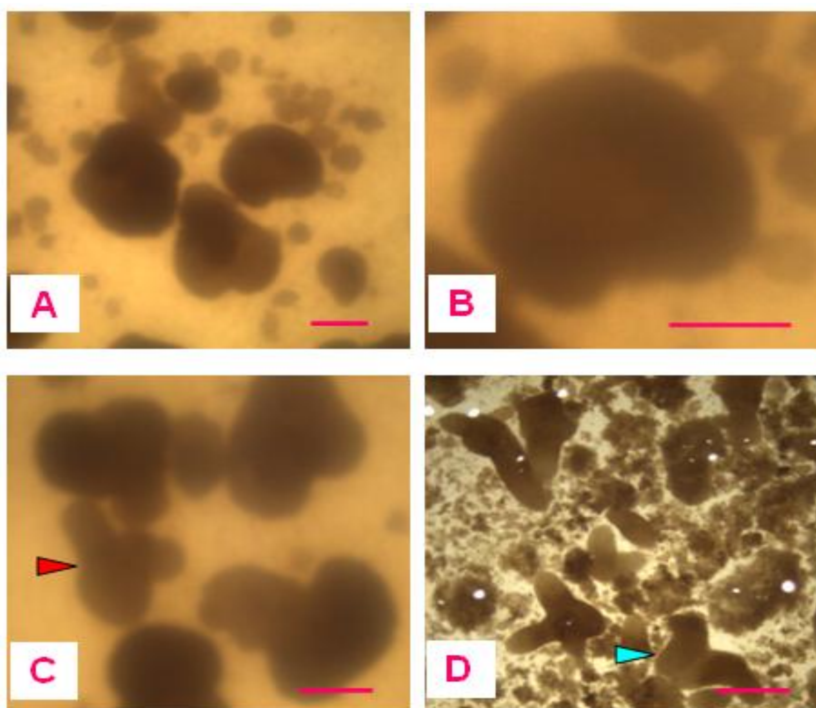

Figure 5. Heart stages of somatic embryos in liquid cell suspension culture (A); Early stages of heart shape somatic embryo (B); Torpedo stages of somatic embryo (red arrow) (D); and Cotyledoneray stages of somatic embryos (blue arrow) in liquid cell suspension culture (E), (Bar = 500 µm).

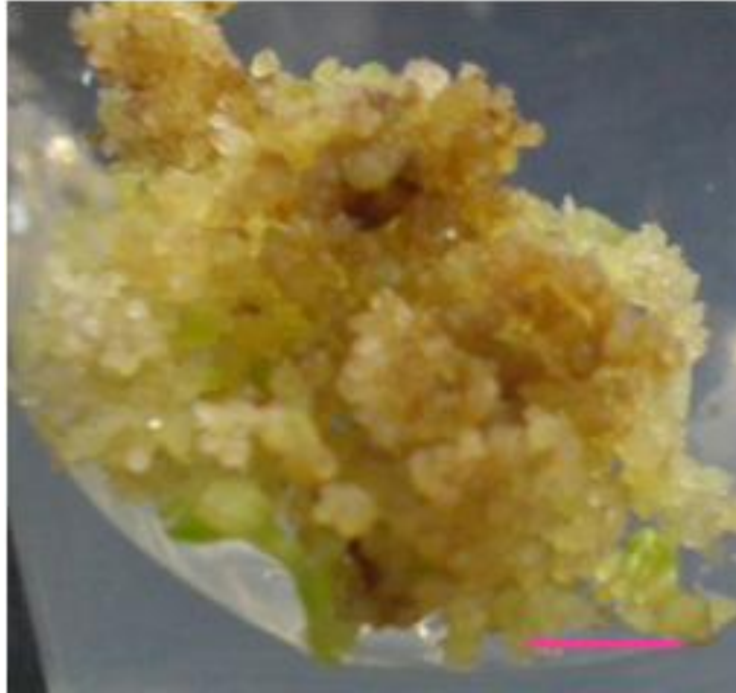

Figure 6. Maturation of somatic embryos derived from cell suspension culture on hormone free MS medium (Bar = 5 mm).

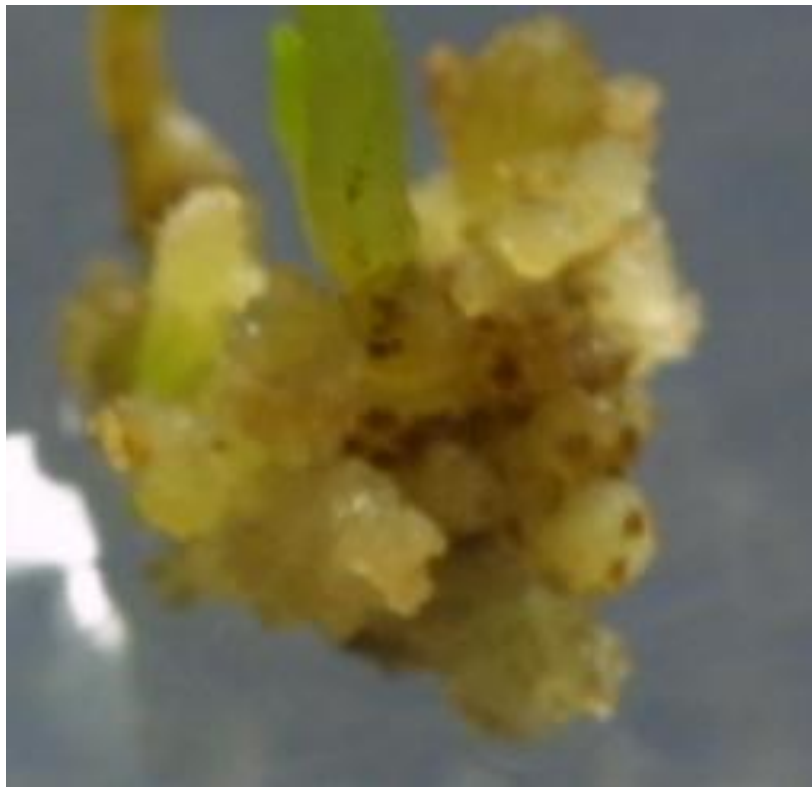

Figure 7. A clump of somatic embryos (consist of 10 to 15 of somatic embryos) which were produced from cell suspension culture system was cultured onto different concentrations of GA_3 for germination (Bar = 1 mm).

Figure 8. Somatic embryos which were produced from cell suspension culture technique started to germinate on free hormone germination medium at the fourth to the fifth week of culture (Bar = 5 mm).

Table 2. Effects of different concentrations of GA_3 on the percentage of germination of somatic embryos produced through cell suspension culture, the percentage of normal plantlet produced and percentage of abnormal plantlets produced after twelve weeks of culture.

Treatments (mgL^{-1})	Code	Somatic embryos germination (%)	Normal plantlets produced (%)	Abnormal plantlets produced (%)
MSO	G1	34^a	29^a	5^a
0.5 GA_3	G2	16^b	8^b	8^a
1 GA_3	G3	11^b	6^b	5^a
1.5 GA_3	G4	10^b	6^b	4^a

Means followed by the same letter(s) are not significantly different using Duncan New Multiple Range Test (DNMRT) at p=0.05.

its very high concentration of nitrate, potassium and ammonia was the most suitable medium used for somatic embryogenesis of *M. champaca*. Many researchers reported that basic MS or modified MS medium combined with additional auxins and cytokinin was suitable in micro-propagation of many plant species. In related species of *M. champaca*, Ibrahim (2006) reported, by using MS medium supplemented with 2,4-D and BA was successful to induce somatic embryogenesis in *Michelia alba*. Kim *et al.* (2007) obtained somatic embryogenesis by culturing immature seed of *Magnolia obovata* explant on MS medium supplemented with 1 mgL^{-1} 2,4-D. Meanwhile, in other plant species, Lee *et al.* (2009) used MS medium containing BAP, Kinetin and GA_3 to improve plantlet conversion and shoot growth from somatic embryo cultures of *Cnidium officinale* Makino.

Conclusion

Plant regeneration of *M. champaca* through cell suspension culture was successfully developed from immature seed. Based on this experiment, liquid MS medium containing 2 mgL^{-1} NAA produced high proliferation of

Figure 9. Normal plantlets produced from cell suspension culture systems germinated on hormone free MS medium after 12th weeks of culture (Bar = 1 cm).

Figure 10. Abnormal plantlets produced from somatic embryos derived from cell suspension culture systems with abnormal shape, stunted growth and somatic embryo growing like a vase (red arrow) germinated on germination medium containing 1.5 mgL-1 GA_3 after 12th weeks of culture (Bar = 8 mm).

somatic embryos.

REFERENCES

Barlow HS, Enoch IA, Rusell LRA (1991). Tropical planting and gardening. Kuala Lumpur. Malayan Nature Society.

Gray DJ (1996). Non zygotic embryogenesis In: Plant Tissue Culture Concepts and Laboratory Exercises (Ed.) Trigiano RN and Gray DJ, CRC Press, Boca Raton, Florida.

Gurel S, Gurel E, Kaya Z (2002). Establishment of cell suspension cultures and plant regeneration in sugar beet (*Beta vulgaris* L.). Turk J. Bot., 26: 197-205.

Ibrahim R (2006). Advanced bioreactor system in the production of fragrance compounds from *Michelia alba*. Abstract of the International Symposium on Molecular Farming in Plants. AsPac J. Mol. Biol. Biotechnol., 14(1): 1-17.

Karami O, Kordestani GK (2007). Proliferation, shoot organogenesis and somatic embryogenesis in embryogenic callus of carnation. J. Fruit Ornam. Plant Res., 15: 167-175.

Kim YW, Park SY, Park IS, Moon HK (2007). Somatic embryogenesis and plant regeneration from immature seeds of *Magnolia obovata* Thunberg. Plant Biotechnol. Rep., 1: 237-242

Lai CY, Lee WC (1994). The initiation of callus culture of *Michelia champaca* for essential oil production. Biotechnol. Lett., 16(1): 85-88.

Lee CY, Kim YK, Kim YS, Suh SY, Lee SY, Park SU (2009). Somatic embryogenesis and plant regeneration in *Cnidium officinale* Makino. J. Med. Plant. Res., 3(3): 96-100

Martin KP (2003). Plant regeneration through somatic embryogenesis on *Holostemma ada-kodien* a rare medicinal plant. Plant Cell Tis. Org. Cult., 72: 79-82.

Mauri PV, Manzanera JA (2003). Induction, maturation and germination of holm oak (*Quercus ilex* L.) somatic embryos. Plant Cell Tiss and Org. Cult., 74: 229-235.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

Phillips GC, Hubstenberger JF, Hansen EE (1995). Plant regeneration by organogenesis from callus and cell suspension cultures. In: Gamborg OL, Phillips GC (eds). Plant Cell Tiss and Org. Cult., pp. 67-78.

Stasolla C, Yeung EC (2003). Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tis. Org. Cult., 74: 15-35.

Wan ZS, Jiang FG, Guan DZ (1990). Occurrence of alkaloids in some Magnoliaceae growing in China. Fitoterapia, 61: 245-247.

Zabala NQ (1990). Silviculture of Michelia champaca In: Silviculture of species. Chittagong, Bangladesh: Chittagong University, Institute of Forestry; Food and Agriculture Organization, Rome, Italy, pp. 68-70.

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Full Length Research Paper

Phytotoxic characterization of various fractions of *Launaea nudicaulis*

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***Launaea nudicaulis* is a local medicinal plant of District Bannu, KPK, Pakistan, used traditionally for various diseases and having the phytotoxic ability. In the present study, allelopathic activity of the various fractions of *Launaea nudicaulis* was studied against radish growth. Two concentrations of the extract: 100 and 1000 ppm were used. Methanolic and ethyl acetate fraction markedly showed inhibition of root and radical growth compared to other fraction. The fresh and dry weight of whole radical and shoot showed significant results. The allelopathic activity of various fractions of *Launaea nudicaulis* may be due to the presence of bioactive constituents.**

Key words: *Launaea nudicaulis*, radish seeds, root inhibition.

INTRODUCTION

Many allelochemicals of plant sources exert their influence by such a mechanism which is not shown by commercial herbicide. Thus for new herbicide discovery, these natural allelochemicals play the role of ideal lead compounds. Therefore recently scientists have focused their great attention on searching for new secondary plant products to develop bio-herbicides and bio-pesticides. To enhance the synthesis and exudation of allelochemicals, the tow major factors, genetic characteristics and environmental conditions have played a very important role in this field (Inderjit, 2003). Many crops such as rice, oat and wheat are being studied. Medicinal plant have are useful in improving human health and amelioration of various diseases (Sahreen et al., 2010; Khan et al., 2009, Khan et al., 2010a, b; Khan et al., 2012a, b), as well as phytoxic effects (Khan et al., 2010; khan et al., 2011). Now days an extensive research has been going on to control weeds worldwide. Medicinal plants are screen for their allelopathic and or medicinal potential and to select the most bioactive ones for chemical analyses (Fujii et al., 2003; Khan et al., 2010c; Khan et al., 2012b, d). *Launaea nudicaulis* is traditionally used in the treatment of various diseases. Therefore the present project is designed to investigate the phototoxic potential of *Launaea nudicaulis* (Figure 1a and b).

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Abbreviations: DMSO, Dimethyl sulfoxide; **LNME,** *Launaea nudicaulis* methanolic extract; **LNHE,** *Launaea nudicaulis* n-hexane extract; **LNEE,** *Launaea nudicaulis* ethyl acetate extract; **LNCE,** *Launaea nudicaulis* chloroform extract; **LNBE,** *Launaea nudicaulis* butanolic extract; **LNWE,** *Launaea nudicaulis* water extract.

MATERIALS AND METHODS

Plant collection and extraction

Ariel parts of *Launaea nudicaulis* was collected from Town Ship, District Bannu at maturity, identified and were shade dried at room temperature, grinded mechanically and extracted with 80 % methanol. The methanolic crude extract is further fractionated with, n-hexane, ethyl acetate, chloroform, butanol and distilled water with increasing order of polarity. After rotary evaporation all the fraction extract are stored at 4°C for further for phytotoxic analysis.

Phytotoxicity bioassay

Protocol of McLaughlin and Rogers (1998) was used for checking the phytotoxic efficacy of the various fractions of plant extract. Two concentrations (100 and 1000 ppm) in respective solvents are

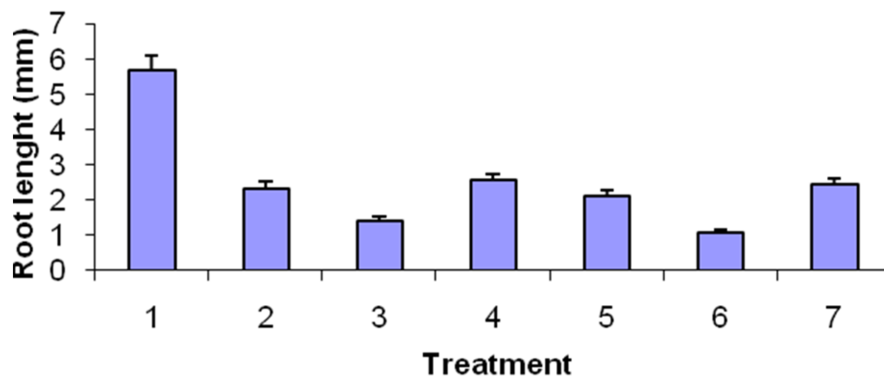

Figure 1a. Root inhibition at 100 ppm concentration of various fractions of *Launaea nudicaulis 1* (non treated control), 2 (n-hexane fraction), 3 (ethyl acetate fraction), 4 (chloroform fraction), 5 (butanol fraction), 6 (methanol fraction), 7 (water fraction).

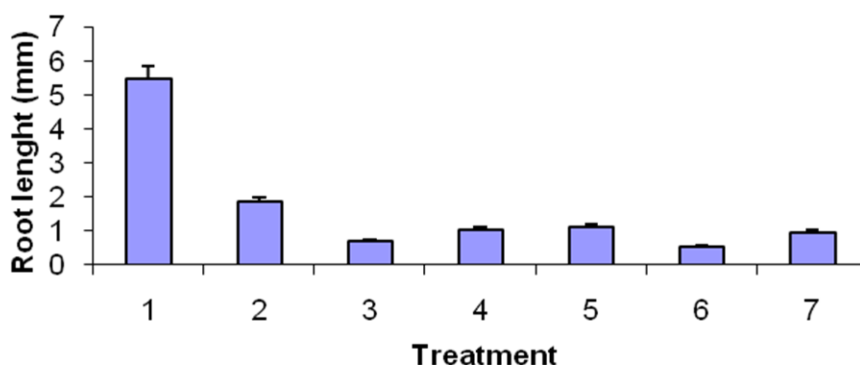

Figure 1b. Root inhibition at 1000 ppm concentration of various fractions of *Launaea nudicaulis 1* (non treated control), 2 (n-hexane fraction), 3 (ethyl acetate fraction), 4 (chloroform fraction), 5 (butanol fraction), 6 (methanol fraction), 7 (water fraction).

prepared and preceded. Radish seed was washed with dH_2O and then with 1% mercuric chloride. Filter paper was put in each autoclaved Petri plates. 5 ml of each fraction was poured in each plate and the respective solvent was evaporated. 10 seeds were placed in each plate and incubated in growth room for five days. After 5 days root and shoot inhibition was noted. Fresh weight and dry weight of whole plant was also recorded.

RESULTS

Phytotoxicity assessment

Effect of various fractions on root growth

Various fractions of *Launaea nudicaulis* revealed methanolic (LNME) and ethyl acetate fraction (LNEE) showed significant (P< 0.01) inhibitory effect on root growth of radish as compare to other fraction of extract. Phytotoxic (allelopathic) effects of *Launaea nudicaulis* were evaluated against radish seed growth under control environmental condition in growth room at both 100 ppm and 1000 ppm, respectively.

Effect of various fractions Shoot growth inhibition

Launaea nudicaulis also effects the shoot growth of radish. Data of the present data revealed that methanolic fraction of *Launaea nudicaulis* as well ethyl acetate fractions of *Launaea nudicaulis* have markedly (P<0.01) the growth of radish at 5th day of treatment both at 100 and 1000 ppm respectively as presented in Figure 2a and b.

Effect of fractions on fresh and dry weight

At the 5th day of treatment, fresh weight of all treatment groups was calculated and observed that LNME and LNEE of *Launaea nudicaulis* significantly reduced (P<0.01)

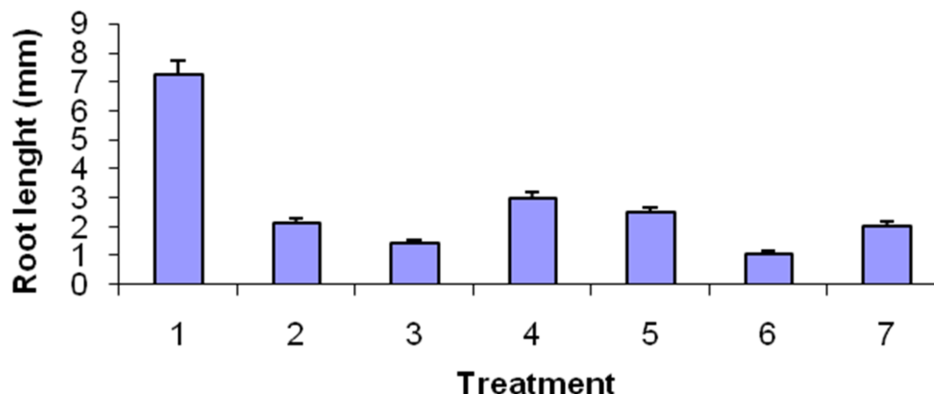

Figure 2a. Represents the shoot inhibition at 100 ppm concentration of various fractions of *Launaea nudicaulis 1* (non treated control), 2 (n-hexane fraction), 3 (ethyl acetate fraction), 4 (chloroform fraction), 5 (butanol fraction), 6 (methanol fraction), 7 (water fraction).

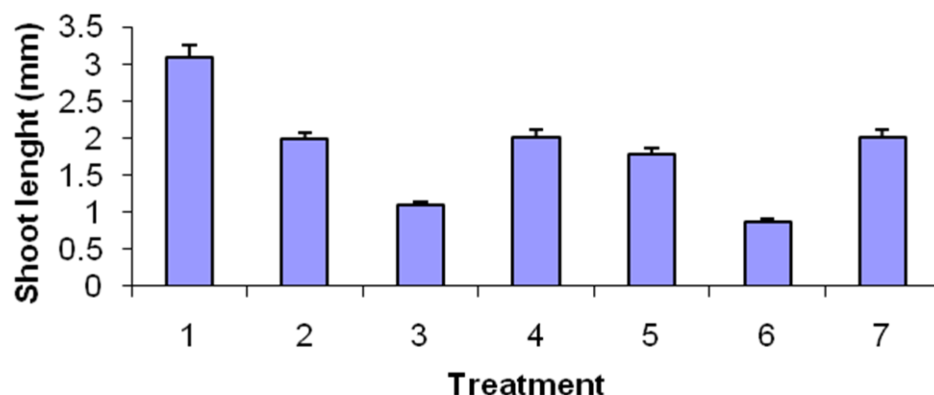

Figure 2b. Represents the shoot inhibition at 1000 ppm concentration of various fractions of *Launaea nudicaulis 1* (non treated control), 2 (n-hexane fraction), 3 (ethyl acetate fraction), 4 (chloroform fraction), 5 (butanol fraction), 6 (methanol fraction), 7 (water fraction).

fresh weight as well as dry weight of the radish plant which might be due the presence of bioactive allelochemicals in these fraction (Table 1).

DISCUSSION

The results of our present screening assays justify the use of the investigated plants against herbicides in Pakistan. Results of the present study revealed that Water, methanolic and butanolic fractions of *Launaea nudicaulis* showed marked inhibition comparatively to other fractions as well as control. Similar results were reported by Khan et al., (2010) and Khan et al., (2011) which justify the present study. Similarly water extract of *Withania somnifera* and *Datura alba* significantly inhibited the root, as well as shoot growth due the presence of bioactive polyphenolic compounds (Javaid, 2009).

Similar investigations was found that essential oil isolated from Turkish *Origanum acutidens* and their phenolic compounds completely inhibited the growth of seedling and roots and possessed antifungal activity when compared to standards compounds. The investigation of Hussain et al. (2010) also in accordance to our findings.

Conclusion

From the present data it is inferred that *L. nudicaulis* methanolic and ethyl acetate fractions have significant herbicidal potency, might be the presence of allelochemicals. Therefore, further study on isolation and purification of these allelochemicals are suggested.

Table 1. Effect of various fractions of *Launaea nudicaulis* on radish whole plant fresh and dry weight.

Treatment	Fresh weight (g)		Dry weight (mg)	
	100 ppm	1000 ppm	100 ppm	1000 ppm
Control	5.67±0.6[a]	5.45±0.2[a]	7.23±0.4[a]	3.09±0.02[a]
LNHE	2.34±0.2[b]	1.87±0.05[b]	2.12±0.02[b]	1.98±0.09[b]
LNEE	1.41±0.02c	0.69±0.001[c]	1.45±0.006[c]	1.09±0.001[c]
LNCE	2.56±0.1[b]	1.02±0.01[b]	3.09±0.1[b]	2.01±0.1[b]
LNBE	2.12±0.5[b]	1.1±0.2[b]	2.54±0.2[b]	1.78±0.3[b]
LNME	1.09±0.06[c]	0.54±0.002[c]	1.08±0.003[c]	0.87±0.001[c]
LNWE	2.45±0.1[b]	0.97±0.3[b]	2.02±0.1[b]	2.01±0.12[b]

Each value in the table is represented as mean ± SD ($n = 3$), means not sharing the same letter are significantly different (LSD) at $P < 0.01$ probability level in each column, LNHE (n-hexane fraction), LNEE (ethyl acetate fraction), LNCE (chloroform fraction), LNBE (butanol fraction), LNME (methanol fraction), LNWE (water fraction).

REFERENCES

Fujii Y, Parvez SS, Parvez MM, Ohmae Y, Iida O (2003). Screening of 239 medicinal plant species for allelopathic activity using sandwich method. Weed Biol. Manag., 3: 233-241.

Hussain F, Hameed I, Dastagir G, Shams-un-Nisa, Khan I, Ahmad B (2010). Cytotoxicity and phytotoxicity of some selected medicinal plants of the family Polygonaceae. Afr. J. Biotech., 9 (5): 770-774.

Inderjit DSO (2003). Ecophysiological aspects of allelopathy. Planta, 217: 529-539.

Javaid A (2009). Role of Effective Microorganisms in Sustainable Agricultural Productivity. In: Advances in Sustainable Agriculture. Springer Publishers (in Press).

Khan RA, Khan MR, Sahreen S, Jan S, Bokhari J, Rashid U (2012). Protective effect of *Launaea procumbens* against KBrO3 induced nephrotoxicity in rats. Afr. J. Pharm. Pharmacol., pp. 317-321.

Khan RA, Khan MR, Sahreen S, Ahmed M (2012b). Evaluation of phenolic contents and antioxidant activity of various solvent extracts of Sonchus asper (L.) Hill. Chem. Cent. J. 6:12 doi:10.1186/1752-153X-6-12.

Khan RA, Khan MR, Sahreen S, Jan S, Bokhari J, Rashid U (2011) Phytotoxic characterization of various fractions of *Launaea procumbens*. Afr. J. Biotech., 10: 5377-5380.

Khan RA, Khan MR, Sahreen S, Jan S, Bokhari J, Rashid U (2011). Protective effects of various fractions of *Launaea procumbens* on molecular markers in rat kidney. Afr. J. Pharm. Pharmacol., 6(3): 157-161.

Khan RA, Khan MR, Sahreen S, Jan S, Bokhari J, Rashid U (2012). Protective effect of *Launaea procumbens* against $KBrO_3$ induced nephrotoxicity in rats. Afr. J. Pharm. Pharmacol., pp. 317-321.

McLaughlin JL, Rogers LL (1998). The use of biological assays to evaluate botanicals. Drug Inform. J., 32: 513-524.

Sahreen S, Khan MR, Khan RA (2010). Hepatoprotective effects of methanol extract of *Carissa opaca* leaves on CCl4-induced damage in rat. BMC Comp. Alter. Med., 11:48 doi: 10.1186/1472-6882-11-48.

Sahreen S, Khan MR, Khan RA (2010). Evaluation of antioxidant activities of various solvent extracts of *Carissa opaca* fruits. Food Chem., 22: 1205-1211.

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Full Length Research Paper

Antioxidant effect of *Zanthoxylum limonella* Alston.

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Zanthoxylum limonella **Alston. (Rutaceae) has been widely used as spice and folk medicine. Different part of this plant, such as ripe fruit was used as a condiment in curries, and its essential oil is quite useful for blood circulation and gastrointestinal tract. The bark was noted for its febrifugal, sudorific and diuretic properties. It has been used for treatment of fever caused by free radical production in the recticulo-endothelial system. The present work investigates the antioxidative potential of *Z. limonella* crude extracts and essential oil on cell-free and cell-based systems. 1,1-diphenyl-2-picrylhydrazyl (DPPH) and trolox equivalent antioxidant capacity (TEAC) assays showed the same ranking order of free radical scavenging activity, methanol extract of stems (SM) > dichloromethane extract of stems (SD) > essential oil of fruits (EO). However, leave extracts exhibited very low radical scavenging activity. Treatment with SD, SM, and EO significantly decreased the malondialdehyde (MDA) level of cell lysates obtained from pretreated prostate cancer cells, while glutathione (GSH) and catalase (CAT) levels increased. The result of the antioxidative potential of *Z. limonella* indicated that crude extracts, SD and SM, from stems and essential oil from fruits of *Z. limonella* exhibited the antioxidant activity possibly related to the regulation of CAT and GSH in prostate cancer cells.**

Key words: *Zanthoxylum limonella* Alston., antioxidant activity, 1,1-diphenyl-2-picrylhydrazyl (DPPH), trolox equivalent antioxidant capacity (TEAC), prostate cancer, lipid peroxidation, glutathione (GSH), catalase (CAT).

INTRODUCTION

The genus *Zanthoxylum*, Family Rutaceae consists of about 250 species of deciduous, evergreen trees and shrubs distributed worldwide around the tropical and subtropical areas. They have been cultivated in North America, South America, Africa, Asia, and Australia. In ancient period, *Zanthoxylum* has been extensively used as a folk medicine for different medical purposes such as stomach ache, toothache, intestinal worms, rheumatism, scabies, snakebites, fever and cholera (Pongboonrord, 1979). *Zanthoxylum limonella* Alston. is widely distributed in the northern part of Thailand and has been traditionally used in food, especially ripe fruits have been commercialized in local markets as a popular spice. Vitamin E has been found in the seed oil (Fish et al., 1975). The essential oil from the fruit affects the gastrointestinal system and initiates smooth muscle contraction by a non-specific mechanism (Ittipanichpong et al., 2002). *Z. limonella* oil has been used as a natural, eco-friendly, and biodegradable mosquito repellent. Antioxidant activity of *Zanthoxylum* has been described. The methanol extract from the fruits of Japanese pepper (*Z. piperitum* DC.) contains hyperoside (quercetin-3-*O*-galactoside), quercitrin (quercetin-3-*O*-rhamnoside) and a glycoprotein (ZPDC). It exhibits a significant antioxidant activity equal to that of α-tocopherol (Yamazaki et al., 2007). ZPDC glycoprotein consists of carbohydrate (18%) and protein (82%). It shows a strong scavenging activity against DPPH radical, superoxide anion, and hydroxyl radical in cell-free system (Lee and Lim, 2007). In addition, the anti-inflammatory activity of some *Zanthoxylum* due to reactive oxygen species (ROS) as a mediator has also been studied (Márquez et al., 2005). Plants in the *Zanthoxylum* genus contain a variety of

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active secondary metabolites, such as alkaloids, lignans, and coumarins, possibly attributed to the ROS in asthma, ulcers, rheumatism, and earache *via* the inflammatory processes (Roig, 1965). *Z. limonella* Alston. has been used in folk medicine for treatment of fever. It was previously found that fever increased the generation of free radicals by cell of the reticulo-endothelial system produced from lipid peroxidation, while decreases the cell antioxidant system (Riedel and Maulik, 1999; Zinchuk, 1999). Familial Mediterranian fever was also correlated with an increment of superoxide radical produced by neutrophils (Sarkisian et al., 1997).

Prostate cancer is the most prevalent type of internal malignancy found in men over the age of fifty. It is the second leading cause of cancer-related deaths among men in Western nations, especially African-American men (Jemal et al., 2007). The causes of prostate cancer are not completely understood. Age is found to be the strongest risk factor. Some epidemiologic studies have suggested that dietary fat closely associated with lipid peroxidation may be an important factor for prostate cancer (Vaca et al., 1988). Wang et al. (1995) suggested that dietary fat content can influence the tumor growth of androgen-sensitive, human prostatic adenocarcinoma cells (LNCaP cells) grown in nude mice. The highest prostate-specific antigen (PSA) levels were found in the high-fat diet group and the lowest in the low-fat group which indicated that prostate cancer progresses according to dietary fat. Extensive research has established a strong relationship between ROS generation and carcinogenesis including cancer progression. ROS can act as secondary messengers to monitor several signaling cascades. They can induce mutations and alter gene function resulting in carcinogenesis *via* oxidation processes (Halliwell, 1994). The mutation of p53 protein has been found associated with the progression of prostate cancer exhibiting various degree of aggressiveness (Navone et al., 1993). ROS played an essential role for migratory/invasiveness phenotypes of prostate cancer. Elimination of excessive ROS may be very effective method to decrease prostate cancer formation and metastasis. This method could be extended to other malignancies due to the strong relation between ROS and tumor formation. Several studies have investigated natural agents or chemopreventive agents from dietary substances to prevent and possible to cure cancer (Syed et al., 2008). Many reports mentions that some natural compounds and dietary agents such as selenium, vitamin E and D, lycopene, soy and isoflavone, low-fat diet, epigallocatechin-3-gallate (EGCG) from green tea, a few compounds from pomegranate reduce the possibility of prostate cancer, both growth and progression (Liao et al.,1995).

In this study, the primary anti-oxidative potential of *Z. limonella* crude extracts and essential oil was evaluated in cell-free system and further extended to prostate cancer cell lines.

MATERIALS AND METHODS

Plant materials

Fresh leaves, stems and fruits of *Z. limonella* Alston. were harvested in the mountain area of Phrae province, Thailand, during January to April 2007. A voucher specimen (BKF No.152276) was submitted to the Herbarium of the Royal Forest Department of Thailand.

Chemicals

1,1-diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxyltoluene (BHT), 3,4,5-(dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), 5,5'-Dithio (bis) nitrobenzoic acid (DTNB) were purchased from Sigma chemical Co. (St. Louis, MO, USA). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), and 2,2'-azinodi-3-ethylbenzthiazoline sulphonate (ABTS) were purchased from Fluka chemical Co. (Switzerland). All organic solvents used were of technical quality, except ethanol (analytical grade) that was purchased from Merck (Germany). Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL.

Extraction procedure

Leaves and stems, 5 kg each, were air-dried and milled to coarse powder. Both powders were separately macerated exhaustively with dichloromethane (DC) and methanol (MeOH), respectively, for 4 weeks. The supernatants were filtered through Whatman No.1 filter paper. Solvents were evaporated from the filtrate by rotary vacuum evaporator (Buchi, R114, Switzerland). The residues were taken to dryness to obtain a viscous mass as the crude extract. The yield of the dichloromethane crude extracts of leave (LD) and stem (SD) were 5.49 and 0.66% dry weight, respectively. The methanol crude extracts of leaves (LM) and stem (SM) yields were also determined and found 6.52 and 4.62% dry weight, respectively.

Dried-ripe fruits of *Z. limonella* were blended by a domestic blender to get fine powdered. Essential oil (EO) was obtained by hydrodistillation using a Clevenger-type apparatus, resulting in light yellow oil with 11.63% yield. Essential oil was stored at 4°C until used.

DPPH assay

The $DPPH^{\bullet}$ test is the conventional $DPPH^{\bullet}$ capacity assay widely use for plant, food on natural product to screen and evaluate the free radical-scavenging effect. The radical form $DPPH^{\bullet}$ with dark-blue color can be protonated by the antioxidant compounds and reduced to the more stable radical $DPPH^{\bullet}$ with the yellow colored diphenylpicrylhydrazine and terminate radical chain reaction. $DPPH^{\bullet}$ assay was able to perform both in thin layer chromatography (TLC)- and cuvette-DPPH radical scavenging assay.

TLC-DPPH radical scavenging assay

All crude extracts, LD, SD, LM, SM, and EO were spotted on to thin layer chromatography (TLC). TLC plates were developed with the appropriate solvent system for each extract and then air-dried for 20 min. The developed plates were sprayed by 0.2% DPPH in methanol and air-dried. The visible yellow spots were observed and photographed.

Cuvette-DPPH radical scavenging assay

Free radical scavenging activity on DPPH radical described by Sithisarn et al. (2006) was used to evaluate the scavenging activity of all extracts. Dimethylsulphoxide (DMSO) was used to dissolve the crude extracts. Serial dilutions of extracts were carried out to obtain a suitable concentration. A 250 µL of BHT or diluted extract was added to 250 µL DPPH ethanolic solution (2.4 mg in 100 ml ethanol). After incubation at ambient temperature for 20 min, the absorbance was monitored against ethanol as a blank at 520 nm by UV-spectrophotometer (Shimadzu, UV-160A, Tokyo, Japan). The percentage of scavenged DPPH was calculated as the percentage of inhibition using the following formula.

$$\% \text{ inhibition} = [(A_{blank}-A_{extract}) / A_{blank}] \times 100$$

where A_{blank} and $A_{extract}$ are the absorbance of blank and extract, respectively.

Ethanolic solution of butylated hydroxyltoluene (BHT) was used as positive control. DPPH, BHT, and reconstituted solution were freshly prepared. All extracts were performed in triplicate and concentration of extracts exhibited 50% inhibition (IC_{50}) obtained from dose response curve was averaged and used to compare the scavenging activity of each extract.

Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was done according to Arts et al. (2004) with some modification. Briefly, ABTS and potassium persulphate solution were dissolved in deionized water to a concentration of 14 and 5 mM, respectively. The reaction mixture was left at ambient temperature for 12 to 16 h in a dark place to get the blue-green coloured $ABTS^{\bullet+}$ radical cation. The radical cation solution was diluted with deionized water to obtain an absorbance of 0.8 ± 0.05 at 734 nm. The water soluble vitamin E analogue, Trolox®, or various concentrations of extracts prepared by serial dilutions were added to the dilute radical cation solution. The decrease in absorbance after 1 min was spectrophotometically read at 734 nm. All of the assays were performed at least in triplicates. The $ABTS^{\bullet+}$ radical cation solution was freshly prepared daily. The decline in absorbance after 1 min caused by Trolox or extracts was plotted against concentration. The TEAC value was obtained from the ratio between the slopes of the linear plot and that of the Trolox.

Cell cultures

Human prostate adenocarcinoma cell lines DU-145 and PC-3 were a generous gift from Professor Thompson EW, Department of Surgery, St. Vincent's Institute, the University of Melbourne, Australia. The cancer cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin as monolayer in 55 cm^2 tissue culture dishes. Both cell cultures were maintained at 37°C in a tissue culture CO_2 incubator at a humidified atmosphere containing 5% CO_2 until 80% confluency and then subcultered twice a week

Lipid peroxidation determination

Whole tumor cell lysates were obtained by repeated freeze-thaw procedures according to the protocol previously described by Ohkawa et al. (1979). Cancer cells were trypsinized, harvested, washed with phosphate buffered saline (PBS), subjected to repeated freeze-thaw procedures in ice-cold bath, and centrifuged. The supernatant was collected and gently vortexed to get a uniform suspension. Homogenous lysates from cancer cells pretreated with various concentrations of crude extracts were used for lipid peroxidation measurement using thiobarbituric acid reactive substance (TBARS). For untreated cancer cells, lysates were combined with various concentrations of crude extract followed by the lipid peroxidation assayed using TBARS.

Thiobarbituric acids tested for malondialdehyde (MDA) were used as a lipid peroxidation following the method previously described by Ohkawa et al. (1979). Briefly, a 500 µL aliquot of cell lysates was combined with a reaction mixture containing 75 µl of 8.1% SDS, 565 µl of 20% acetic acid, and 565 µl of 0.8% thiobarbituric acid (TBA). The resulting mixture was vigorously mixed, incubated in a water bath at 95°C for 1 h, and cooled to room temperature with tap water. A 500 µl of *n*-butanol and pyridine (15:1, v/v) mixture was added to each sample, shaken vigorously, and centrifuged at 1200 g for 10 min. The supernatant fraction with the pink color of MDA-TBA complex was isolated and the absorbance at 532 nm was measured against mixture of *n*-butanol and pyridine as a blank. The same procedure was repeated with malonaldehyde bis(dimethyl acetal) as a positive control. The content of lipid peroxidation was expressed as nM MDA per mg protein by interpolation in a standard curve in water covering a concentration range of 0 to 200 mM. Protein concentration was estimated by Bradford's method. All samples were conducted independently in triplicate.

Catalase (CAT) assay

CAT catalyzes the decomposition of H_2O_2 molecules into water and oxygen. The reaction kinetics of CAT activity was conducted at 25°C using 50 mM phosphate buffer of pH 7.0 containing H_2O_2 as a substrate. After the 1-min incubation with cell lysates, the decrease of absorbance at 240 nm was monitored as the CAT activity to decompose hydrogen peroxide molecules. A molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$ for H_2O_2 was used.

Glutathione (GSH) assay

The total GSH contents of the cell lysates were estimated by colorimetric assay using the reaction between sulphydryl group of DTNB to produce a yellow-color of 5-thio-2-nitrobenzoic acid (TNB). TNB production is directly proportional to the concentration glutathione in cell lysates. All experiments were done in triplicate. Total glutathione was determined by measuring the absorbance 410 nm after 30 min incubation. The results were expressed in µmole per mg protein.

Statistical analysis

The statistic analysis of the obtained data was performed by SPSS (version 11.5) software for window. Data were statistically analyzed using one-way ANOVA and Turkey's multiple comparison testes in which the significance level was defined as $p < 0.05$.

RESULTS

DPPH assay

TLC chromatogram of crude extracts (Figure 1) was used for a qualitative screening of antioxidant activity. $DPPH^{\bullet}$ scavenging activity was visualized as yellow spots against purple background. LD extract gave no visualized

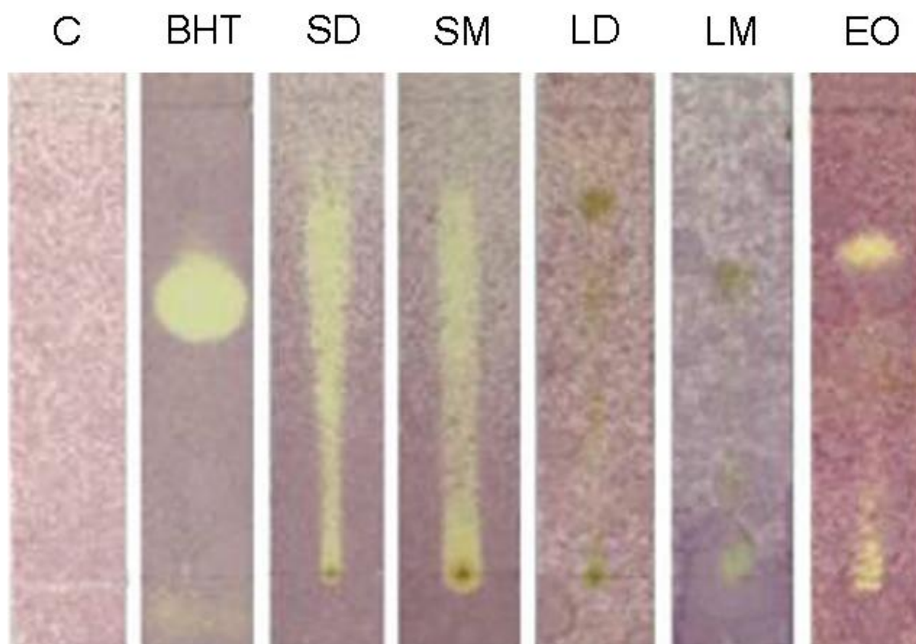

Figure 1. TLC plate sprayed with 0.2% DPPH$^{\bullet}$ solution in methanol, air dried, and visualized under visible light, and photographed under visible light. (C) control, (BHT) standard antioxidant butylated hydroxytoluene, (SD) dichloromethane extract of stem, (SM) methanol extract of stem, (LD) dichloromethane extracts of leave, (LM) methanol extract of leave, and (EO) essential oil.

spot on one-dimensional TLC analysis which means absence of antioxidant activity. LM extract showed a pale yellow area at the spotted point of extract which perhaps indicated the polar antioxidant in this crude extract. The last lane of EO slowly turned yellow after methanolic DPPH$^{\bullet}$ solution was sprayed, suggesting some degree of DPPH$^{\bullet}$ scavenging activity. The rapid development of yellow and long tail from SD and SM extracts showed the higher antioxidant activity than LM and EO extracts due to many antioxidant compounds present. The order of DPPH$^{\bullet}$ scavenging potency of the crude extracts from *Z. limonella* was as follow: Stem extracts > essential oil > leave extracts. Therefore, stem extracts and essential oil were further studied in prostate cancer cell lines.

The principle of quantitative analysis of DPPH antioxidant assay is based on a reaction in which a blue solution of stable free radical DPPH accepts an electron from a free radical scavenger, antioxidant compounds, and decolorizes. The amplitude of decolorization can be quantitatively determined by reading the absorbance. A large antioxidant capacity is revealed by low IC_{50} value (Table 1). IC_{50} of SD, SM, and EO are 54.63 $\pm$ 2.89, 117.47 $\pm$ 4.66, and 5,764.67 $\pm$ 6.45 μg/ml, respectively. As previously found in TLC chromatogram, SD and SM extracts still exhibited higher scavenging activity than EO. However, the IC_{50}'s of leave extracts, LD and LM was not determined due to the unability to decolorize the methanolic DPPH solution.

Trolox equivalent antioxidant capacity (TEAC) assay

Percentage inhibition of ABTS$^{\bullet+}$ absorbance as a function of the concentration for each crude extract was determined at 1-min time point. It was found that the percentage inhibition, from which the antioxidant activity could be inferred, varied in a dose-dependent fashion. TEAC values listed in Table 1 as μM Trolox/g crude extract is closely related to IC_{50} from DPPH assay with the same ranking order of free radical scavenging activity. Both values show that the crude extracts from stem have higher antioxidant activity than essential oil. TEAC values of SM, SD, and EO were 15.47 $\pm$ 0.34, 14.34 $\pm$ 0.31, and 7.05 $\pm$ 0.34 μM respectively.

Cell lysates from PC-3 and DU-145 at 24 h-pretreated with SD, SM, and EO were also tested by TEAC assay. In all treatments, TEAC values were found to increase with difference magnitude compare to the control (Figure 3).

Lipid peroxidation determination

In the present study, lipid peroxidation products, that is, MDA, were analyzed using TBAR assay, which consists of spectrophotometically measured color intensity of MDA-TBA complex. MDA-TBA complex nearly was absence in all extracts themselves, SD, SM, or EO after

Table 1. Antioxidative capacities of the several crude extract of *Z. limonella*.

Plant extract	50% DPPH scavenging activity (IC_{50}, µg ml^{-1})	Trolox equivalent antioxidant capacity (µM Trolox/g crude extract)
SM extract	54.6 ± 2.9	15.5 ± 0.3
SD extract	117.5 ± 4.7	14.3 ± 0.3
EO	5,764.7 ± 6.5	7.1 ± 0.3
BHT	19.7 ± 0.2	-

SM, Stem of methanol extract; SD, stem of dichloromethane extract; EO, essential oil of fruit.

been reacted with TBAR reagent. The MDA level of PC-3 untreated cell lysates slightly decreased, approximately 5 to 10% of control, by SM and EO. Moderately reduction of MDA, approximately 20% of control, was found by SD at 0.5 µg/ml. All extracts at 5 and 10 µg/ml seemed to exert no effect. In addition, all extracts at 0.5, 5 and 10 µg/ml exhibited no effect to MDA levels in DU-145 untreated cell lysates.

Cell lysates obtained from pretreated PC-3 and DU-145 with different concentrations: 0, 0.5 and 5 µg/ml of those three extracts was also used to test the antioxidant activity by TBAR assay. The concentrations of the extract at 0.5 and 5.0 µg/ml were previously proved not to influence cell growth via the proliferation assay (not shown). For PC-3 untreated cell lysates, MDA levels were significantly decreased by SD, SM and EO at dose 0.5 µg/ml at 78.80 ± 1.38, 88.89 ± 1.50, and 93.68 ± 2.67%, respectively. SM and EO expressed higher lipid peroxidation inhibition than SD at concentration of 5.0 µg/ml. Both concentrations of EO seemed to display no effect on the MDA level in DU-145 pretreated cell lysates. The same characteristic lipid peroxidation inhibitions of SD and SM were found in DU-145 and PC-3.

Catalase activity

CAT activity from cell lysates pretreated with SD and SM in both PC-3 and DU-145 increased significantly in a dose dependent manner. This was also found in cell lysates pretreated with EO in PC-3. The same magnitude of CAT elevation was observed in cell lysates pretreated with EO in DU-145 as shown in Figure 3.

Glutathione level

Reduced GSH levels of the cell lysates from PC-3 24 h-pretreated with SD and SM at concentration of 0.5 µg/ml seemed to significantly increase ($p < 0.05$) compared to the control group 111.97 ± 3.77 and 112.48 ± 3.23%, respectively. The increase percentage was lower when the concentration of SD and SM was increased 10-times, 104.83±4.85 and 104.20±3.11%, respectively. The same pattern of higher increase in reduced GSH could be observed in DU-145 pretreated cell lysates (Figure 3). GSH in PC-3 cell lysates pretreated with EO at 0.5 µg/ml was slightly decreased and significantly decreased with EO at 5 µg/ml.

DISCUSSION

Several compounds were isolated from *Z. limonella*. Somanabandhu et al. (1992) isolated five compounds from the bark of *Z. limonella*: Ubiquitous lupeol, alkaloid rutaecarpine and three coumarins, xanthoxyletin, osthol and scopoletin. In addition, there has been no report regarding antioxidant compound in *Z. limonella*. However, some of these compounds previously isolated from other plants were studied to possess antioxidant potential. Kim et al. (1997) reported that one of the components of *Artemisia iwayomogi*, scopoletin was unable to scavenge the DPPH$^{\bullet}$. In contrast, scopoletin was found to inhibit lipid peroxide and generation of superoxide and hydroxyl radicals. Kang et al. (1998) reported that the isolation of scopoletin from *Solanum lyratum* protects hepatocyte from CCl_4-induced toxicity by maintaining the GSH content, the activity of superoxide dismutase (SOD), and inhibiting the production of MDA as a result of its antioxidation and free radical-scavenging effect. Lupeol, a triterpene found in many fruits and vegetables was found to contain antioxidant, antimutagenic and antiimflammatory effect in *in vitro* and *in vivo* systems (Saleem et al., 2001; Geetha and Varalakshmi, 2001). Lupeol and its ester lupeol linoate effectively scavenge free radicals and reduce oxidative stress indices by enhancing the antioxidant capacity of the liver of cadmium treated rats (Sunitha et al., 2001).

Many reports have shown the antioxidant activity of extracts, synthetic, or natural compounds by DPPH and TEAC assays. These methods are based on similar redox reactions of stable free radicals and give information regarding the quality and the activity, both of which indicate the content to the radical scavenging compounds present in the tested sample. TLC chromatogram indicated that the extracts from fruits and stems exhibited a higher antioxidant activity than that of leaves. This has been attributed to terpenoids in the essential oil of fruit and coumarins and alkaloids in stem

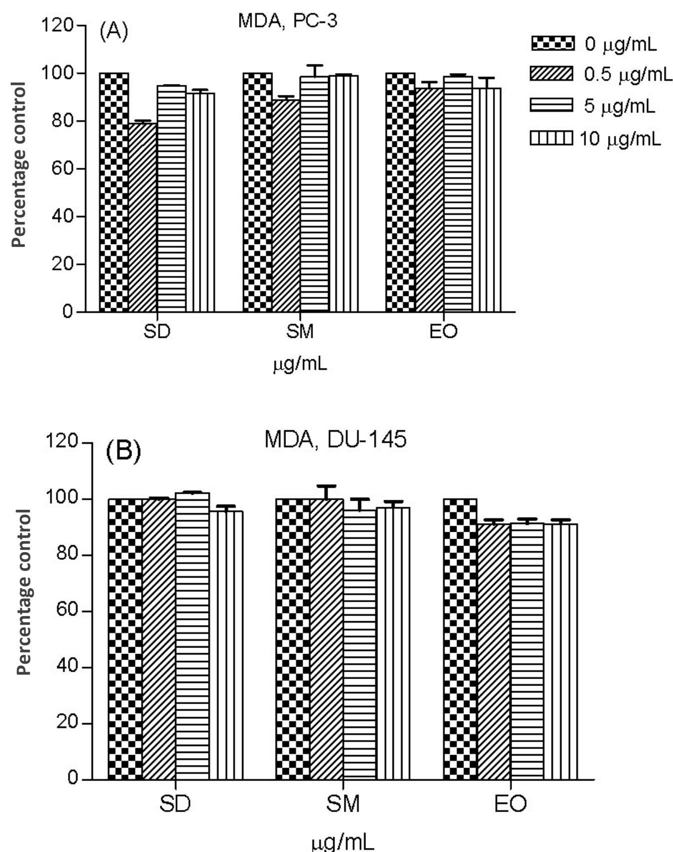

Figure 2. Change on MDA levels with SD, SM extract and EO in PC-3 (A) and DU-145 (B) cell lysates at 0 (control), 0.5, 5 and 10 µg/ml.

reported by Ittipanichpong et al. (2002) and Somanabandhu et al. (1992). The same ranking order of free radical scavenging activity among SD, SM, and EO was found, with a high correlation between TLC chromatogram, IC_{50} and TEAC values as shown in Figure 1 and Table 1. The data from DPPH and TEAC assays of crude extracts from *Z. limonella*, leaves, fruits, and stems revealed a wide range of antioxidant activity. Palasuwan et al. (2005) has reported a moderate degree of antioxidant activity of *Z. limonella* crude extract from seed. Since SD and SM showed a higher scavenger activity than EO, we extended the study to test the antioxidant capability on prostate cancer cell lines, PC-3 and DU-145.

In the erythrocytes of patients with prostate cancer, MDA levels have been found to be significantly higher (Aydin et al., 2006). It was previously mentioned that there is a higher oxidative stress in benign epithelium of prostate cancer patients than normal men. On the other hand, it is known that the generation of ROS is able to trigger many processes of lipid peroxidation, pro-carcinogenic processes, and protein damage. These cause considerable damages and disturb physiological functions of cellular essential components including lipid, protein and genetic materials. Several diseases were developed, especially many kinds of cancers and aging (Bandyopadhyay et al., 1999). Normally, the counteracting defense system to control excess free radicals was established intracellular as the antioxidant systems composed of enzymatic and nonenzymatic groups. The function of both is linked to each other and generates a balance between oxidative stress from ROS and antioxidant capacity to maintain an optimal health. Three important enzymes are involved as the effective antioxidants, superoxide dismutase (SOD), CAT, and glutathione peroxidase (GPX). SOD catalyses the conversion of $O_2^{\bullet-}$ to H_2O_2 and O_2, while CAT converts H_2O_2 to H_2O and O_2, and GPX reduces H_2O_2 to H_2O (Leewenburgh and Heinecke, 2001). Many compounds that function differently as nonenzymatic antioxidants include lipid-soluble and water-soluble compound, such as GSH, vitamin C, vitamin E, lipoic acid, uric acid, ubiquinol, etc. The action of antioxidants have been mentioned in four groups; chain breaking antioxidant, scavenging radicals, radical reduction, and chelating agent for transition metal catalysts (Pokorny et al., 2001). Many kinds of natural antioxidants such as vitamin E and lycopene are expected to play an important role for prostate cancer prevention and progression, especially when the patients are suffering and feel hopeless from conventional chemotherapy (Syed et al., 2007).

The information stated in Figure 2 showed quite low ability of SD, SM, and EO to change the MDA concentration in untreated cell lysates of PC-3 and DU-145. These results show that SD, SM, and EO had weak activity against MDA level in both cell lysates and can not provide direct protection against free radical to protect the cell membrane from the damage caused by lipid peroxidation. However, the indirect effect via intracellular antioxidant system might be possible.

Furthermore, the intracellular antioxidant system, GSH and CAT, was done in PC-3 and DU-145 pretreated with SD, SM, and EO at the concentration of 0, 0.5, and 5 µg/ml (Figure 3). SD and SM seemed to regulate GSH level and CAT activity, leading to the control of intracellular antioxidant power via an increasing of TEAC value. These observations may support the hypothesis that SM and SD might have antioxidant capacity by regulating the thiol-regulated cellular activity. GSH, the major low-molecular-mass thiol in the cytoplasm, acts as a free radical scavenger, trapping ROS that would otherwise interact with cellular thiols through an enzyme-catalysed reaction. GPX is used as an electron donor in the reduction of peroxidase, including lipid peroxides (Ghezzi, 2005).

On the other hand, EO has high possibility to regulate CAT activity more than GSH level in both cell lines. The intracellular antioxidant power of cell lines exposed to EO might largely depend on the CAT activity. CAT prevents the hydrogen peroxide from harming the cell itself. CAT is

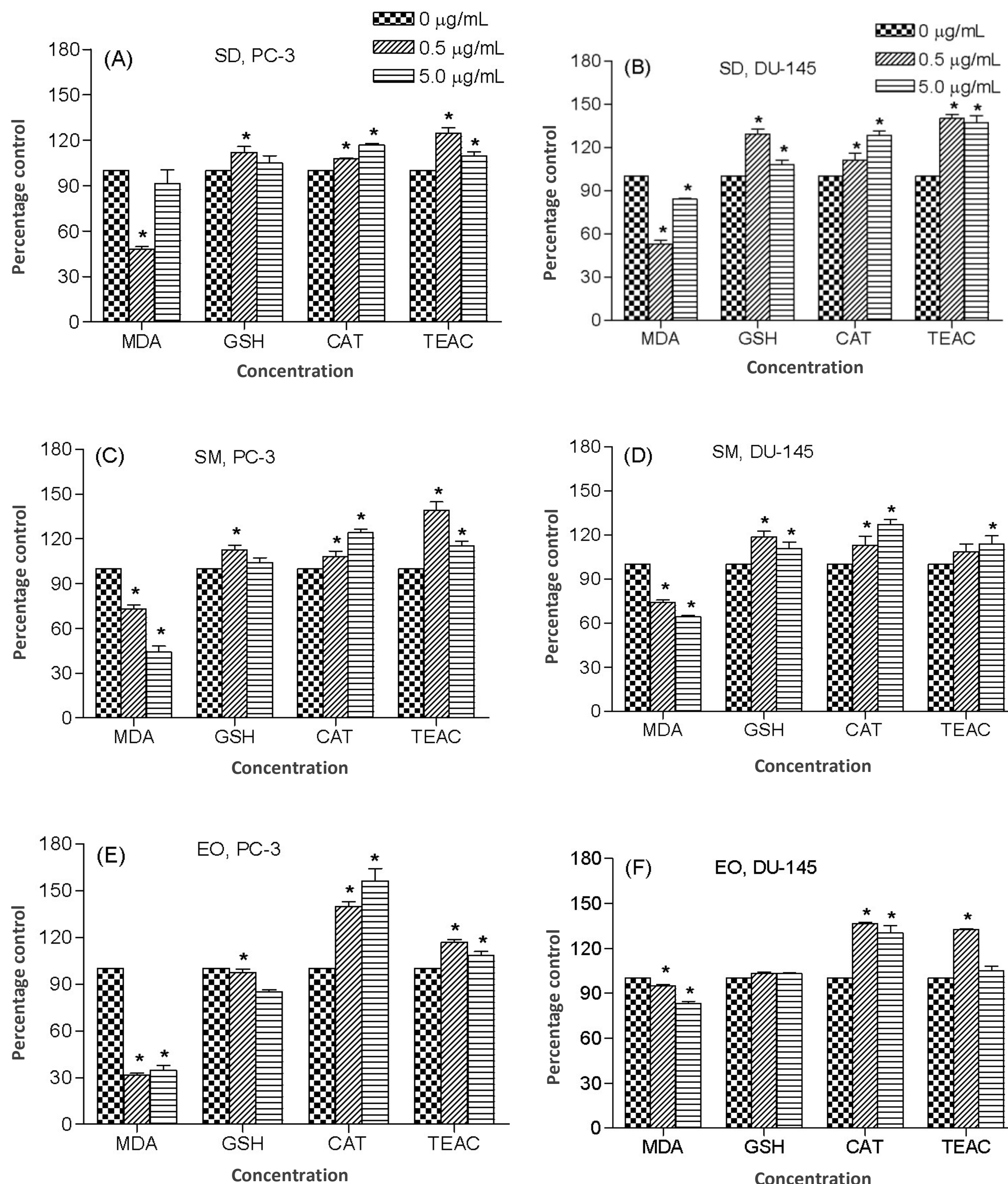

Figure 3. Change on MDA levels (ng μg^{-1} protein), GSH levels (mg mg^{-1} protein) and CAT (μmoles mg^{-1} protein min^{-1}), TEAC activities (μM Trolox mg^{-1} protein) with SD extract, SM extract and EO administration in PC-3 and DU-145 prostate cancer cells (A-F) at 0 (control), 0.5 and 5 μg/ml for 24 h.* $p<0.05$ versus untreated control as analyzed by one way ANOVA and Turkey's multiple-comparison test.

frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules (Gaetani et al., 1996). Gene expression of CAT and enzymes involved in thiol-regulated cellular activity is very interesting. Further investigation will determine gene expression in animal and human. The chemical analysis and compound isolation should be focused in searching effective natural compounds for the adjuvant therapy of prostate cancer.

Conclusion

The antioxidant capability of the extracts from *Z. limonella* was found in a wide range depended on various parts of plant. SD, SM, and EO expressed quite and interesting

indirect action via intracellular antioxidant content, GSH and CAT, to monitor the lipid peroxidation process.

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REFERENCES

Arts MJTJ, Dallnga JS, Voss HP, Haenen GRMM, Bast A (2004). A new approach to assess the total antioxidant capacity using the TEAC assay. Food Chem., 88: 567-570.

Aydin A, Arsova-Sarafinovska Z, Sayal A, Eken A, Erdem O, Erten K, Özgök Y, Dimovski A (2006). Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia. Clin. Biochem., 39: 176-179.

Bandyopadhyay U, Das D, Banerjee RK (1999). Reactive oxygen species-oxidative damage and pathogenesis. Curr. Sci., 77: 658-666.

Fish F, Gray AI, Waterman PG (1975). Coumarin, alkaloid and flavonoid constituents from the root and stem bark of *Zanthoxylum avicennae*. Phytochemistry, 14: 841-842.

Gaetani G, Ferraris A, Rolfo M, Mangerini R, Arena S, Kirkman H (1996). Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood, 87: 1595-1599.

Geetha T, Varalakshmi P (2001). Anti-inflammatory activity of lupeol and lupeol linoleate in rats. J. Ethnopharmacol., 76: 77-80.

Ghezzi P (2005). Oxidoreduction of protein thiols in redox regulation. Biochem. Soc. Trans., 33: 1378-1381.

Halliwell B (1994). Free radicals, antioxidants and human disease: curiosity, cause or consequence? Lancet, 344: 721-724.

Ittipanichpong C, Ruangrungsi N, Pattanaautsahakit, C (2002). Chemical compositions and pharmacological effect of essential oil from the fruit of *Zanthoxylum limonella*. J. Med. Assoc. Thail., 85: S344-S353.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007). Cancer statistics. CA Cancer J. Clin., 57: 43-66.

Kang SY, Sung H, Park JH, Kim YC (1998). Hepatoprotective activity of scopoletin, a constituent of *Solanum lyratum*. Arch. Pharmacal. Res., 21: 718-722.

Kim SS, Lee CK, Kang SS, Jung HA, Choi JS (1997). Chlorogenic acid, an antioxidant principle from the aerial parts of *Artemisia iwayomogi*. Arch. Pharmacal. Res., 20: 148-154.

Lee SJ, Lim KT (2007). Glycoprotein of *Zanthoxylum piperitum* DC has a hepatoprotective effect via anti-oxidative character *in vivo* and *in vitro*. Toxicol. *In Vitro*, 22: 376-385.

Leewenburgh C, Heinecke JW (2001). Oxidative stress and antioxidants in exercise. Curr. Med. Chem., 8: 829-838.

Liao S, Umekita Y, Guo J, Kokontis JM, Hiipakka RA (1995). Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. Cancer Lett., 96: 239-243.

Márquez L, Agüero J, Hernández I, Garrido G, Martínez I, Diéguez R, Prieto S, Rivas Y, Molina-Torres J, Curni M, Delgado Y (2005). Anti-inflammatory evaluation and phytochemical characterization of some plant of the *Zanthoxylum* genus. Acta Farm. Bonaer., 24: 325-330.

Navone NM, Troncoso P, Pisters L, Goodrow TL, Palmer JL, Nichols WW, Von Eschenbach AC (1993). p53 accumulation and gene mutation in the progression of human prostate carcinoma. J. Natl. Cancer Inst., 85: 1657-1661.

Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Chem., 95: 351-358.

Palasuwan A, Soogarun S, Lertlum T, Wiwanitkit V, Pradniwat P (2005). Inhibition of Heinz body induction in vitro model and total antioxidant activity of medicinal Thai plants. A.P.J.C.P., 6: 458-463.

Pokorńy J, Yanishlieva N, Gordon M (2001). Antioxidants in food. In Yaanishlieva-Maslarova NV (ed) Inhibiting oxidation, Woodhead Publishing Ltd and CRC Press LLC, pp. 22-25.

Pongboonrord S (1979). Mai-tet-muang-Thai. Bangkok: Kasem Bannakich Press.

Riedel W, Maulik G (1999). Fever: An integrated response of the central nervous system to oxidative stress. Mol. Cell Biochem., 196: 125-132.

Roig JT (1965). Diccionario Botánico de Nombres Vulgares Cubanos. In: Consejo Nacional de Universidades, (ed) Cuba: La Habana, pp. 846-847.

Saleem M, Alam A, Arifin S, Shah MS, Ahmed B, Sultana S (2001). Lupeol, a triterpene, inhibits early responses of tumor promotion induced by benzoyl peroxide in murine skin. Pharm. Res., 43: 127-134.

Sarkisian T, Emerit I, Arutyunyan R, Levy A, Cernjavski L, Filipe P (1997). Familial Mediterranian fever: clastogenic plasma factors correlated with increased O^{2-} – production by neutrophils. Hum. Genet., 101: 238-242.

Sithisarn P, Supabphol R, Gritsanaparn W (2006). Comparison of free radical scavenging activity of Siamese neem tree (*Azadirachta indica* A. Juss var. *siamensis* Valeton) leaf extracts prepared by different methods of extraction. Med. Princ. Pract., 15: 219-222.

Somanabandhu A, Ruangrungsi N, Lange GL, Organ MG (1992). Constituents of the stem bark of *Zanthoxylum limenella*. J. Sci. Soc. Thai., 18: 181-185.

Sunitha M, Nagaraj M, Varalakshmi P (2001). Hepatoprotective effect of lupeol and lupeol linoleate on tissue antioxidant defense system in cadmium-induced hepatotoxicity in rats. Fitoterapia, 72: 516-523.

Syed DN, Khan N, Afaq F, Mukhtar H (2007). Chemoprevention of prostate cancer through dietary agents: progress and promise. Cancer Epidem. Biomar., 16: 2193-2203.

Syed DN, Suh Y, Afaq F, Mukhtar H (2008). Dietary agents for chemoprevention of prostate cancer. Cancer Lett., 265: 167-176.

Vaca CE, Wilhelm J, Harm-Ringdahl M (1988). Interaction of lipid peroxidation products with DNA. A Review. Mutat. Res., 195: 137-144.

Wang Y, Corr JG, Thaler HT, Tao Y, Fair WR, Heston WDW (1995). Decreased growth of established human prostate LNCaP tumors in nude mice fed a low-fat diet. J. Natl. Cancer Inst., 87: 1456-1462.

Yamazaki E, Inagaki M, Kurita O, Inoue T (2007). Antioxidant activity of Japanese pepper (*Zanthoxylum piperitum* DC) fruit. Food Chem., 100: 171-177.

Zinchuk V (1999). Effect of nitric oxide synthase inhibition on hemoglobin-oxygen affinity and lipid peroxidation in rabbits during fever. Respiration, 66: 448-454.

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Full Length Research Paper

Rapid method for simultaneous determination of 11 chemical constituents in the traditional Chinese medicinal prescription Wu-Ji-San by reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD)

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In the present paper, a simple and sensitive high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) method was investigated for simultaneous determination of 11 components in Wu-Ji-San (WJS). The 11 identified components were ephedrine hydrochloride, paeoniflorin, liquiritin, ferulic acid, naringin, hesperidin, cinnamaldehyde, imperatorin, glycyrrhizic acid, honokiol and magnolol. The method was established using an Inertsil ODS-2 (250 mm × 4.6 mm i.d. with 5.0 µm particle size, GL Sciences) column. The 11 components were separated in less than 75 min with gradient elution using acetonitrile and 0.35% phosphoric acid in water at a flow rate of 1 ml/min. All calibration curves showed good linear regression (r^2 > 0.9993) within the test ranges. The method was validated for specificity, accuracy, precision, and limits of detection. The proposed method not only enables in a single run the simultaneous identification and determination of the 11 multi-structural components from WJS which form the basis of its therapeutic effect for quality control, but can also be used as a reference for the other prescription.

Key words: Wu-Ji-San, traditional Chinese medicinal prescription, reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD), chemical constituents.

INTRODUCTION

Wu-Ji-San (WJS) has been used in China for approximately one thousand years, and originates from 1115 A.D. into the pharmacopoeia of China's first "Taiping Benevolent Dispensary side". The prescription has been developed and evolved by ancient Chinese medical experts of the Song, Yuan, Ming and Qing Dynasties. Now, WJS is widely used for exogenous cold or dietary inadvertently caused by headache, body pain, abdominal pain, vomiting, anorexia, and irregular menstruation embolism with high usability and efficacy. WJS has become a standard medicine in many Chinese families (Rao et al., 2009).

As a traditional Chinese medicine (TCM), WJS contains the following 15 traditional Chinese medicines: Herba ephedrae (*Ephedra sinica* Stapf), Radix Angelicae dahuricae (*Angelica dahurica* (Fisch. ex Hoffm.) Benth. et Hook.f.), Ramulus Cinnamomi (*Cinnamomum cassia* Presl), Rhiaoma Zingiberis (*Zingiber officinale* Rosc.), Poria (*Poria cocos* (Schw.) Wolf), Radix Angelicae sinensis (*Angelica sinensis* (Oliv.) Diels), Radix Paeoniae alba (*Paeonia lactiflora* Pall.), Radix Platycodi (*Platycodon grandiflorum* (Jacq.) A.DC.), Rhizoma

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Atractylodis (*Atractylodes lancea* (Thunb.) DC.), Cortex Magnoliae officinalis (*Magnolia officinalis* Rehd. et Wils.), Pericarpium Citri Reticulatae (*Citrus reticulata* Blanco), Fructus Aurantii (*Citrus aurantium* L.), Rhizoma Pinelliae Praeparatum (*Pinellia ternata* (Thunb.) Breit.), Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.) and Rhizoma Ligusticum (*Ligusticum chuanxiong* Hort.).

Modern chemistry and pharmacology studies have shown that there are five scattered plot of 11 main active ingredients, respectively, with the relaxation of bronchial smooth muscle, blood vessels and uterine contraction (Tong and Eisenach, 1992; Bilcíková et al., 1987), anti-virus (Hirabayashi et al., 1995; Chan et al., 1995; Lee, 1999; Bae, 2000; Kim et al., 2000; Pompei et al., 1979), anti-bacterial (Tsou et al., 2000; Chang et al., 2001; Widelski et al., 2009; Rosselli et al., 2007; Clark et al., 1981), anti-inflammatory (Lee et al., 2009; Jayaprakasam et al., 2009; Shi and Wei, 2004), antioxidant (Kim et al., 2009; Deon et al., 2002; Lo et al., 1994; Bao et al., 2004; Yao et al., 2009), sedative (Zhang et al., 2009), anti-thrombosis (Teng et al., 1988), anti-tumor and other pharmacological effects (Teng et al., 1988; Moon et al., 1983). Keeping in view the current reports, it is believed that the main components of WJS are primarily responsible for its anti-viral, anti-bacterial, anti-inflammatory, analgesic and other pharmacological effects.

The currently available assay procedure for WJS detects only a single compound or a few marker compounds. In the present study, a simple, rapid and accurate HPLC-DAD coupled method was successfully developed for the simultaneous determination of 11 compounds in WJS. The developed method, provided grounds for its use in quality control studies on WJS, and it also added to characterize chemical constituents responsible for the therapeutic effect of WJS.

MATERIALS AND METHODS

The fifteen Chinese herbs that comprise WJS were supplied by a TCM dispensary store in the China Hospital (Hunan, China) and identified. Voucher specimens (No. 200505) were deposited at the Laboratory of Ethnopharmacology in Hunan Medical University No. 1 Affiliated Hospital. Authentic standards of geniposide, puerarin, paeoniflorin, ferulic acid, liquiritin, hesperidin, naringin, paeonol, daidzein, glycyrrhizic acid, honokiol and magnolol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany). Phosphoric acid (analytical grade) was purchased from Gaojing Chemical Industry Company (Hangzhou, China). Other reagents were of analytical grade.

Instrumentation and analytical conditions

The high performance liquid chromatography (HPLC) system 1200 series (Agilent Technologies, Palo Alto, CA, USA) was equipped with ChemStation software (Agilent Technologies) and comprised of a quaternary solvent delivery pump, an online vacuum degasser, an autosampler, a thermostated compartment and a diode array detector. All separations were carried out on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d. with 5.0 µm particle size, GL Sciences, Tokyo Japan) from Hanbang Science and Technology (Hunan, China).

Mobile phase A was 0.35% (v/v) phosphoric acid aqueous solution. Phase B was pure acetonitrile. The elution was performed using a linear gradient of (0 to 5 min, 7 to 18% A; 15 to 38 min, 18 to 30% A; 38 to 42 min, 30 to 41% A; 42 to 45 min, 41 to 55% A; 45 to 65 min, 55 to 62% A; 65 to 75 min, 62 to 75%). The flow-rate was 1.0 $ml \cdot min^{-1}$, column temperature was maintained at 30°C. The effluent was monitored at 200 to 400 nm, and the injection volume was 5 µl. The peak identification was based on the retention time and comparison with the DAD spectrum of the standard.

Preparation of standard solutions

Standard stock solutions of the 11 reference standards (geniposide, puerarin, and others) were directly prepared by accurately weighing appropriate amounts of the standard compounds and dissolving them in methanol. They were then diluted to six concentrations for construction of calibration plots. The standard stock solutions were all prepared in dark brown calibrated flasks and stored at 4°C in a refrigerator till used. Empower software was used to prepare the standard curves from the peak area of each compound. The contents of these constituents in the test samples were calculated using the regression parameters obtained from the standard curves.

Preparation of sample solutions

A total of 4 g of Herba ephedrae, Radix Angelicae dahuricae, Ramulus Cinnamomi, Rhizoma Zingiberis, Poria, Radix Angelicae sinensis, Radix Paeoniae alba, Radix Platycodi, Rhizoma Atractylodis, Cortex Magnoliae officinalis, Pericarpium Citri Reticulatae, Fructus Aurantii, Rhizoma Pinelliae Praeparatum, Radix Glycyrrhizae and Rhizoma Ligusticum at a weight ratio of 0.4: 0.3: 0.5: 0.3: 0.3: 0.3: 0.3: 0.6: 0.8: 0.4: 0.5: 0.5: 0.3: 0.3: 0.2 were crushed into small pieces. The mixture was placed in a refrigerator at 4°C in 50 ml pure methanol, soaked overnight and passed through a filter paper. The final extraction was equal to 0.04 g crude drug/ml methanol. The methanolic extract was sealed in sterile bottles and retained in a refrigerator (4°C) until used. The sample injection volume for HPLC analysis was 5 µl. The samples were filtered through a 0.45 µm filter before HPLC analysis.

RESULTS AND DISCUSSION

Optimization of extraction procedure

Various extraction methods, solvents and times duration for extraction, were evaluated to obtain the best possible extraction efficiency. The results showed that soaking overnight at low temperature was better than using ultrasonic extraction. Therefore, in all further experiments, the procedure of soaking overnight at low temperature was employed. Various solvents including water, methanol-water (50:50, v/v) methanol and ethanol were screened. Methanol exhibited complete extraction of all the major constituents. It is a convenient and economical method that the sample was placed in the refrigerator overnight for cold soak extraction.

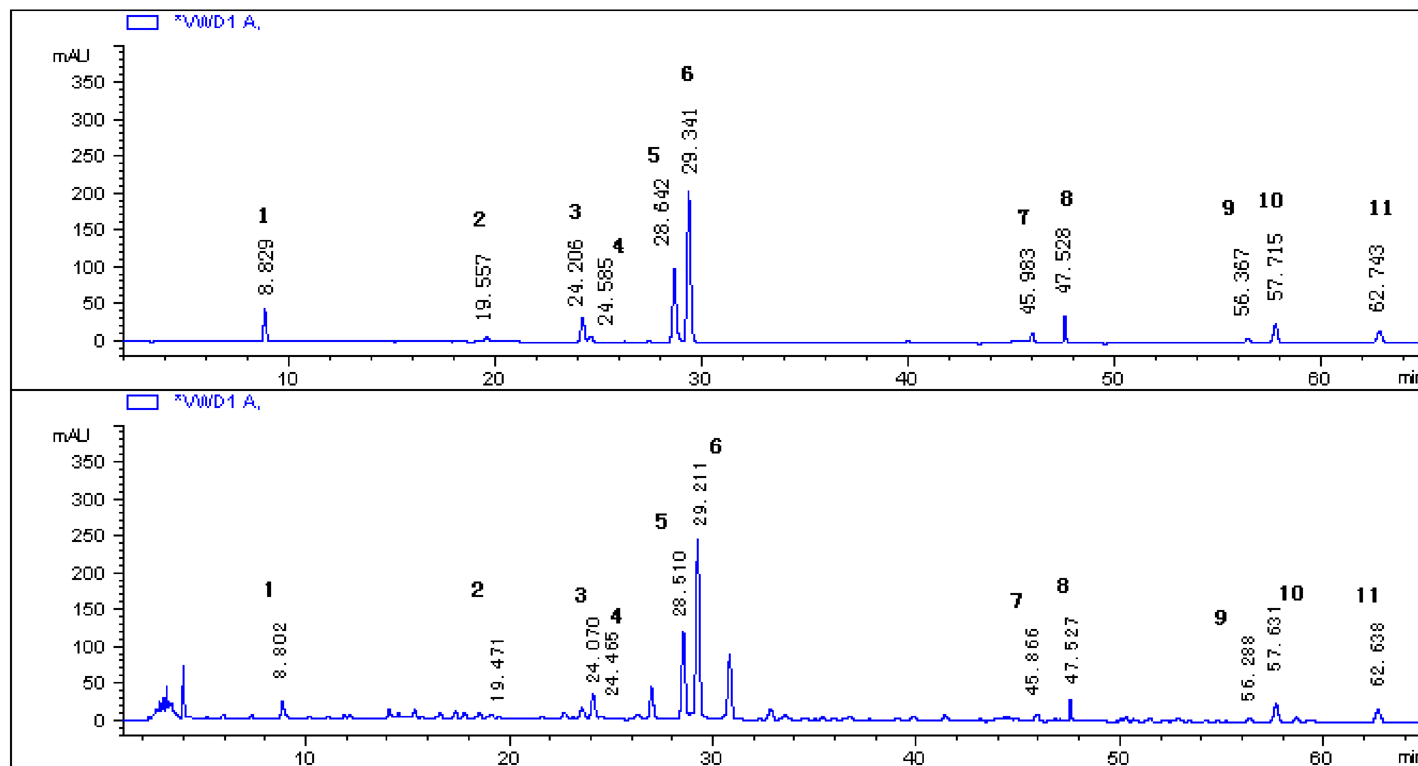

Figure 1. Typical chromatograms of the standard mixture (A) and Wu-Ji-San (B) at different detection wavelengths. (1) Ephedrine hydrochloride; (2) Paeoniflorin; (3) Liquiritin; (4) Ferulic acid; (5) Naringin; (6) Hesperidin; (7) Cinnamaldehyde; (8) Glycyrrhizic acid; (9) Imperatorin; (10) Honokiol; (11) Magnolol.

Optimization of chromatographic conditions

A common limitation of multiple component analysis is the low sensitivity of detection for some analytes at the selected single monitoring wavelength. Hence in the current study diode array detection (DAD) was used providing the facility of using multi wave-length monitoring facilities. Different detection wavelengths used to monitor different compounds simultaneously in a single run were as follows: λ 207 nm (for 1, 2, 3, 4, 5); 237 nm (for 6 and 8), 300 nm (for 9), 294 nm (for 7, 10 and 11) to provide sufficient sensitivity for each analyte.

With the DAD, the ultraviolet (UV) spectra of the bioactive constituents could be compared with those of the authentic standards. The desired compound from WJS was identified by comparing both the retention times and UV spectra with those of the authentic standard. The identity of each analyte was further confirmed by spiking the actual sample with the standard. The excellent agreement between the standard and sample spectra found in all analyzed samples of WJS indicated that under the proposed analytical conditions, the 11 marker constituents were sufficiently resolved. The peak for each compound was separated successfully by gradient elution in less than 75 min, and there was no interference by one or the other components in the matrix. Typical chromatograms of the authentic standards and WJS recorded at different detection wavelengths are depicted in Figure 1.

Method validation

Standard stock solutions containing 11 analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. The assay linearity was determined by the analysis of six different concentrations of the standard solutions. Table 1 shows the regression data and LODs (S/N = 3) of the components. All calibration curves showed good linear regression (r^2 > 0.9994) within the test ranges.

The relative standard deviation (R.S.D.) was considered to be a measurement of precision and accuracy. The intra- and inter-day precisions were determined by analyzing working solutions at three concentrations in 5 replicates during a single day and by duplicating the experiments on 3 successive days. As shown in Table 2, the overall intra- and inter-day variations was less than 5% for all 11 analytes. These results demonstrated that the developed method is reproducible with good precision.

The stability test was performed with sample solutions

Table 1. Regression data and LODs for the 11 components determined (n = 6).

Components	Regression equation	Correlation coefficient (r^2)	Linear range (μg/ml)	LOD (μg/ml)
Ephedrine hydrochloride	y = 2018.6x + 46.011	0.9994	2.52-75.11	0.19
Paeoniflorin	y = 1.332x + 12.813	0.9993	3.43-102.16	0.52
Liquiritin	y = 3564x - 34.343	0.9994	3.15-93.37	0.74
Ferulic acid	y = 378.21x - 3.285	0.9995	3.85-114.72	0.79
Naringin	y = 3264.6x - 65.26	0.9998	8.56-255.28	0.76
Hesperidin	y = 3493.2x + 61.495	0.9998	13.54-405.62	0.15
Cinnaldehydum	y = 3083x - 9.238	0.9995	0.69-20.74	0.43
Glycyrrhizic acid	y = 549.05x - 23.338	0.9998	9.96-298.80	0.40
Ammidin	y = 5503.4x + 3.7606	0.9996	0.26-7.82	0.17
Honokiol	y = 2307.1x + 1.5637	0.9999	2.95-88.55	0.15
Magnolol	y = 1499.5x + 86.085	0.9999	3.27-96.21	0.17

Table 2. The precision data of the proposed HPLC method.

Components	Nominal concentration (μg/ml)	Precision			
		Intra-day (n = 5)		Inter-day (n = 3)	
		Mean±SD (μg/ml)	R.S.D (%)	Mean±SD (μg/ml)	R.S.D (%)
	9.53	9.25±0.25	2.66	9.34±0.23	2.50
Ephedrine hydrochloride	19.97	20.05±0.08	0.39	20.49±0.55	2.70
	34.14	33.96±0.45	1.32	34.76±0.66	1.91
	8.96	8.66±0.37	4.25	8.25±0.21	2.57
Paeoniflorin	17.58	17.85±0.21	1.16	18.12±0.32	1.78
	30.76	30.52±0.20	0.66	31.29±0.67	2.13
	16.89	16.23±0.70	4.31	15.56±0.51	3.29
Liquiritin	33.07	33.25±0.17	0.51	34.42±1.19	3.46
	56.76	57.09±0.26	0.45	58.58±1.58	2.69
	11.27	10.45±0.79	4.55	9.76±0.68	4.92
Ferulic acid	23.11	23.33±0.90	3.85	24.28±0.60	2.45
	44.34	44.71±1.81	4.04	46.96±3.03	4.45
	51.81	49.48±2.44	4.93	46.13±1.29	2.79
Naringin	98.62	98.79±0.21	0.21	101.65±3.18	3.13
	167.33	167.95±0.47	0.28	171.38±3.71	2.16
	83.19	79.93±3.77	4.72	74.32±1.59	2.14
Hesperidin	160.86	161.22±0.38	0.23	164.84±5.07	3.08
	276.09	276.25±0.61	0.22	281.54±5.42	1.93
	3.27	3.37±0.21	4.32	3.24±0.20	4.30
Cinnamaldehyde	6.92	6.77±0.39	4.75	6.72±0.29	4.26
	9.93	9.61±0.52	4.43	10.14±0.56	4.56
	47.47	44.49±2.63	4.91	40.78±0.77	1.90
Glycyrrhizic acid	87.11	87.32±0.30	0.35	89.72±2.80	3.12
	144.43	144.87±0.46	0.32	147.22±2.54	1.73

Table 2. Contd.

	1.10	1.56±0.07	4.69	1.50±0.06	4.19
Imperatorin	2.13	2.06±0.12	4.67	1.93±0.08	3.89
	2.43	2.47±0.03	1.42	2.52±0.04	1.49
	16.05	15.45±0.73	4.74	14.30±0.22	1.57
Honokiol	31.61	31.76±0.11	0.33	32.64±1.06	3.24
	55.19	55.33±0.14	0.26	56.29±1.06	1.88
	14.01	13.40±0.74	4.51	12.42±0.38	3.05
Magnolol	27.58	27.49±0.16	0.57	28.00±0.95	3.40
	48.01	47.96±0.25	0.51	48.97±1.00	2.05

Table 3. Statistic results of recovery for extraction of analytes in LDP.

Components	Original (mg)	Spiked (mg)	Detected (mg)	Calculated recovery (%)	R.S.D (%)
Ephedrine hydrochloride	0.249	0.113	0.364	102.19	4.21
	0.250	0.225	0.473	99.18	2.03
	0.251	0.450	0.688	97.11	1.98
Paeoniflorin	0.192	0.153	0.341	97.35	3.02
	0.193	0.306	0.501	100.89	1.59
	0.193	0.612	0.820	102.37	2.25
Liquiritin	0.425	0.140	0.563	99.04	2.79
	0.426	0.279	0.701	98.71	1.45
	0.427	0.558	0.974	97.95	0.89
Ferulic acid	0.133	0.171	0.311	103.85	4.87
	0.133	0.342	0.477	100.59	3.47
	0.134	0.684	0.798	97.12	2.66
Naringin	1.202	0.383	1.581	98.95	2.64
	1.205	0.765	1.994	103.14	1.90
	1.208	1.530	2.731	99.53	0.69
Hesperidin	2.012	0.608	2.618	99.73	2.45
	2.017	1.215	3.256	101.92	2.06
	2.022	2.430	4.364	96.35	1.20
Cinnamaldehyde	0.078	0.031	0.110	101.16	4.69
	0.079	0.062	0.140	98.26	3.77
	0.079	0.124	0.204	100.65	4.51
Glycyrrhizic acid	1.084	0.448	1.540	101.76	3.04
	1.087	0.896	1.955	96.86	2.52
	1.090	1.793	2.874	99.55	1.33
Imperatorin	0.013	0.012	0.024	96.56	4.38
	0.013	0.023	0.037	102.39	2.71
	0.013	0.047	0.060	101.23	2.48

Table 3. Contd.

Honokiol	0.400	0.133	0.531	98.42	3.27
	0.401	0.266	0.668	100.47	1.48
	0.402	0.531	0.948	102.76	2.36
Magnolol	0.345	0.144	0.485	97.04	3.72
	0.346	0.288	0.633	99.55	2.95
	0.347	0.576	0.919	99.23	1.47

Table 4. Amount of the 11 main components found in WJS (*n* = 3).

Components	Contents (ng/mg)	R.S.D (%)
Ephedrine hydrochloride	700 ± 13	1.86
Paeoniflorin	539 ± 18	2.34
Liquiritin	1193 ± 20	1.71
Ferulic acid	374 ±35	2.92
Naringin	3377 ± 71	2.11
Hesperidin	5652 ± 91	1.60
Cinnamaldehyde	220 ± 6	2.79
Glycyrrhizic acid	3045 ±44	1.44
Imperatorin	36 ± 1	1.49
Honokiol	1125 ±16	1.43
Magnolol	970± 16	1.66

placed at 4°C and these were analyzed at 0, 24, and 48 h. The R.S.D. values of the peak area and retention times were no more than 4.9 and 2.0%, respectively. The solution was therefore considered to be stable for at least 48 h at 4°C.

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels of the mixed standard solutions to known amounts of the working samples. The resultant samples were then extracted and analyzed using the described method. The average percentage recoveries were evaluated by calculating the ratio of detected amount versus added amount. The recovery of the method was in the range of 96.56 to 103.85%, with R.S.D. less than 4.87% as shown in Table 3. Considering the results, the method was deemed to be accurate.

Determination of 11 components in WJS

The developed assay was subsequently applied for the simultaneous determination of eleven major compounds in WJS samples. A representative chromatogram of the extracts is shown in Figure 1B and the quantity of each compound identified is summarized in Table 4. These data indicate that the proposed method is suitable for the simultaneous determination of 11 compounds in WJS. Therefore, this assay method provided the option to be used in the quality control of WJS and other drugs containing such active biological molecules.

Conclusion

This is the first report of accurate and reliable analytical method for the simultaneous determination of 11 major bioactive constituents in Chinese medicine WJS by using reverse phase high-performance liquid chromatography coupled with diode array detection (RP-HPLC-DAD). This method is validated for good accuracy, repeatability and precision. Hence, this method can be used as a reference to evaluate the quality of WJS and other related herbal drug products. It is therefore very significant for further experiments that focus on evaluating the ancient prescription with China and Japanese origin.

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REFERENCES

Bae EA (2000). *In vitro* inhibitory efects of some flavonoids on rotavirus infectivity. Biol. Pharm. Bull., 23: 1112.

Bao ZJ, Yang XQ, Ding ZT (2004). Simultaneous determination of magnolol and honokiol by UV spectrophotometry and study on free radical searenger activity. Nat. Prod. Res. Dev., 16: 435-438.

Bilcíková L, Bauer V, Kolena J (1987). The effects of methylxanthines, ethymizol, ephedrine and papaverine on guinea pig and dog trachea. Gen. Physiol. Biophys., 6: 137-148.

Chan WS, Wen PC, Chiang HC (1995). Structure-activity relationship of caffeic acid analogs on xanthine oxidase inhibition. Anticancer Res., 15: 703.

Chang ST, Chen PF, Chang SC (2001). Antibacterial activity of leaf essential oils and their constituents Cinnamomum osmophloeum. Ethnopharmacology, 77: 123-127.

Clark AM, EI-Feraly FS, Li WS (1981). Antimicrobial activity of phenolic constituents of *Magnolia grandiflom* L. J. Pham. Sci., 70: 951-952.

Deon SM, Bok SH, Jang MK (2002). Comparison of antioxidant effects of naringin and p robucol in cholesterol-fed rabbits. Clin. Chim. Acta, 317: 181-190.

Hirabayashi T, Ochiai H, Sakai S (1995). Inhibitory effect of fertulic acid and isoferulic acid on murine interleukin- 8 production in response to influenza virus infections *in vitro* and *in vivo*. Planta Med., 61: 221.

Jayaprakasam B, Doddaga S, Wang R, Holmes D, Goldfarb J, Li XM (2009). Licorice flavonoids inhibit eotaxin - 1 secretion by human fetal lung fibroblasts *in vitro*. J. Agric. Food Chem., 57: 820-825.

Kim DH, Song MJ, Bae EA (2000). Inhibotory effects of herbal medicines on rotavirus infectivity. Biol. Pharm. Bull., 23: 356.

Kim SH, Lee MK, Lee KY, Sung SH, Kim J, Kim YC (2009). Chemical constituents isolated from Paeonia lactiflora roots and their neuroprotective activity against oxidative stress *in vitro*. Med. Chem., 24: 1138-1140.

Lee JH (1999). Antiviral activity of some flavonoids on Heroessimplex virus. Korean Pharnuog., 30: 49.

Lee JY, Kang HS, Park BE, Moon HJ, Sim SS, Kim CJ (2009). Inhibitory effects of Geijigajakyak- Tang on trinitrobenzene sulfonic acid-induced colitis. J. Ethnopharmacol., 126: 244-251.

Lo YC, Teng CM, C, hen CF, Chen CC, Hong CY (1994). Magnolol and honokiol isolated from Magnolia oficinalis pmtect rat heart mitochondriaagainst lipid peroxidation. Biochem. Pharmacol., 47: 549-553.

Pompei R, Flore O, Marccialis MA, Pani A, Loddo B (1979). Glycyrrhizic acidinhibits virus growth and inactivates virus particles. Nature, 281: 689-690.

Rao J, Cai GX, Li DD, Wang YH, Chen L (2009). Progress of Wujisan in clinic and pharmacological research of each drug in it. J. T.C.M Univ. Hunan, 29: 75-77.

Rosselli S, Maggio A, Bellone G (2007). Antibacterial and anticoagulant activities of coumarins isolated from the flowers of Magydaris tomentosa. Planta Med., 73: 116-120.

Shi HC, Wei YH (2004). Magnolol alter the course of endotoxin tolerance and provides early protecion against endotoxin challenge following sublethal hemorrhage in rats. Shock, 22: 358-363.

Tong C, Eisenach JC (1992). The vascular mechanism of ephedrine's beneficial effect on uterine perfusion during pregnancy. Anesthesiology, 76: 792-798.

Tsou MF, Hung C F, Lu HF (2000). Effects of caffeic acid, chlorogenic acid and ferulic acid on growth and arylamine N- acetyltransfera se activity in Shigellasonne i(group D). Microbes, 101: 37.

Widelski J, Popova M, Graikou K (2009). Coumarins from Angelica lucida L. *Antibacterial activities*. Antibacterial act., 14: 2729-2734.

Yao K, Zhang L, Ye PP (2009). Protective effect of magnolol against hydrogen peroxide-induced oxidative stress in human lens epithelial cells. Am. J. Chin. Med., 37: 785-796.

Zhang XJ, Chen HL, Li Z, Zhang HQ, Xu HX, Sung JJ, Bian ZX (2009). Analgesic effect of paeoniflorin in rats with neonatal maternal separation-induced visceral hyperalgesia is mediated through adenosine A(1) receptor by inhibiting the extracellular signal-regulated protein kinase (ERK) pathway. Pharmacol. Biochem. Behav., 94: 88-97.

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Full Length Research Paper

Some biochemical parameters and histopathological features following prolonged administration of aqueous pod extract of *Acacia nilotica* in albino rats

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The effects of aqueous pod extract of *Acacia nilotica* was investigated in rats. The pod extract of *A. nilotica* was obtained by Soxhlet extraction using distilled water as a solvent. The aqueous extract was administered for a period of 21 days at 50, 100 and 200 and 400 mg/kg body weight, respectively. The liver enzymes and some biochemical parameters were evaluated. The tissue samples were also taken for histopathological preparations. The effect of the extract significantly ($P<0.05$) increased values of Aspartate amino transferase (AST), Alanine aminotransferase (ALT) and Creatinine. While, significantly ($P>0.05$) decreased values of serum albumin, total protein, glucose and triglyceride at the end of the 21 days. While alkaline phosphatase (ALP) and Urea values were not affected. The histopathological study revealed marked haemorrhages of lung, heart and kidney with renal tubular degeneration. Liver revealed hepatocellular necrosis and mononuclear cell infiltrations, mild congestion of the stomach and mild sub mucosal mononuclear cell infiltrations of the small intestine, respectively. It was therefore concluded that *A. nilotica* extract has the potential to produce some level of toxicities and it consumption for medicinal purposes should be discourage.

Key words: Histopathological features, *A. nilotica* aqueous pod extract, biochemical parameters.

INTRODUCTION

Acacia nilotica: (English Names; thorn tree, wattles, Babul, Black babul, Indian Arabic gum), a member of the Family; *Fabaceae.* Tender pods and shoots are used as vegetables and are fed to camels, sheep and goats especially in Sudan, where it is said to improve milk production from these animals. In South Africa, the Zulu′s take the stem bark extract for cough and the Chipi1 use root bark for tuberculosis treatment, while the Masai use the stem bark and root decoction, to alleviate mood. In Ayurvedic medicine, the stem bark is considered a remedy for treating premature ejaculation (Pande et al., 1981). In Nigeria local traditional healers used the bark and pods extract for the treatment of ailments such diarrhoea and stomach ache. Although, the world health organization (WHO) in recognition of the increased value of herbal medicine to primary health care, has advocated for the proper identification, sustainable exploitation n, scientific development and appropriate utilization of herbal medicine which provide safe and effective remedies in Medicare (Wambebe, 1998). Many herbs including the decoction from the pods of *A. nilotica* have been used in folk medicine for the control of diarrhoea. *A. nilotica* although widely used in north eastern Nigeria for treatment purposes, it has not been scientifically evaluated for its use and toxicity. The scientific investigation of *Acacia nilotica* could support its reported efficacy in herbal medicine.

MATERIALS AND METHODS

Plant collection, identification and extract preparation

Fresh pods and leaves of *A. nilotica* were collected from Lai –Lai grazing reserve, Potiskum Local Government Area of Yobe State, Nigeria and submitted for confirmation to Dr. S.S Sanusi, Department of Biological Sciences, University of Maiduguri and a

Table 1. Effect of the aqueous pod of *A. nilotica* on mean[b] liver enzymes.

Parameter	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
AST (μ/L)	154.0±8.10	171.0±5.90*	171.0±6.50*	177.0±9.90*	182.0±11.2*
ALT (μ/L)	22.8±6.5	47.0±3.4*	54.0±7.4*	48.6±3.7*	52.0±8.9*
ALP (μ/L)	177.0±16.7	172.0±47.0	170.0±36.0	161.0±15.0	192.0±9.40

b = Mean±Standard deviation based on five observations. *P<0.05 means significantly different from the control.

voucher specimen was deposited at the Department of Veterinary Physiology and Pharmacology herbarium, University of Maiduguri, Nigeria. The pods were air dried at room temperature for three weeks. The crushing of the pods was done in laboratory using pestle and mortar, after which it was ground into powder. About (200 gm) of the powdered pod was weighed and introduced into a conical flask and 1 litre of distilled water was added thereafter. The mixtures was then shaken and allowed to stand for 30 min, after which it was boiled for 1 h, cooled and shaken vigorously, before filtration using whatman No. 1 filter paper. The filtrate was concentrated in a rotatory evaporator and stored at 4°C until used, and the yield was 6.75% (w/w).

Sub acute toxicity studies

Twenty five (25) Wister albino rats of both sexes (weighing between 140 to 160 g) were randomly selected and divided into five groups of five rats each, were used for the studies. Group I was used as control and Groups II, III, IV and V were treated orally with 50, 100 and 200 and 400 mg/kg of aqueous pod extract of *A. nilotica,* respectively for a period of 21 days. At the end of the treatment period, the rats were humanely sacrificed and blood samples collected to obtain serum following centrifugation. Serum samples harvested were used for the determination of the liver enzymes and biochemical parameters.

Estimation of liver enzymes and some biochemical parameters

ALT and AST were assayed using kits based on the method of **Reitiman** and Frankel (1957). Alkaline phosphatase was determined using the method of Deutsche and Kelinsche (1972). The estimations of some biochemical parameters were also carried out using standard procedures according to manufacturer's instructions.

Histopathology

The tissue samples of heart, lungs, small intestine, stomach, liver and kidney obtained were fixed in 10% formalin. The tissues were dehydrated through graded concentration of ethanol (70, 95% and absolute), cleared in xylene and embedded in paraffin wax. The embedded tissues were stained with hematoxylene and eosin (H and E) for light microscopic examination. The lesions observed were photographed using Vanox T Olympus photographing microscope (Drury and Wallingthon, 1979).

Statistical analysis

All values were expressed as Mean±Standard Deviation. While analysis of variance (ANOVA) was used to analyse the extent of variation between groups and P values equal to or less than 0.05 were considered significant (Mead and Curnow, 1982). The computer soft ware Graph pad instat was used to analyse the data.

RESULTS

Extraction

The aqueous pod extract was light green and have slight bitter taste. The yield was 6.75% (w/w).

Effect of prolonged oral administration of aqueous pod extract of *A. nilotica* on liver enzymes

The result of the effect of prolonged oral administration of *A. nilotica* water extract was presented in Table 1. Administration of the extract orally for 21days significantly (P<0.05) increased the levels of the liver enzymes (AST and ALT) in the treated rats when compared with the control. The levels of ALP were not affected by the extract treatments (Table 1).

Effect of prolonged administration of *A. nilotica* extract on some biochemical parameters

Total protein, albumin, cholesterol and triglyceride concentrations in the serum of extract treated rats were significantly (P<0.05) decreased when compared to the control (Table 2). Urea values was unaffected by the extract treatments, however, creatinine levels were significantly (P<0.05) increased in the treated animals when compared with the control group.

Histopathological studies

Treatment of rats with *A. nilotica* pod extract for 21 days resulted in the presence of lesions in the organs and tissues (Figures 1 to 6). Treatment of the rats with the extract at 400 mg/kg resulted in a mild congestion (Figure 1) of the mucous membrane of the stomach. Extract treatment at 200, and 400 mg/kg produced moderate sub mucosal mononuclear cell infiltrations of the intestine (Figure 2). The kidneys of rats treated with the extract showed marked congestion, tubular degeneration and

Table 2. Effect of the aqueous pod extract of *A. nilotica* on mean[b] on some biochemical parameters.

Parameter	Control	50 mg/kg	100 mg/kg	200 mg/kg	400 mg/kg
Albumin (mmol/L)	35.6±1.10	27.6±4.40*	31.0±1.60*	30.0±1.80*	30.0±1.20*
T. Protein (mmol/L)	62.0±3.40	49.0±5.0*	50.0±0.8*	50.0±0.8*	52.0±3.8*
T.Bilirubin (μmol/L)	4.60±1.50	3.80±0.840	4.60±1.10	4.40±1.10	4.20±0.840
Cholesterol (mmol/L)	2.50±0.28	2.20±0.230	2.23±0.10	2.20±0.44	1.50±0.40*
Urea (mmol/L)	5.14±0.16	6.54±1.00	6.36±1.00	5.70±0.54	5.24±1.90
Creatinine (μmol/L)	37.8±4.50	58.2±3.70*	53.2±6.10*	54.4±3.40*	56.6±3.60*
Glucose (mmol/L)	8.28±0.42	6.90±0.99*	6.46±0.62*	6.16±0.43*	6.28±0.94*
Triglyceride (mmol/L)	2.10±0.16	1.30±0.58*	1.04±0.09*	1.18±0.27*	0.88±0.23*

b = Mean±Standard deviation based on five observations. *P<0.05 means significantly different from the control.

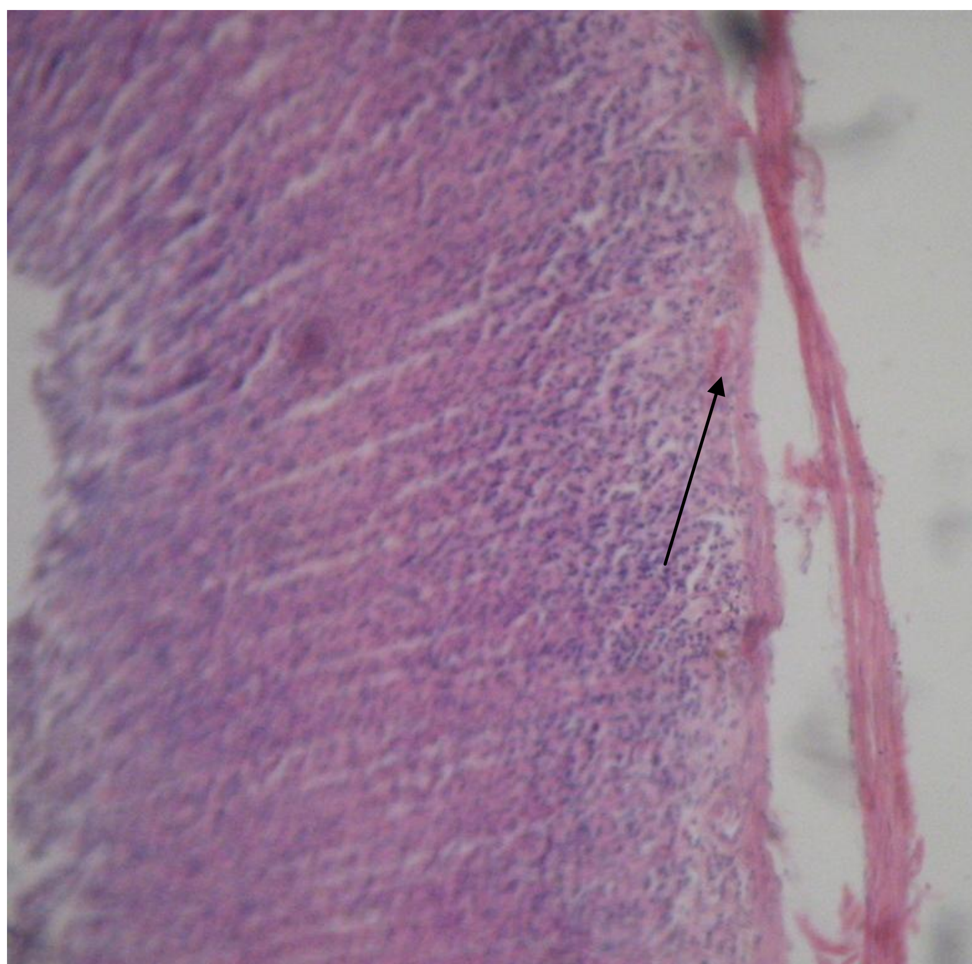

Figure 1. Stomach of rat treated with aqueous pod extract of *A. nilotica*at 400 mg/kg showing mild congestion (arrow). H and E×200.

necrosis (Figure 3), with mononuclear cell infiltrations. The lesions observed in the kidney were more severe in the groups treated with higher doses of the extract. There was marked interstitial mononuclear cell infiltration and haemorrhages of the lungs in the rats treated with 200 mg/kg dose, while those treated with the highest doses of the extract also had pulmonary oedema (Figure 4) and congestion of the lungs. In the liver there was hepatocellular

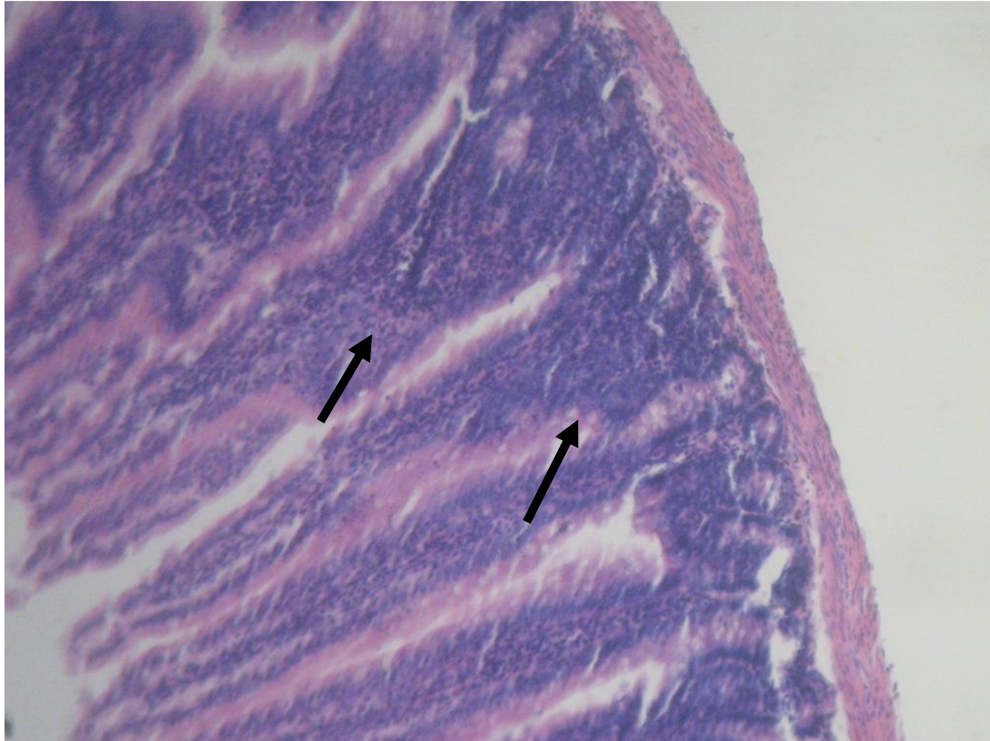

Figure 2. Small intestine of rat treated with aqueous pod extract of *A. nilotica* at 400 mg/kg showing sub mucosal mononuclear cell infiltrations (arrows).H and E×100.

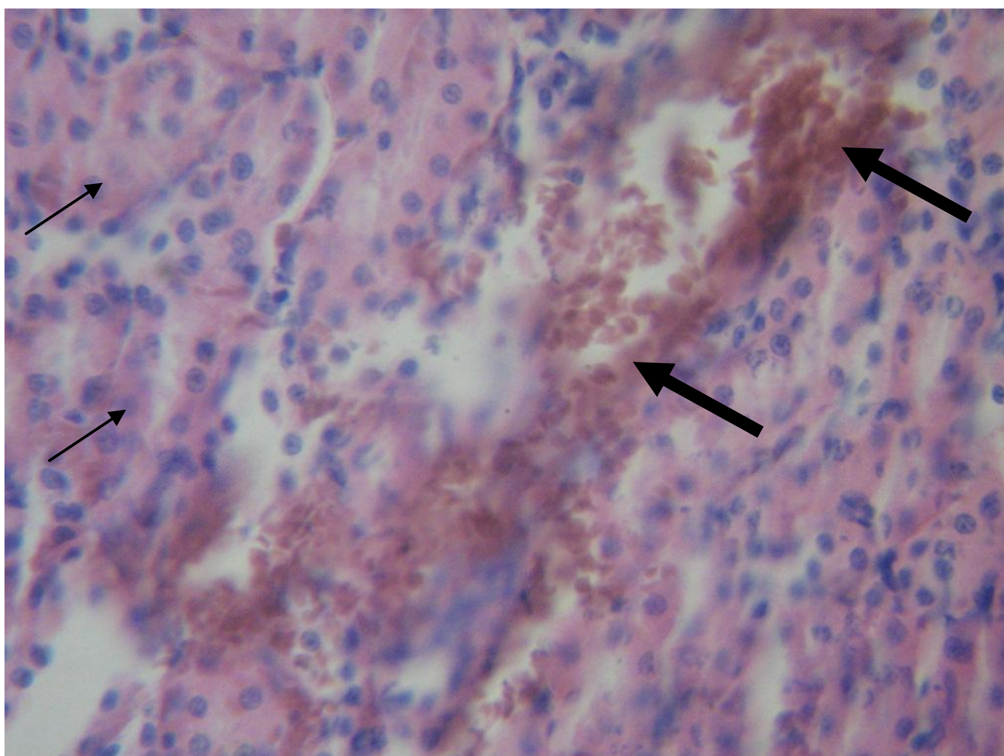

Figure 3. Kidney of rat treated with 200 mg/kg of the aqueous pod extract of *A. nilotica* showing haemorrhage (thick arrows) and Extensive renal tubular degeneration and necrosis (thin arrows). H and E ×400.

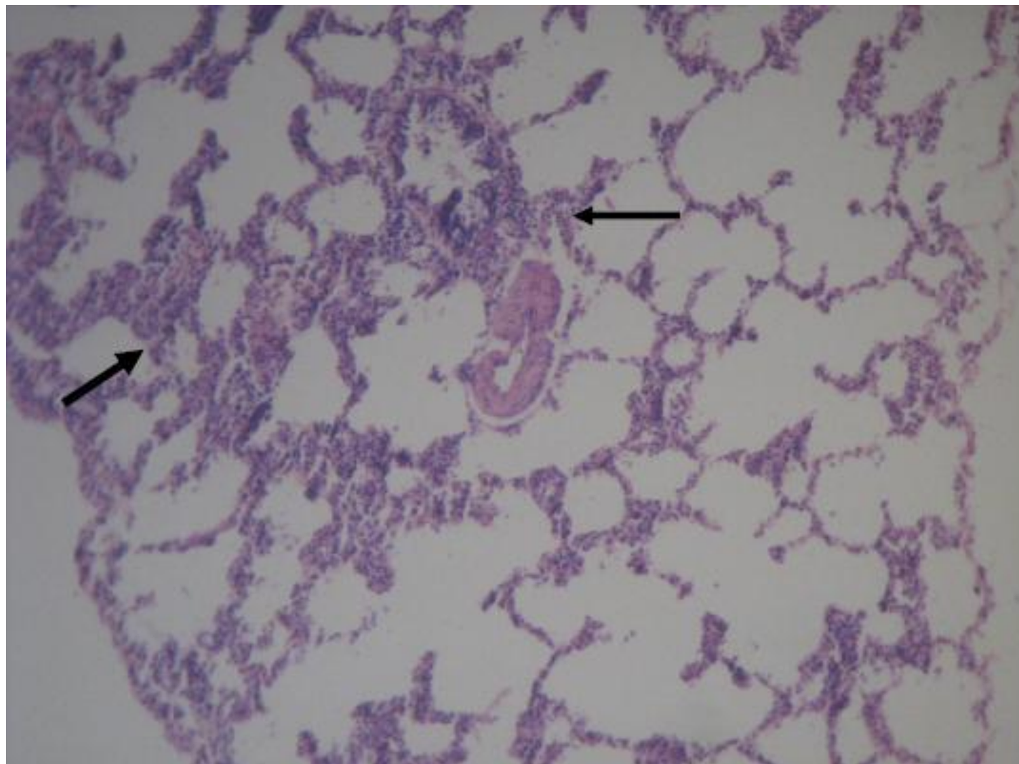

Figure 4. Lungs of rat treated at 200 mg/kg with aqueous pod extract of *A. nilotica* showing marked interstitial mononuclear infiltration (arrows). H and E ×100.

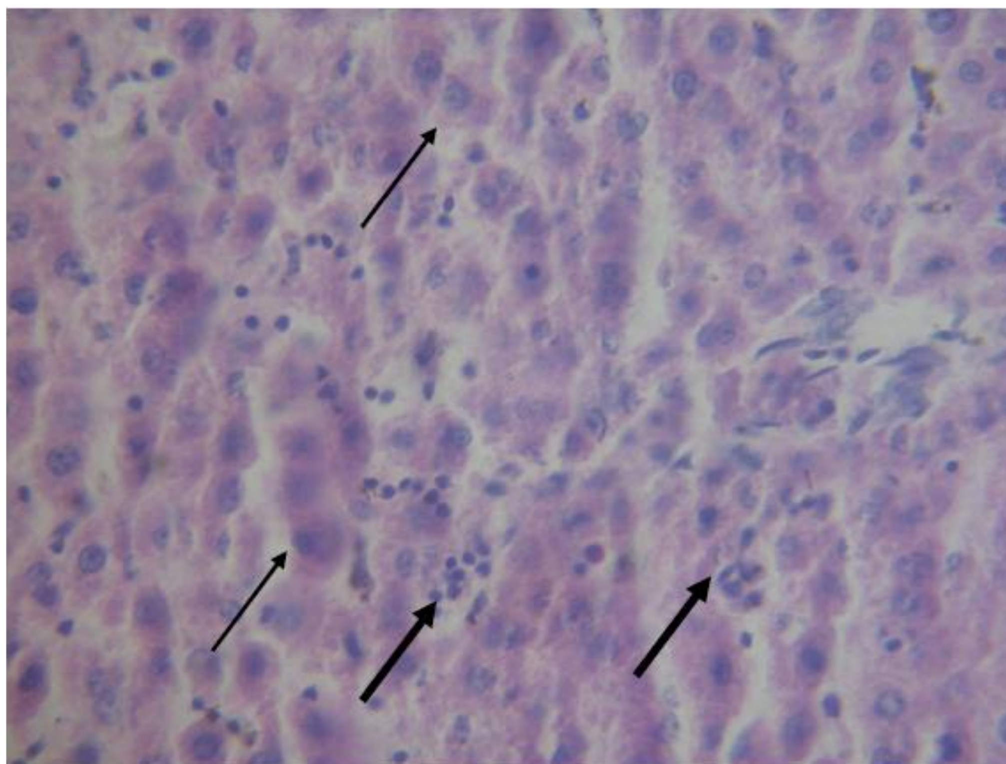

Figure 5. The liver of rat treated with aqueous pod extract of *A. nilotica*At 200 mg/kg, showing mononuclear cell infiltration (thick arrows) and hepatocellular necrosis (thin arrows) H and E × 400.

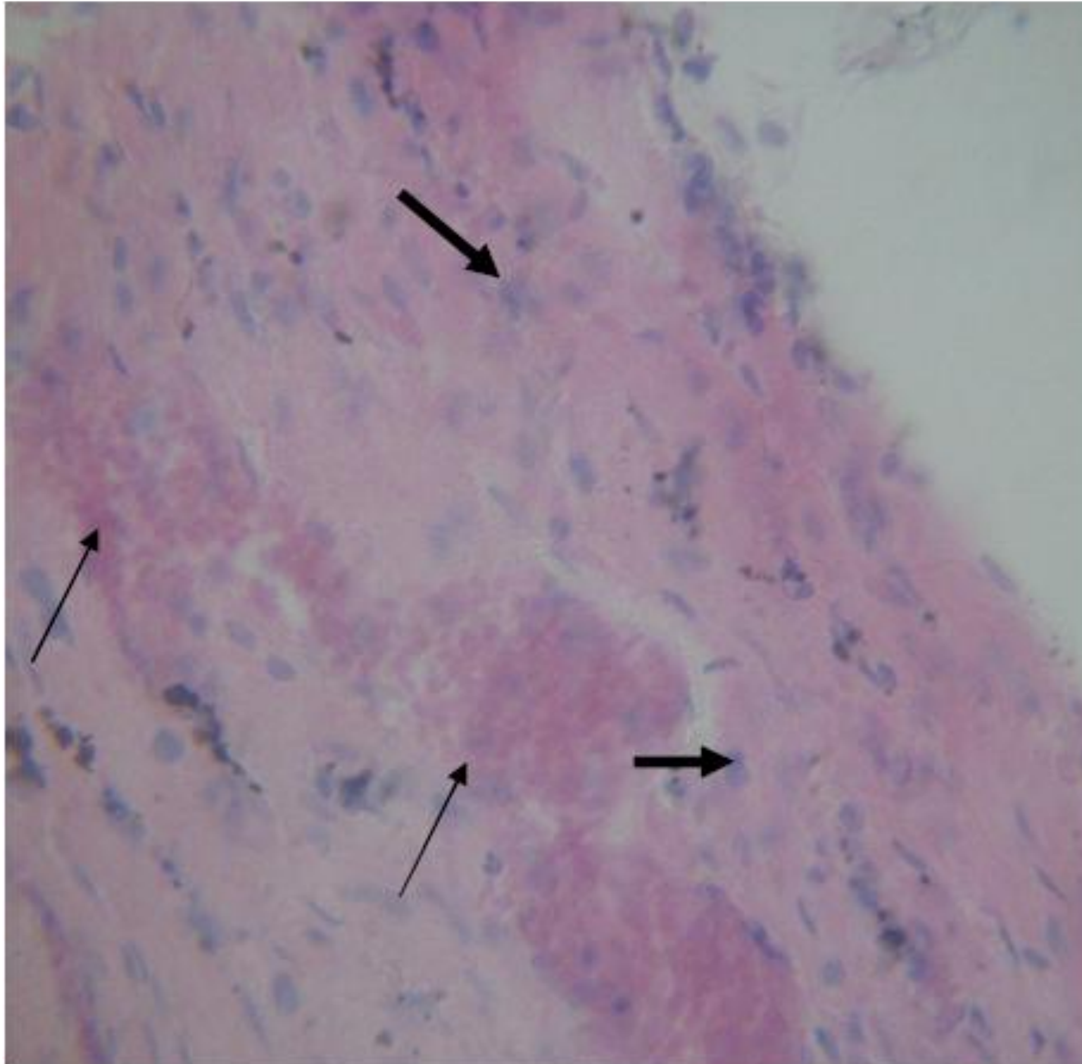

Figure 6. The heart of the rats treated with aqueous pod extract of *A. nilotica* at 400 mg/kg, showing haemorrhages (thin arrows), myocardial necrosis (thick arrows). H and E ×200.

necrosis and diffused mononuclear cell infiltrations (Figure 5). The observed lesions appear to be dose dependent. The heart showed intramuscular haemorrhages, mononuclear cell infiltrations, myocardial degeneration and necrosis (Figure 6) in the extract treated rats. The severity of the lesion observed varied with the administered dose. The lesions were very severe in animals treated with the highest dose (400 mg/kg) of the extract.

DISCUSSION

Administration of *A. nilotica* pod extract to rats for 21 days resulted in increased AST and ALT values of treated animals. This may be indicative of liver damage (Schalm et al., 1975; Teizt, 1994). The extract also significantly ($P<0.05$) decreased levels of serum total protein and albumin, which further strengthens the suggestion of liver damage (Jubb et al., 1995).The increase in the value of ALT may also be suggestive of the involvement of other organ and tissues like the heart in the degeneration process (Teizt, 1994). The ALP in this study was not elevated, which may be an indication of non involvement of the bone tissue. The decrease in urea levels and significant increases in creatinine values in this study may suggest that the kidney was adversely affected. Creatinine is produce in the body in proportion to the muscle mass, and its high level indicates renal damage or failure of the kidney to excrete creatinine owing to renal failure (Teizt, 1994). The administration of the extract significantly reduced the cholesterol and triglyceride values of the blood. This could be an indication that the extract affected the cardiovascular

system. The extract decreased the blood sugar levels significantly (P<0.05). It also induced anorexia in the treated rats. The decrease in blood glucose and triglyceride values following extract administration is an interesting finding which may suggest that this extract could be utilized in treating atherosclerotic and diabetic problems (Taiwo et al., 2005).

The histopathological examination of tissues showed presence of dose dependent severe lesions in the liver, kidney, heart and lungs. The lesion found in the stomach and intestines were milder. The presence of lesions in all these organs and the tissues may be an indication that the active /toxic principle presence in the extract once absorbed was extensively distributed throughout the body (Buxton, 2005). The presence of the lesions in the liver and the kidney may not be unexpected since the liver is the primary organ of biotransformation and the kidney is the main organ of excretion (Baggot, 1977). The extract is known to contain some chemical compounds that are capable of exerting pronounced physiological/ pharmacological effects including alkaloids, tannins, saponins and glycosides. It was concluded that the prolonged administration of the aqueous extract of *A. nilotica* for medicinal purposes should be discourage since it has potential to produce some level of toxicities.

REFERENCES

Baggot JD (1977). Principles of Drugs Dispositions in the Domestics Animals. The Basis of Veterinary Clinical Pharmacology. WB Sunders Company. Philadelphia, pp. 40-110.

Buxton ILO (2005). Pharmacokinetics and Pharmacodynamics: The dynamics of drug absorption, distribution, action and elimination. In: The Pharmacological Basics of Therap. 11th ed. MacGraw- Hill medical publishing Division New York, Pp. 1-40.

Deutsche GCF, Kelinshe Z (1972). A study of optimum buffer conditions for traditional medicine 2017 SC Hamthome Portland or 97214 Monograph, p. 80.

Drury RAB, Willington EA (1979). Carlethon Histopathological Techniques; 4th ed. Oxford University Press London, pp. 21-70.

Jubb KVF, Kennedy PC, Palmer N (1995). Pathology of Domestic Animals. 3rd Edition, Academic Press Inc. New York.

Mead R, Curnow RN (1982). A simple Experiment in Statistical Method in Agriculture Biology. Chapman Hall, London, pp. 33-46.

Pande MB, Talpada PM, Pebel JS, Shukla PC (1981). Note on the nutritive value of babul (Acacia nilotica Linn) Seeds (extracted) In: Indian J. Anim. Sci., 51(1): 107-108.

Reitman S, Frankel S (1957). A method of assaying liver enzymes in human serum. Am. J. Clin Pathol., 28: 56-58.

Schalm OW, Jain NC, Carrol EJ (1975). Veterinary Haematology, 3rd edition. Lae and Febiger Philadelphia, pp. 340-470.

Taiwo VO, Olukunle OA, Ozor IC, Oyejobi AT (2005). Consumption of Aqueous Extract of Raw Aloe Vera leaves: Histopatholgical and Biochemical Studies in Rats and Tilapia. Afr. J. Biomed. Res., 8: 169-178.

Teizt NW (1994). Fundamentals of Clinical Chemistry with Clinical Correlation, 1st ed. Baellaire Tindal, Ltd., London.

Wambebe C (1998). Development and Production of Standardized Phytomedicines. West Afr. J. Parm., 12(2): 13-24.

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Full Length Research Paper

Kenaf seed oil from supercritical carbon dioxide fluid extraction inhibits the proliferation of WEHI-3B leukemia cells *in vivo*

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Hibiscus cannabinus **(kenaf) seed oil is a rich source of bioactive phytochemicals with high anti-oxidative and cancer chemopreventive properties. However, the seeds are disposed as waste material during the harvesting or processing of kenaf. Preliminary study revealed that kenaf seed oil from supercritical carbon dioxide fluid extraction (SFE) induced apoptosis in WEHI-3B leukemia cells. Thus, this study was carried out to investigate the effects of kenaf seed oil from SFE on WEHI-3B cells *in vivo*. Acute toxicity study revealed that kenaf seed oil is practically non-toxic by oral route. Treatment with kenaf seed oil increased the population of T cells, but decreased the populations of immature monocytes and granulocytes in the peripheral blood of WEHI-3B/BALB/c mice. The weights of the spleen and liver of WEHI-3B/BALB/c mice decreased after the treatment with kenaf seed oil. Moreover, infiltration of leukemic cells into the splenic red pulp reduced after the treatment. In conclusion, kenaf seed oil reduced the severity of leukemia in WEHI-3B/BALB/c mice. These results provide information to industrialists and farmers to fully utilize and develop kenaf seed oil as a novel bio-health product.**

Key words: *Hibiscus cannabinus* (kenaf), oil, supercritical carbon dioxide fluid extraction, WEHI-3B; BALB/c mice, leukemia.

INTRODUCTION

Leukemia is a malignant hematopoietic disorder characterized by an abnormal increase of immature white blood cells. As of 2010, leukemia is diagnosed 10 times more often in adults than in children, and more common in male than female in the US (American Cancer Society, 2010). Even though there are a few means for treatment of the disease such as chemotherapy, blood transfusion, radiotherapy and bone marrow transplantation. None is yet satisfactory as the side effects especially generated from the chemotherapy are difficult to handle (Chiang et al., 2004). Thus, there is a need to seek for alternatives for management of leukemia.

Natural products play an important role in the current cancer treatment with substantial numbers of anticancer agents used in the clinic being either natural or derived from natural products such as plants (Ghavami et al., 2010; Jing et al., 2010; Moon, 2010; Nobili et al., 2009). The advantage of using plant-derived anticancer agents is that the produced adverse effects are lesser as

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compared to the synthetic drugs (Kinghorn et al., 2003). *Hibiscus cannabinus* (kenaf) is a natural product and valuable traditional medicinal plant that has often been used to treat bilious conditions, bruises and fever in India and Africa (Agbor et al., 2005). Kenaf contains large amounts of bioactive components such as tannins, saponins, polyphenolics, alkaloids, fatty acids, phospholipids, tocopherol and phytosterols (Mohamed et al., 1995). Kenaf seed oil, which has been characterized in our lab recently, is a unique and rich source of bioactive compounds with high anti-oxidative properties (Chan and Ismail, 2009; Mariod et al., 2011; Nyam et al., 2009). However, the seeds are disposed as waste material during the harvesting or processing of kenaf.

Recently, the ethanolic extract of kenaf leaves is reported to have potential immunomodulatory effect (Lee et al., 2007). In addition, kenaf seed oil has been reported to prevent the chemical carcinogen-induced colon cancer in rats (Ghafar et al., 2010) as well as induce apoptosis in the ovarian cancer cells (Yazan et al., 2011). Preliminary study (unpublished data) showed that kenaf seed oil induced apoptosis in murine myelomonocytic leukemia WEHI-3B, human leukemia HL-60 and K562 cells therefore raising the possibility that kenaf seed oil could affect WEHI-3B leukemia cells *in vivo.* The WEHI-3B cell line was originally derived from the BALB/c mice (Warner et al., 1969). It is a good animal model that has been extensively used to induce leukemia in BALB/c mice for evaluation of anti-leukemia effects of drugs and herbal plants (He and Na, 2001; Lin et al., 2009; Mohan et al., 2010; Tsou et al., 2009; Wen et al., 2010; Yu et al., 2010).

Supercritical fluid extraction (SFE) is a process to extract bioactive components by using supercritical fluid as the solvent. A supercritical fluid is any substance at a temperature and pressure above its thermodynamic critical point. The dissolving power of a supercritical fluid is adjustable by changing the pressure or/and temperature.

It has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent (Wang and Weller, 2006). Carbon dioxide is often selected as the supercritical fluid as this gas is non-toxic, non-explosive, environmental friendly and easily to be removed from the product at the end of the extraction (Pourmortazavi and Hajimirsadeghi, 2007). This ensures the safe consumption of the product. In this study, the effects of kenaf seed oil extracted by SFE on WEHI-3B leukemia cells *in vivo* were investigated.

MATERIALS AND METHODS

Materials and reagents

Kenaf seeds variety V36 were purchased from the National Tobacco Board, Pasir Putih, Kelantan, Malaysia. RPMI-1640 with L-glutamine, fetal bovine serum and penicillin–streptomycin were purchased from PAA, Austria.

Animal management

Male BALB/c mice (20 to 28 g) at 8 weeks of age were housed in a well ventilated room with a 12/12 h light/dark cycle at the ambient temperature of 25 to 30°C. The mice were acclimatized for 1 week. Food and water were given *ad libitum.* The use of animals and the experimental protocol were approved by the Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, University Putra Malaysia (ACUC no: UPM/FPSK/PADS/BR-UUH/00350).

Cell culture

The murine myelomonocytic leukemia WEHI-3B cells from the American Type Culture Collection (ATCC, USA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 5% carbon dioxide at 37°C.

Kenaf seeds oil extraction

Kenaf seeds were extracted by using supercritical carbon dioxide extractor (Thar 1000 F, USA) at 600 bars 40°C as reported previously (Yazan et al., 2011).

Preparation of kenaf seed oil emulsion

Kenaf seed oil was slowly added with gentle stirring to distilled water and tween 80 (Sigma, USA) as an emulsifier. Emulsion was prepared at room temperature using homogenizer (Ultra-turax T25 basic IKA®-WERKE, Germany) at 13,000 rpm for 3 to 5 min.

Acute toxicity study

Mice were divided into 6 groups (3 mice per group). The mice were administered orally through gavage with the freshly prepared emulsion (10 ml/kg). The first group (control group) received tween 80 in distilled water (vehicle). Group 2 to 6 were administered orally with kenaf seed oil emulsion at the dose of 1.0, 2.0, 3.0, 4.0 and 5.0 g/kg, respectively. Body weight of the mice was recorded daily for 14 days following single oral dose of kenaf seed oil emulsion. The percentage of weight loss (relative to the initial starting weight) was calculated and a loss of greater than 15% is considered toxic (Mohan et al., 2010; Phillips et al., 2004).

Kenaf seed oil treatment

Sixty BALB/c mice were divided into 6 groups (10 animals per group). Group 1 was the control (animals without leukemia, treated with distilled water only). The remaining groups of mice were injected intraperitoneally with WEHI-3B cells at 1×10^6 cells in 100 µl of PBS on day 0. Group 2 was treated with distilled water only. Group 3, 4 and 5 were treated with kenaf seed oil emulsion at 0.5, 1.0 and 1.5 g/kg, respectively. Group 6 was treated with tween 80 in distilled water (vehicle). The treatment was administered orally to the mice daily through gavage at day 14th after WEHI-3B cells injection for 2 weeks (Wen et al., 2010).

Immunofluorescence staining of blood cells

Whole blood was collected (approximately 1 ml) from each animal at the end of the experiment. The blood samples were incubated

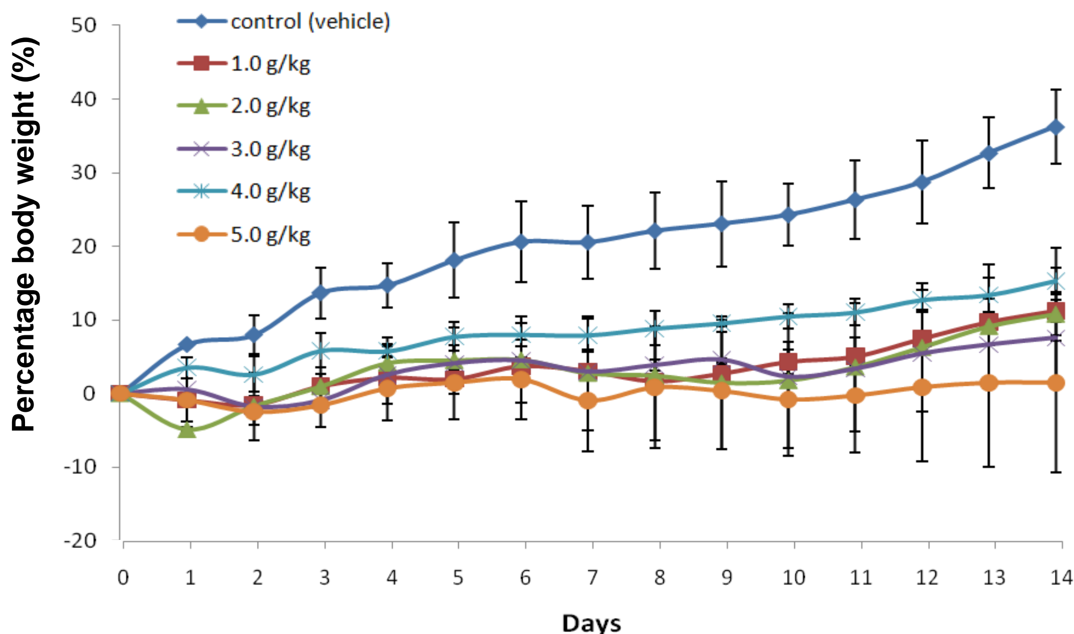

Figure 1. Changes in body weight of BALB/c mice treated with single dose of kenaf seed oil emulsion (n=3). Weight loss of greater than 15% was not observed in all treated groups.*, Significantly different ($p<0.05$).

with anti-CD16 for 5 min at 4°C and examined for cell surface markers of T cells (CD3), B cells (CD19) and monocytes as well as granulocytes (CD11b) by staining with anti-CD3-FITC, CD19-PE and CD11b-PE antibodies (Beckman Coulter, USA) for 20 min at 4°C, respectively. Following staining, the cells were treated immediately with Versalyse Lysing Solution (Beckman Coulter, USA) for 10 min at room temperature to lyse the red blood cells, followed by centrifugation for 5 min at 150 x g at 4°C. After removal of the supernatant, the isolated white blood cells were washed with 3 ml of cold PBS and fixed with 0.1% formaldehyde of 10X concentrated IOTest3 Fixative Solution (Beckman Coulter, USA). The cell surface marker level of isolated white blood cells was analyzed by flow cytometry (CyAn ADP, USA).

Organ samples (liver and spleen)

The livers and spleens were isolated, weighed and photographed.

Histopathological analysis

Spleen tissues were flushed with PBS, fixed with $RCL_2^{®}$ and embedded in paraffin. Sections of 5 µm were stained with hematoxylin and eosin according to the standard procedures (Ghafar et al., 2010).

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) version 16.0. Results were analyzed by one-way analysis of variance (ANOVA). Data were expressed as mean ± standard deviation (mean ± SD). A difference was considered to be significant at $p<0.05$.

RESULTS

Yield of kenaf seed SFE extraction

Kenaf seed yielded 15 to 19% of oil after 150 min extraction.

Acute toxicity of kenaf seed oil in BALB/c mice

Administration of kenaf seed oil emulsion did not cause any mortality at all the doses. It was found to be safe even at the highest concentration (5.0 g/kg) whereby no weight loss is greater than 15% (relative to initial starting weight) was observed in all the treated groups (Figure 1). Therefore, the LD_{50} could not be estimated, and it is possible for more than 5.0 g/kg.

Kenaf seed oil affects the cell surface markers of WEHI-3B/BALB/c mice

The population of T cells (Figure 2A), monocytes (Figure 2C) and granulocytes (Figure 2D) increased and decreased ($p<0.05$), respectively, after the treatment with kenaf seed oil emulsion at 1.0 and 1.5 g/kg. There is no significant changes ($p>0.05$) of the B cell population in the peripheral blood of kenaf seed oil treated WEHI-3B/

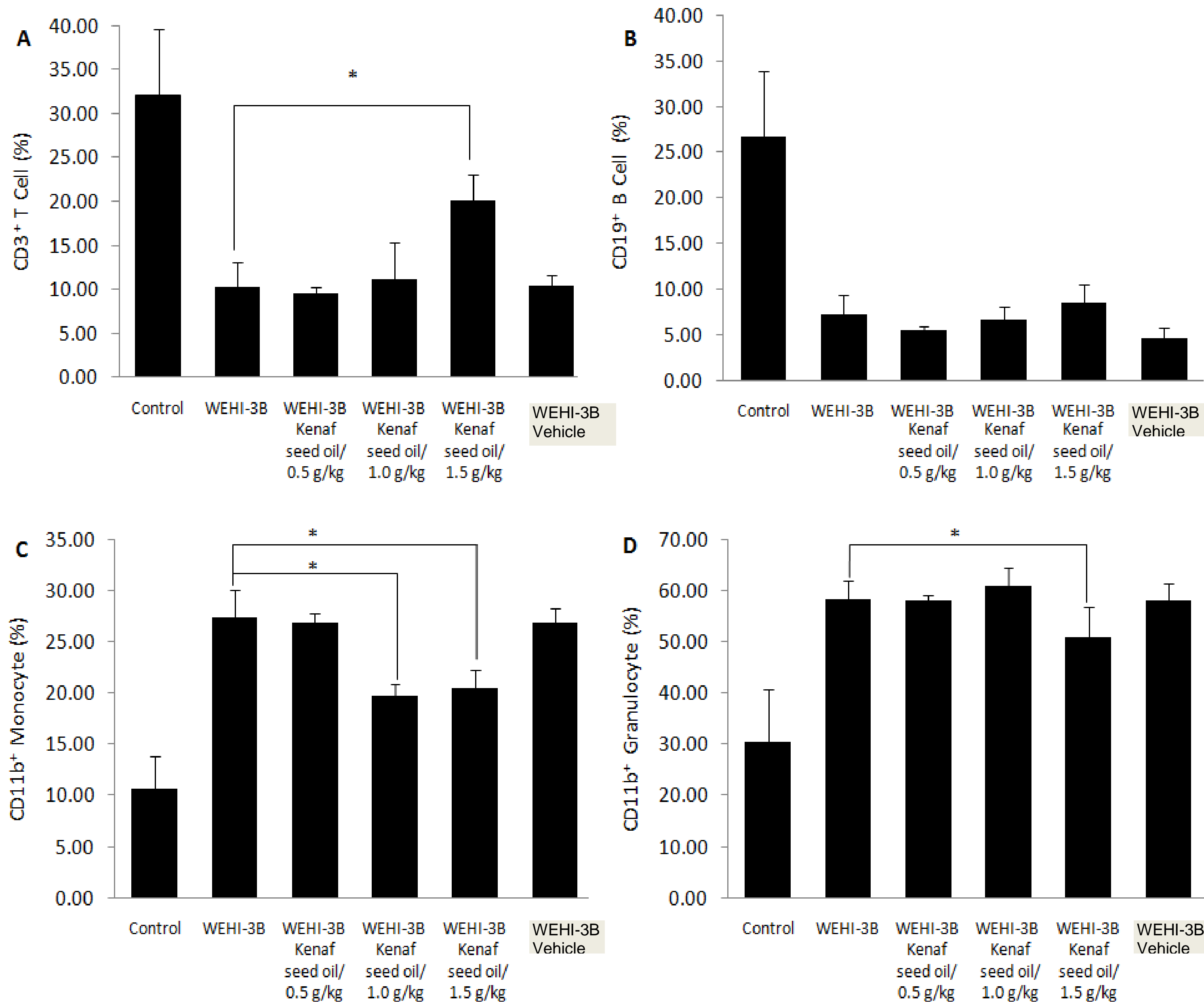

Figure 2. Percentage of white blood cell surface markers of T cell (A), B cell (B), monocyte (C) and granulocyte (D) (n=4). *, Significantly different ($p<0.05$).

BALB/c mice (Figure 2B).

Kenaf seed oil reduced the weight of liver and spleen of WEHI-3B/BALB/c mice

Figure 3A shows a reduction in size of the liver and spleen of WEHI-3B/BALB/c mice treated with kenaf seed oil. The weight of liver (Figure 3B) and spleen (Figure 3C) of WEHI-3B/BALB/c leukemic mice reduced significantly ($p<0.05$) after the treatment with kenaf seed oil emulsion at 1.0 and 1.5 g/kg. The reduction was in a dose-dependent manner.

Kenaf seed oil reduced the infiltration of leukemic cells into the splenic red pulp

The examined spleen tissues demonstrated a pattern ranging from minimal histopathological changes to scanty small neoplastic cell nests present in the sinusoid (Figure 4B) as compared to the control (Figure 4A). The infiltration of immature myeloblastic cells into the splenic red pulp of WEHI-3B/BALB/c leukemic mice reduced in the

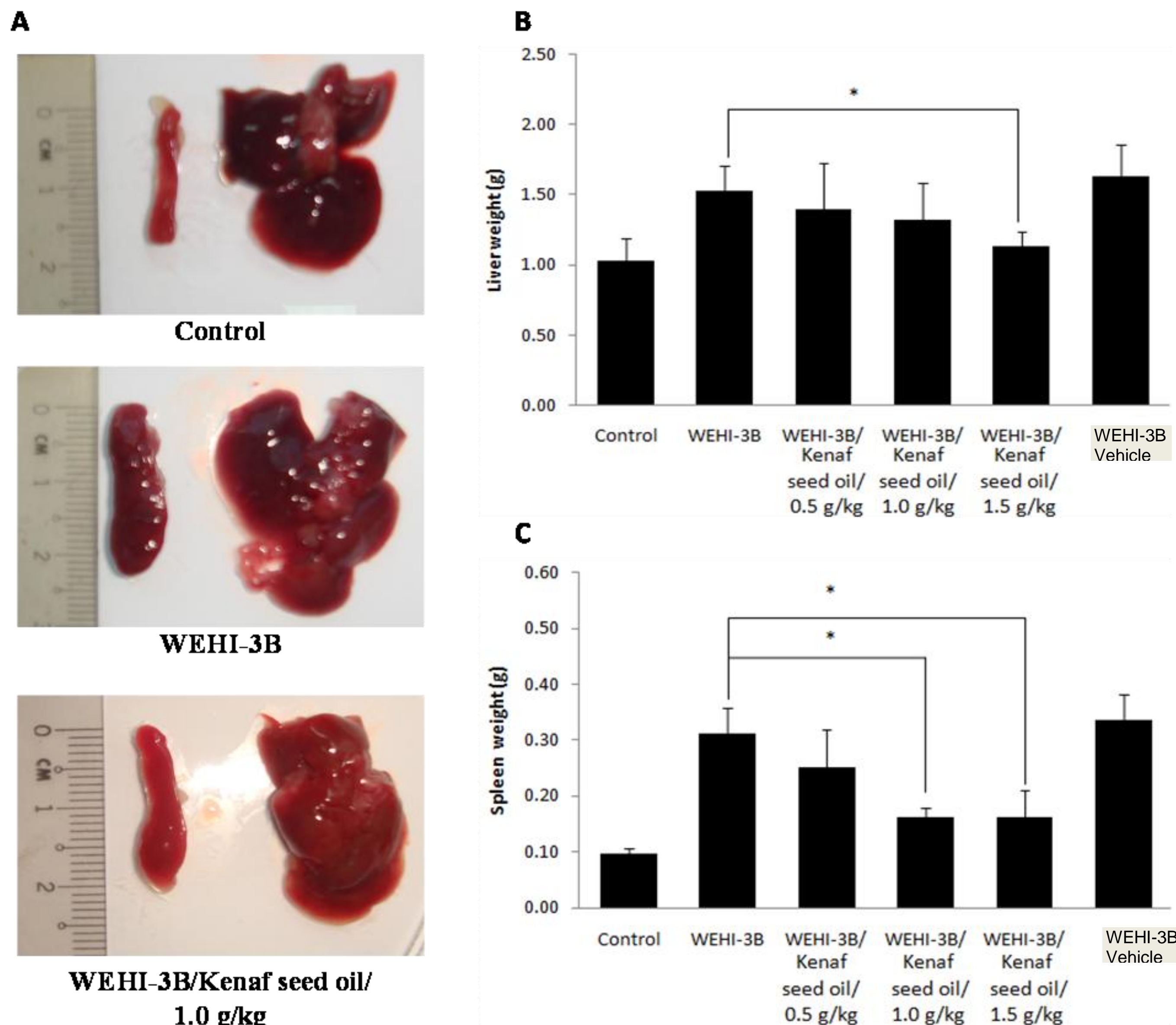

Figure 3. Liver and spleen tissues (A), liver weight (B) and spleen weight (C) of WEHI-3B/BALB/c leukemic mice treated with or without kenaf seed oil for 2 weeks (n=10). *, Significantly different ($p<0.05$).

treatment at 1.0 g/kg (Figure 4C). The neoplastic cells in the examined spleen tissues presented large irregular nuclei with clumped chromatin, prominent nucleoli and abundant clear and light eosinophilic cytoplasm.

DISCUSSION

There are many efforts to find a suitable extraction method with the highest oil yield and yet taking into consideration the safety of the oil. SFE is claimed as the non-toxic, non-explosive and environmental friendly extraction technique to extract the bioactive components from natural products including kenaf seed (Chan and Ismail, 2009). In any extraction method technique, the yield is an important criterion for commercialization of the product of interest. High kenaf seed oil yield from this study (19%) after 150 min of extraction suggested that kenaf seed oil might be a profitable product to be commercialized.

Acute toxicity study revealed that administration with kenaf seed oil did not produce any death or clinical signs of toxicity in mice even at the highest dose (5.0 g/kg) during 14 days of observation, suggesting a LD_{50} above 5.0 g/kg by oral route (Figure 1). This finding was consistent with other studies (Costa-Silva et al., 2008;

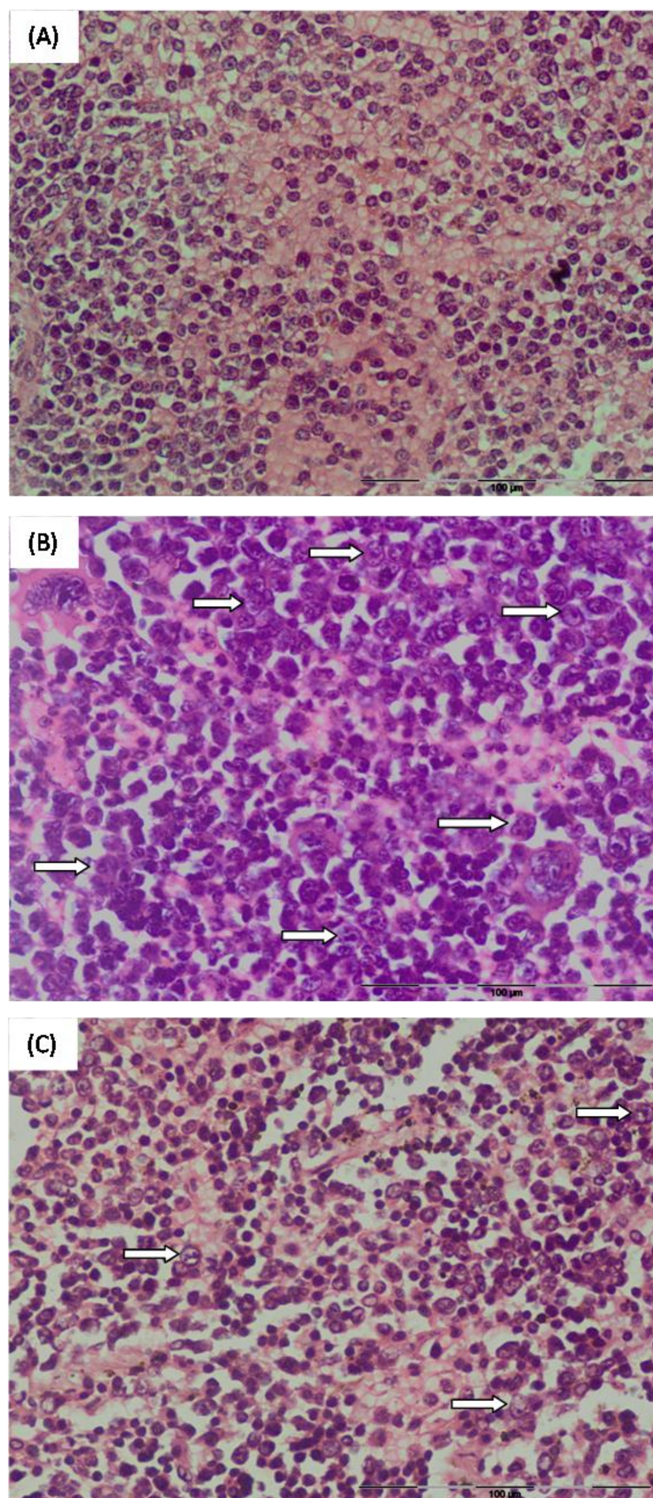

Figure 4. Histopathological examination of the spleen from the control BALB/c mice not injected with WEHI-3B (A), injected with WEHI-3B but not treated (B) and treated with kenaf seed oil at 1.0 g/kg (C). White arrows represent neoplastic cells (400X).

Krishna et al., 2009). It is suggested that kenaf seed oil is practically non-toxic by oral route (Kennedy et al., 1986). Recently, kenaf seed oil has been reported to prevent the formation of aberrant crypt foci, the precursor for colon cancer at the dosages of 0.5, 1.0 and 1.5 g/kg (Ghafar et al., 2010). Therefore 0.5 (one tenth) and 1.5 g/kg (three tenth) were selected as the minimum and maximum dosages for our actual *in vivo* experiment, respectively.

An increase in the percentage of peripheral monocytes (Figure 2C) and granulocytes (Figure 2D) were observed in the untreated WEHI-3B/BALB/c mice as compared to the control. However, both populations of the cells decreased after treatment with kenaf seed oil. The reduction might be due to the induction of apoptosis or differentiation of immature cells into the mature one (He and Na, 2001; Mohan et al., 2010). CD11b antigen has a wide tissue distribution such as macrophages, bone marrow, spleen, natural killer cells and blood monocytes and granulocytes. Thus it is a good marker to measure the percentage of blood monocytes and granulocyte in our study (Ho and Springer, 1982). The elevation of these two populations as detected by flow cytometry in the WEHI-3B/BALB/c mice as compared to the control is actually the immature one (He and Na, 2001; Warner et al., 1969). Moreover, the population of T cells significantly increased ($p<0.05$) after treatment with kenaf seed oil (Figure 2A). However, there is no significant changes ($p>0.05$) of the B cell population in the peripheral blood of kenaf seed oil treated WEHI-3B/BALB/c mice (Figure 2B). It has been found that cytotoxic T cells destroy virally infected cells as well as tumor cells (Xue et al., 2005). It is speculated that cytotoxic T cells were activated in this study to eliminate the leukemic cells.

The average weight of liver (Figure 3B) and spleen (Figure 3C) of WEHI-3B/BALB/c mice decreased ($p<0.05$) after the treatment with kenaf seed oil emulsion. The enlarged size of liver and spleen was observed in WEHI-3B/BALB/c mice (Figure 3A). The WEHI-3B leukemia animal model is characterized by elevated peripheral monocytes and granulocytes with immature morphology and by apparently enlarged and infiltrated spleens as compared to the normal mice (He and Na, 2001; Wen et al., 2010; Yu et al., 2010). From the histopathological examination on spleen, it is confirmed that the treatment with kenaf seed oil reduced the infiltration of immature myeloblastic cells into the splenic red pulp (Figure 4C) as compared to the untreated WEHI-3B/BALB/c mice (Figure 4B). Spleen plays an important role in the removal of blood-borne microorganisms and cellular debris as well as old red blood cells from the circulation. Red pulp of the spleen is made up of several types of blood cells, including granulocytes and red blood cells (Mebius and Kraal, 2005). Recently, it has been found that red pulp is the reservoir for half of the body's monocytes (Swirski et al., 2009). This explains the enlargement of the spleen tissue of WEHI-3B/BALB/c mice.

Phytosterols and linoleic acid are speculated to be

responsible for the anti-leukemia properties in our study. Kenaf seed oil contains various active components such as fatty acid, phytosterols, tocopherols and other antioxidants (Mariod et al., 2011; Mohamed et al., 1995; Nyam et al., 2009). Phytosterols are the bioactive components in kenaf seed SFE extract. They have structure similar to cholesterol but with some modify-cations on the side chain and include the addition of double bond and/or methylorethyl group (Awad et al., 2007). The most common dietary phytosterols are β-sitosterol, campesterol and stigmasterol. Kenaf seed oil contains high amount of phytosterols with β-Sitosterol (70%) are the most abundant phytosterols in the oil (Mariod et al., 2011; Mohamed et al., 1995; Nyam et al., 2009). Pharmacological studies revealed that phyto-sterols showed growth inhibitory and apoptosis effects on breast (Awad et al., 2007), leukemia (Moon et al., 2008; Park et al., 2007), lung (Schabath et al., 2005), ovarian (McCann et al., 2003), stomach (De-Stefani et al., 2000) and prostate cancer (McCann et al., 2005). Meanwhile, another bioactive component in kenaf SFE oil is α-linoleic acid, an essential ω-3 fatty acid that is metabolized to eicosapentaenoic acid, a precursor of eicosanoids with anti-inflammatory (Ruiz et al., 2002). α-Linoleic acid has been reported to inhibit the proliferation of MOLT-4 leukemia *in vitro* (Phoon et al., 2001).

In conclusion, kenaf seed edible oil reduced the severity of leukemia in WEHI-3B cells *in vivo*. These results provide information to industrialists and farmers to fully utilize and develop kenaf seed oil as a novel bio-health product.

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REFERENCES

Agbor GA, Oben JE, Ngogang JY (2005). Haematinic activity of *Hibiscus cannabinus*. Afr. J. Biotechnol., 4(8): 833-837.

American Cancer Society (2010). *Cancer Facts and Figures 2010*. Atlanta: American Cancer Society. www.cancer.org (accessed November 30, 2010).

Awad AB, Chinnam M, Fink CS, Bradford PG (2007). *β*-Sitosterol activates Fas signaling in human breast cancer cells. Phytomedicine, 14: 747-754.

Chan KW, Ismail M (2009). Supercritical carbon dioxide fluid extraction of *Hibiscus cannabinus* L. seed oil: A potential solvent-free and high antioxidative edible oil. Food Chem., 114: 970-975.

Chiang LC, Cheng HY, Liu MC, Chiang W, Lin CC (2004). *In vitro* evaluation of antileukemic activity of 17 commonly used fruits and vegetables in Taiwan. LWT-Food Sci. Technol., 37: 539-544.

Costa SJH, Lima CR, Silva EJR, Araújo AV, Fraga MCCA, Ribeiroe RA, Arruda AC, Lafayette SSL, Wanderley AG (2008). Acute and subacute toxicity of the *Carapa guianensis* Aublet (Meliaceae) seed oil. J. Ethnopharmacol., 116: 495-500.

De-Stefani, Boffetta P, Ronco AL, Brennan P, Deneo PH, Carzoglio JC, Mendilaharsu M (2000). Plant Sterols and Risk of Stomach Cancer: A Case-Control Study in Uruguay. Nutr. Cancer, 37: 140-144.

Ghafar SAA, Yazan LS, Tahir PM, Ismail M (2010). Kenaf seed supercritical fluid extract reduces aberrant crypt foci formation in azoxymethane-induced rats. Exp. Toxic. Pathol., doi:10.1016/j.etp.2010.08.016.

Ghavami G, Sardari S, Shokrgozar MA (2010). Anticancerous potentials of *Achillea* species against selected cell lines. J. Med. Plant Res., 4(22): 2411-2417.

He Q, Na X (2001). The effects and mechanisms of a novel 2-aminosteroid on murine WEHI-3B leukemia cells *in vitro* and *in vivo*. Leuk. Res., 25: 455-461.

Ho M, Springer T (1982). Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen. J. Immunol., 128: 2281.

Jing LJ, Mohamed M, Rahmat A, Bakar MFA (2010). Phytochemicals, antioxidant properties and anticancer investigations of the different parts of several gingers species (*Boesenbergia rotunda*, *Boesenbergia pulchella* var *attenuata* and *Boesenbergia armeniaca*). J. Med. Plant Res., 4(1): 27-32.

Kennedy GL, Ferenz RL, Burgess BA (1986). Estimation of acute oral toxicity in rats by determination of the approximate lethal dose rather than the LD_{50}. J. Appl. Toxicol., 6: 145-148.

Kinghorn AD, Farnsworth NR, Soejarto DD, Cordell GA, Swanson SM, Pezzuto JM, Wani MC, Wall ME, Oberlies NH, Kroll DJ, Krame RA, Rose WC, Vite GD, Fairchild CR, Peterson RW, Wild R (2003). Novel strategies for the discovery of plant-derived anticancer agents. Pharm. Biol., 43: 53-67.

Krishna KL, Mruthunjaya K, Patel JA (2009). Antioxidant and Hepatoprotective Activity of Leaf Extract of *Justicia gendarussa* Burm. Int. J. Biol. Chem., 3: 99-110.

Lee YG, Byeon SE, Kim JY, Lee JY, Rhee MH, Hong S, Wu JC, Lee HS, Kim MJ, Cho DH, Cho JY (2007). Immunomodulatory effect of *Hibiscus cannabinus* extract on macrophage functions. J. Ethnopharmacol., 113: 62-71.

Lin JP, Yang JS, Lu CC, Chiang JH, Wu CL, Lin JJ, Lin HL, Yang MD, Liu KC, Chiu TH, Chung JG (2009). Rutin inhibits the proliferation of murine leukemia WEHI-3 cells *in vivo* and promotes immune response *in vivo*. Leuk. Res., 33: 823-828.

Mariod AA, Matthaus B, Ismail M (2011). Comparison of supercritical fluid and hexane extraction methods in extracting kenaf (Hibiscus cannabinus) seed oil lipids. J. Am. Oil Chem. Soc., 88: 931-935.

McCann SE, Ambrosone CB, Moysich KB, Brasure J, Marshall JR, Freudenheim JL, Wilkinson GS, Graham S (2005). Intakes of Selected Nutrients, Foods, and Phytochemicals and Prostate Cancer Risk in Western New York. Nutr. Cancer, 53: 33-41.

McCann SE, Freudenheim JL, Marshall JR, Graham S (2003). Risk of Human Ovarian Cancer is Related to Dietary Intake of Selected Nutrients, Phytochemicals and Food Groups. Nutr. J., 133: 1937-1942.

Mebius RE, Kraal G (2005). Structure and function of the spleen. Nat. Rev. Immunol., 5: 606-616.

Mohamed A, Bhardwaj H, Hamama A, Webber C (1995). Chemical composition of kenaf (*Hibiscus cannabinus* L.) seed oil. Ind. Crop. Prod., 4: 157-165.

Mohan S, Abdul AB, Abdelwahab SI, Al ZAS, Aspollah SM, Abdullah R, Taha MME, Beng NK, Isa NM (2010). *Typhonium flagelliforme* inhibits the proliferation of murine leukemia WEHI-3 cells *in vitro* and induces apoptosis *in vivo*. Leuk. Res., 34: 1483-1492.

Moon DO, Kim MO, Choi YH, Kim GY (2008). β-Sitosterol induces G_2/M arrest, endoreduplication, and apoptosis through the Bcl-2 and PI3K/Akt signaling pathways. Cancer Lett., 264: 181-191.

Moon HI (2010). Studies of the anticancer effect of sesquiterpene lactone from *Carpesium rosulatum*. J. Med. Plant Res., 4(18): 1906-1909.

Nobili S, Lippi D, Witort E, Donnini M, Bausi L, Mini E, Capaccioli S (2009). Review: Natural compounds for cancer treatment and prevention. Pharmacol. Res., 59: 365-378.

Nyam KL, Tan CP, Lai OM, Long K, Che MYB (2009). Physicochemical properties and bioactive compounds of selected seed oils. LWT-Food Sci. Technol., 42: 1396-1403.

Park C, Moon DO, Rhu CH, Choi BT, Lee WH, Kim GY, Choi YH (2007). β-Sitosterol Induces Anti-proliferation and Apoptosis in Human Leukemic U937 Cells through Activation of Caspase-3 and Induction of Bax/Bcl-2 Ratio. Biol. Pharm. Bull., 30(7): 1317-1323.

Phillips RM, Jaffar M, Maitland DJ, Loadman PM, Shnyder SD, Steans G, Cooper PA, Race A, Patterson AV, Stratford IJ (2004). Pharmacological and biological evaluation of a series of substituted 1,4-naphthoquinone bioreductive drugs. Biochem. Pharmacol., 68: 2107-2116.

Phoon MC, Desbordes C, Howe J, Chow VTK (2001). Linoleic and linolelaidic acids differentially influence proliferation and apoptosis of MOLT-4 leukaemia cells. Cell Bio. Int., 25: 777-784.

Pourmortazavi SM, Hajimirsadeghi SS (2007). Supercritical fluid extraction in plant essential and volatile oil analysis. J. Chromatogr. A, 1163: 2-24.

Ruiz M, Castillo D, Dobson D, Brennan R, Gordon S (2002). Genotypic variation in fatty acid content of black currant seeds. J. Agric. Food Chem., 50: 332-335.

Schabath MB, Hernandez LM, Wu X, Pillow PC, Spitz MR (2005). Dietary Phytoestrogens and Lung Cancer Risk. J. Am. Med. Assoc., 294: 1493-1504.

Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortezn RV, Panizzi P, Figueiredo JL, Kohler RH, Chudnovskiy A, Waterman P, Aikawa E, Mempel TR, Libby P, Weissleder R, Pittet MJ (2009). Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. Science, 325: 612-616.

Tsou MF, Peng CT, Shih MC, Yang JS, Lu CC, Chiang JH, Wu CL, Lin JP, Lo C, Fan MJ, Chung JG (2009). Benzyl isothiocyanate inhibits murine WEHI-3 leukemia cells *in vitro* and promotes phagocytosis in BALB/c mice *in vivo.* Leuk. Res., 33: 1505-1511.

Wang L, Weller CL (2006). Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. Technol., 17: 300-312.

Warner N, Moore M, Metcalf D (1969). A transplantable myelomonocytic leukemia in BALB-c mice: cytology, karyotype, and muramidase content. J. Nat. Cancer Inst., 43: 963-982.

Wen YF, Yang JS, Kuo SC, Hwang CS, Chung JG, Wu HC, Huang WW, Jhan JH, Lin CM, Chen HJ (2010). Investigation of anti-leukemia molecular mechanism of ITR-284, a carboxamide analog, in leukemia cells and its effects in WEHI-3 leukemia mice. Biochem. Pharmacol., 79: 389-398.

Xue SA, Gao L, Hart D, Gillmore R, Qasim W, Thrasher A, Apperley J, Engels B, Uckert W, Morris E, Stauss H (2005). Elimination of human leukemia cells in NOD/SCID mice by WT1-TCR gene-transduced human T cells. Blood, 106: 3062-3067.

Yazan LS, Foo JB, Ghafar SAA, Chan KW, Tahir PM, Ismail M (2011). Effect of kenaf seed oil from different ways of extraction towards ovarian cancer cells. Food Bioprod. Process., 89: 328-332.

Yu CS, Lai KC, Yang JS, Chiang JH, Lu CC, Wu CL, Lin JP, Liao CL, Tang NY, Wood WG, Chung JG (2010). Quercetin inhibited murine leukemia WEHI-3 cells *in vivo* and promoted immune response. Phytother. Res., 24: 163-168.

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Full Length Research Paper

Limonene synthase gene expression under different concentrations of manganese in *Cuminum cyminum* L.

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SQ-RT-PCR was applied to study Limonene synthase (LS) gene expression pattern and response to different concentrations of manganese in different organs of cumin (*Cuminum cyminum* L.). The results revealed that the gene is expressed in very small (< 2 mm) and small (3 to 4 mm) flowers and also in shoots, while it is not expressed in roots, leaves, medium (4 to 5 mm) and larger flowers. The highest level of gene expression was observed in shoot and very small flowers. Partial sequencing of LS gene in cumin revealed 68 to 85% identical to that of some other plants. To determine the effect of manganese on LS gene expression, the plants were exposed to different concentrations of manganese (0, 40, 80 and 160 ppm) as spray by applying two methods: T_1- alternatively spraying at late vegetative stage besides blooming; T_2-spraying only at blooming. LS gene expression was increased considerably under 80 ppm concentration of Mn at blooming and reduced remarkably less than 40 ppm concentrations in both methods and at 160 ppm in T_1 method in comparison with controls. Anatomical studies indicate that essential oil ducts are located not only on the fruits tissue but also surprisingly on shoot. Overall, results of this research reveal for the first time that limonene synthase gene is expressed in cumin. In addition, use of 80 ppm concentration of Mn at blooming could be considered as optimum to increase LS gene expression. Existence of oil ducts on shoot of this plant is a remarkable finding for further studies.

Key words: *Cuminum cyminum* L., limonene synthase gene, manganese.

INTRODUCTION

Medicinal and aromatic herbs have been receiving increased interest all over the world due to their efficacy as alternative medicine for curing human ailments without major known side effects. To date, very little is known about the genetics of plant secondary metabolism, as the genes of most pathways have not been identified and little is understood of their regulation and function. Cumin (*Cuminum cyminum* L.) is valued for its aroma, medicinal and therapeutic properties. The seeds contain 3 to 4% volatile oil and about 15% fixed oil (Spices Board Stastistics, 2006). In traditional medicine, cumin has various uses: it is used to treat hoarseness, jaundice, dyspepsia and diarrhoea. It is effective on gastrointestinal (Amin, 2000), reproductive (Weiss, 2002) and nervous (Janahmadi et al., 2006) systems. Cumin also has hypoglycaemic (Aslam et al., 2003), hypolipidaemic (Aruna et al., 2005) and chemoprotective (Gagandeep et al., 2003) properties. Terpenoids compose the largest and most diverse family of natural products. Of the more than 30,000 individual terpenoids now identified (Buckingham, 1998), at least half are synthesized by plants. *C. cyminum* has limonene (Kan et al., 2007) which is a terpenoid. The synthesis of limonene, in providing the first committed intermediate of the pathway, represents a possible rate-limiting step of monoterpene production (Croteau and Gershenzon,1994; Gershenzon and Croteau, 1990). It is assumed that monoterpenes (such as limonene) are primarily synthesized in the plastids via the MEP pathway-derived IPP and DMAPP (Mahmoud and Croteau, 2002). Limonene serves as the common olefinic precursor of the essential oil terpenes (Kjonas

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Abbreviations: **GPP,** Geranyl pyrophosphate; **LS,** limonene synthase; **MRE**s, metal-responsive elements.

and Croteau, 1983) through a series of secondary, largely redox, transformations (Croteau and Gershenzon 1994). Limonene synthase enzyme catalyzes the stereo-specific cyclization of GPP to form the monocyclic monoterpene limonene (Munoz-Bertomeu et al., 2008). LS gene has a key role in the beginning of biosynthesis pathway of terpenoids derivative compounds.

Manganese is an essential micronutrient throughout all stages of plant development (Marschner, 1995). This essential trace element plays an important role in several physiological processes as almost every compartment of the cell carries at least one enzyme whose activity is dependent on Mn^{2+}. This metal acts as cofactor for oxidases, dehydrogenases, DNA and RNA polymerases, kinases, decarboxylases and sugar transferases. (Crowley et al., 2000; Culotta et al., 2005; Keen et al., 2000). Many investigators have confirmed the role of elements on growth and yield of many aromatic and medicinal plants such as *Ruta graveolens* L. (El-Khateeb et al., 1994), *Mentha viridis* L. (El-Ghadban, 1994), Rosemary (Kassem, 2002) and *Trachyspermum ammi* L. (Swaefy, 2002). Manganese has been shown to be the most effective single micronutrient enhancing oil production (Nandi and Chatterjee, 1991). Manganese application as foliar spray (50 ppm) has been reported to increase the yield of essential oil and its main constituents in cumin (El-Sawi and Mohamed, 2002). Manganese-dependent gene expression is not fully understood in eukaryotic organisms. Existence of metal- or manganese-responsive sequences on promoter sequence of genes is possible (Gutiérrez et al., 2008). Several heavy metal-inducible genes have been reported in plants (Berna and Bernier, 1999; Hagen et al., 1988; Lescure et al., 1991), but surprisingly, little is known about the transcriptional regulation of gene expression in response to heavy metals. Whilst there are some reports of the manganese effects on percent and components of oil in different plants, few researches has been carried out on expression of precursor genes in this regard. The present investigation is the first attempt to study the limonene synthase gene expression in different organs as well as the effect of manganese on expression of this gene in *C. cyminum*. Also, this paper studies existence of oil ducts on cumin shoot.

MATERIALS AND METHODS

Plant materials and experimental design

Seeds of cumin (*C. cyminum* L.), a native to Neishabour city (Iran), were placed on water flow for 12 to 15 h. Then, they were surface-sterilized by their immersion in 2% (v/v) NaOCl for 3 min and sulphur 80% DF fungicide for 2 min, followed by three times of rinsing in sterile water after any step. The seeds were next transferred to plastic pots containing (Klasmann-Deilmann, potgrond H) peat moss under equal greenhouse conditions (period of day/night, 16/8, at 26 ± 3°C) and irrigated three times per week. Plants were affected by three methods: T_0, without any treatment; T_1, sprayed with different concentrations of manganese as $MnSO_4.4H_2O$ (0, 40, 80 and 160 ppm) both at late vegetative stage and blooming; T_2, identical to T_1 except for one spray only at blooming. Treatments were as follow: PBAF0, spray with distilled water (0 ppm of Mn) at late stages of vegetative phase and blooming; PAF0, spray with distilled water (0 ppm of Mn) only at blooming; PBAF40, spray of Mn (40 ppm) at late stages of vegetative phase and blooming; PAF40, spray of Mn (40 ppm) only at blooming; PBAF80, spray of Mn (80 ppm) at late stages of vegetative phase and blooming; PAF80, spray of Mn (80 ppm) only at blooming; PBAF160, spray of Mn (160 ppm) at late stages of vegetative phase and blooming; PAF160, spray of Mn (160 ppm) only at blooming. For limonene synthase gene expression analysis in different organs, samples of root, stem, leaf and flower at different developmental stages (F_1<2 mm; F_2, 3 to 4 mm; F_3, 4 to 5 mm and F_4>5 mm in size) were taken from T_0 method. These samples were immediately frozen in liquid nitrogen and stored frozen at -80°C until use. For LS gene expression analysis under manganese treatment, only F_2 flowers (3-4 mm) of plants in T_1 and T_2 methods were studied.

Determination of Mn content in *C. cyminum*

Aerial parts of plants in different treatments were rinsed twice with deionized water. Samples for each treatment were dried for 24 h at 60°C, weighed and ashed in a 480°C oven for 16 h. After cooling, the ash was digested with 2 ml HNO_3 (65%) and heated to dryness. The sample was then dissolved in 5 ml 3 N HCl and brought to volume in a 25 ml volumetric flask using 0.1 N HCl. The Mn contents were determined by atomic absorption system (GVC 902, Australia).

RNA extraction

Total RNA of various organs and treatments was extracted using RNX-plus kit (RN7713C, CinnaGen, Iran) according to the manufacturer's instruction with slight optimization. Approximately, 200 mg tissue from at least three different individual plants was used per sample and two RNA samples were extracted for each. Frozen samples were ground to a fine powder in liquid nitrogen with a pestle and mortar. About 100 to 200 mg of this powder was re-suspended in 500 µl cold (4°C) RNX-plus kit solution. After mixing thoroughly, the samples were incubated at room temperature for 5 min. Then, 200 µl of chloroform was added to samples. After 15 s shaking and 5 min incubation at 4°C, samples were centrifuged (Hettich, Mikro 200, Germany) at 4°C for 15 min at 13680×g. 250 µl of the aqueous phase was recovered and total RNA was precipitated with an equal volume of ice-cold isopropyl alcohol for 25 min at 4°C followed by a centrifugation step at 4°C for 15 min at 13680×g. The pellet was subsequently washed with ice-cold 75% ethanol, air-dried and dissolved in 40 µl of RNase–free water. The quality and concentration of RNA samples were examined by EB-stained agarose gel electrophoresis and spectrophotometer analysis.

cDNA synthesis

One µl of oligo (dT_{18}), 100 pmol, (K1621, Fermentas) was added to 5 µl total RNA and then reached 13 µl with RNase–free water. After being mixed, it was incubated at 70°C for 5 min and briefly chilled on ice. 4 µl of ×5 buffer (for ×1 final concentration) and 2 µl dNTP (10 mM) for 1 Mm final concentration were added to the mixture and the total volume was incubated at 37°C for 5 min. Afterwards, 1 unit RT-enzyme (K1621, Fermentas) was added and incubated at 42°C for 1 h. Inactivation of the reverse transcriptase accomplished by incubating the mixture at 70°C for 10 min. The cDNAs were

Table 1. Primers used for semi-quantitative RT-PCR. *α-Tubulin synthase* accession number EC930869.

Gene	Primers 5' - 3'	Product (bp)
Limonene synthase	Forward: GATGATATTTACGATGTCTATGGTAC Reverse: GAATTGATTTCGGCACATCGCCTC	490
α-Tubulin synthase	Forward: CAGCCAGATCTTCACGAGCTT Reverse: GTTCTCGCGCATTGACCATA	119

then stored at -20°C until use.

PCR primer design

Since limonene synthase gene has not yet been sequenced in *C. cyminum*, degenerate primers were designed based on the highly conserved nucleotide sequences among several plants such as citrus, mentha and perilla genus. By doing experiment with a few pairs of primers, eventually one pair producing bund was chosen (Table 1). The housekeeping gene α-tubulin was used as the standard for checking the quantity and quality of cDNA and/or RNA templates.

Semi- quantitative RT-PCR

1 µl of a four-fold dilution of the first-strand reaction (variable according to organs) was used as template for PCR amplification together with 1.3 µl of each primer and 12.5 µl Master Mix (PR8252C, CinnaGen, Iran) to a total volume of 25 µl. PCR amplification was performed using a thermocycler (Techne TC515, England) under the following conditions: 3 min at 94°C, followed by 28 or 35 cycles (for α-tubulin or LS respectively) of 45 s at 94°C, 1 min at the annealing temperature at 54.8°C for α-tubulin and 42.5°C for LS, 1 min at 72°C and the final extension of 10 min at 72°C. After PCR, the samples were separated on 1.7% agarose gel and stained with ethidium bromide. Gel pictures were obtained using Gel-Doc Transilluminator (UVP Bioimaging system, USA). Densitometric evaluation of DNA bands was performed with the totalLab software version 1.10. All PCR analysis was repeated three times.

Verification of amplified products and sequencing

250 µl of PCR product was sequenced directly after 1% agarose gel fractionation and purification using the PCR gel extraction kit (K0513, fermentase) according to the manufacturer's instruction. Sequencing of purified PCR fragments was carried out by Genservice Company (England). LS gene sequencing was performed both for F_1 flower (<2 mm) and shoot.

Anatomical study

For anatomical observations, shoots and flowers (3 to 4 mm in size) were fixed in FAA. Cross-sections of the shoots and flowers were made with a Reichert sliding microtome and by hand–cutting. Sections were cleared in sodium hypochlorite and stained by carmine-vest (1% w/v in 50% ethanol) and methyl green (1% w/v, aqueous). They were mounted in gelatin and observed by optical microscope (Nikon YS 100).

Statistical analysis

Statistical analysis was performed using one–way ANOVA. The F-ratio test indicated the existence of differences among treatments at $P \leq 0.05$. Different letters indicate significant differences among the treatments in a multiple range analysis for 95% confidence level. Each value is the mean ± S.E of three replicates.

RESULTS

Sequence analysis of the *C. cyminum* limonene synthase gene

Partial sequences of 492 bp amplicons for LS gene of cumin flower and shoot were sequenced and compared. BLAST analysis revealed perfect similarity between nucleotides according to ClustalW alignment. Figure 1A shows partial sequence of nucleotide and the deduced amino acid of LS gene of *C. cyminum* (492 bp). Nucleotide sequence of this gene revealed 68 to 85% identity to that of some other plants (Figure 1B). Partial sequence of limonene synthase gene of *C. cyminum* showed the highest homology of 88% to that of LS from *Mentha longifolia*. It also shared high homology with those of known LS from other plants (*Mentha spicata*, 85%; *Agastache rugosa,* 76%; *Perilla citriodora,* 71% and *Perilla frutescens*, 70%).

C. cyminum limonene synthase tissue-specific expression

To examine the expression patterns of the limonene synthase gene in *C. cyminum* organs, the level of corresponding mRNA was monitored in different organs by semi- quantitative RT-PCR. As figure 2 shows, there are large differences in expression levels of the LS gene in different organs. The highest expression was identified in the shoot and F_1 flower (<2 mm). Weaker expression was detected in F_2 flower (3 to 4 mm). No expression from F_3 (4 to 5 mm) and F_4 (>5 mm) flowers, roots and leaves was observed.

Expression pattern of limonene synthase gene in response to different concentrations of manganese

To characterize whether the *C. cyminum* limonene synthase gene expression is induced by different concentrations of manganese, the expression levels of

(A)

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TGAATTGATTTCGGCACATCGCCTCTCTTCAACTCATTCGATGAAGTTCCATAGTCGTCCGCTAGTCTAAA
 -   I D F G T S P L F N S F D E V P - S S A S L K

AATTTCTGACGCCAAGCGTACTATATCCGGAATGCCCTCTAAGAATTCCATTGCTTCTTTCTTGATTGGAT
 I S D A K R T I S G M P S K N S I A S F L I G

TTGATGAGAAGATATAAGAATGAAGTACTATCGAAGGCCCCGTTATACTCACCAGTCCATTGCTCAAGTAC
F D E K I - E - S T I E G P V I L T S P L L K Y

TCCTGAAATGTAGGTTTATATCCACTGTAATACCACCGAGCCTCCACCATGTAGCGTTTCGACAATTCAAT
 S - N V G L Y P L - Y H R A S T M - R F D N S I

CCATGCATTTCTTAGTTGCGGGAAAACATTGACATTCTGCTCGCTGAGGACGTCGAAAGCAATCTCGCTGG
 H A F L S C G K T L T F C S L R T S K A I S L

CGAAATTGTTAAGGGACAGAAAACATAATTTCATGTAGTGTGGAAGCTCCGTCATTGCATTTAAATCCCAT
A K L L R D R K H N F M - C G S S V I A F K S H

CTCTCGATAACACTTGTGAAGAGTTCAAGTTCATCCAGTGTACCATAGACATCGTAAATATCATCA
 L S I T L V K S S S S S S V P - T S -I S S
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B

```
L13459.1     TGAACGAGGGTGGTGTTGATGGC------------------GACCTTTTAACAAGAATCG 755
AF175323.1   TGAACGAGGGTGGTGTTGATGGC------------------GACCTTTTAACAAGAATCG 764
AF233894.1   TCAATGACAAAACAATCGATGATGATGATGATGCAGACACTAATCTTATATCGTGTGTGC 785
D49368.1     TCAATGAGAAAACAATCGATGATGATGATGATGCAGACACTAATCTTATATCGTGTGTGC 760
AY055214.1   TGAAGGGTGATGAAATTGATGAC------------------AACCTTTTATCATCCATTA 816
cumin        ---------------------------------------------------------TGA 3
                                           *

L13459.1     CATATTCTTTGGACATCCCTCTTCATTGGAGGATTAAA--AGGCCAAATGCACCTGTGTG 813
AF175323.1   CATATTCTTTGGACATCCCACTTCATTGGAGGATTAAA--AGGCCAAATGCACCTGCGTG 822
AF233894.1   GCCACTCTTTGGACATCCCAATTCATTGGAGGATTCAA--AGGCCAAATGCAAGCTGGTG 843
D49368.1     GCCACTCTTTGGACATCCCAATTCATTGGAGGATTCAA--AGGCCAAATGCAAGCTGGTG 818
AY055214.1   GATATTCTTTAGAGATCCCAAACTATTGGAGTGTTGTA--AGGCCGAATGTAAGTGTGTG 874
cumin        ATTGATTTCGGCACATCGCCTCTCTTCAACTCATTCGATGAAGTTCCATAGTCGTCCGCT 63
             * *    * *** *      *      **  *  * *    **       *

L13459.1     GATCGAATGGTATAGGAAGAGGCCCGACATGAATCCAGTAGTGTTGGAGCTTGCCATACT 873
AF175323.1   GATCGAATGGTATAGGAAGAGGCCCGACATGAATCCAGTAGTGTTGGAGCTTGCCATACT 882
AF233894.1   GATTGATGCATATAAGAGGAGAAGTCACATGAATCCACTTGTGTTGGAGCTTGCCAAACT 903
D49368.1     GATTGATGCCTATAAGAGGAGAAGTCACATGAATCCACTTGTGTTGGAGCTTGCCAAACT 878
AY055214.1   GATTGATGAATATAGGAAGAGATCAGACATGAATCCAGTGGTGTTGGAGCTCGCCATACT 934
cumin        AGTCTAAAAATTTCTGACGCCAAGCGTACTATATCCGG-AATGCC----CTCTAAGAATT 118
           *  *    * *  ** *         *  ****     **      **      * *

L13459.1     CGACTTAAATATTGTTCAAGCACAATTTCAAGAAGAGCTCAAAGAATCCTTCAGGTGGTG 933
AF175323.1   CGACTTAAATATTGTTCAAGCACAGTTTCAAGAAGAGCTTAAAGAATCCTTCAGGTGGTG 942
AF233894.1   CGACTTAAATATTTTTCAAGCACAGTTTCAACAAGAACTCAAACAAGACTTAGGGTGGTG 963
D49368.1     CGACTTAAATATTTTTCAAGCACAGTTTCAACAAGAACTCAAACAAGACTTAGGGTGGTG 938
AY055214.1   GGACGCAAATATTGTTCAAGCACAATTGCAACTAGAACTAAAAGAATCACTAAGGTGGTG 994
cumin        CCA-TTGCTTCTTTCTTGATTG-GATTTGATGAGAAGATATAAGAA----TGAAGTACTA 172
           *      * **  *  *      **  *     *  *  ** **    *   **  *

L13459.1     GAGAAATACTGGGTTTGTTGAGAAGCTGCCCTTCGCAAGGGATAGACTGGTGGAATGCTA 993
AF175323.1   GAGAAATACTGGGTTTGTTGAGAAGCTGCCCTTCGCAAGGGATAGACTGGTGGAATGCTA 1002
AF233894.1   GAAAAATACATGCCTTGCTGAGAAGCTCCCCTTTGCAAGGGATAGGCTTGTGGAATGCTA 1023
D49368.1     GAAAAATACATGCCTTGCTGAGAAGCTCCCCTTTGTAAGGGATAGGCTTGTGGAATGCTA 998
AY055214.1   GAGAAATACGTGCTTTGTGGAGAAGCTCCCGTTCGCGAGGGATAGGCTTATAGAGAGCTA 1054
cumin        TCGAAGGCCCCGTTATACTCACCAG-TCCATTGCTCAAGT-----ACTCCTGAAATGTAG 226
            **   *  *   *    *  ** * *  *     **      **  *  *  *

L13459.1     CTTTTGGAATACTGGGAT--CATCGAGCCACGTCAGCATGCAAGTGCAAGGATAATGATG 1051
AF175323.1   CTTTTGGAATACTGGGAT--CATCGAGCCACGTCAGCATGCAAGTGCAAGGATAATGATG 1060
AF233894.1   CTTTTGGTGTACTGGGAT--CATTCAGCCTCTTCAACATGAAAATGCAAGGGTAACGCTG 1081
D49368.1     CTTTTGGTGTACTGGGAT--CATTCAGCCTCTTCAACATGAAAATGCAAGGGTAACGCTG 1056
AY055214.1   CTTCTGGAGTACAGGGAT--GGTAGAGCCCCGTCAGCATGCAAACGCAAGGATAATTATG 1112
cumin        GTTTATATCCACTGTAATACCACCGAGCCTC--CACCATGTA-GCGTTTCGACAATTCAA 283
           **       ** *  **       **** *  ** **** *   *     *  **

L13459.1     GGCAAAGTCAACGCTCTGATTACGGTGATCGATGATATTTATGATGTCTATGGCACCTTA 1111
AF175323.1   GGCAAAGTCAACGCTCTGATTACGGTGATCGATGATATTTATGATGTCTATGGCACCTTA 1120
AF233894.1   GCCAAAGTTAACGCTCTAATAACCACGTTAGATGATATTTACGATGTCTATGGTACCTTA 1141
D49368.1     GCCAAAGTTAACGCTCTAATAACCACGTTAGATGATATTTACGATGTCTATGGTACCTTA 1116
AY055214.1   GCCAAAGTTATCGCTCTTATAACAGTCATGGATGATATTTACGATGTCTATGGTACCTTA 1172
cumin        TCCATG--CATTTCTTAGTTGCGGGAAAACATTGACATTCTGCTCGCTGAGGACGTCGAA 341
           **     *   **     *            *** ***      *    * *     * *

L13459.1     GAAGAACTCGAACAATTCACTGACCTCATTCGAAGATGGGATATAAACTCAATCGACCAA 1171
AF175323.1   GAAGAACTCGAACAATTCACTGACCTCATTCGAAGATGGGATATAAACTCAATCGACCAA 1180
AF233894.1   GAAGAACTCGAGCTATTCACCGAGGCGATTCGGAGATGGGATGTTAGTTCAATTGACCAT 1201
D49368.1     GAAGAACTCGAGCTATTCACCGAGGCGATTCGGAGATGGGATGTTAGTTCAATTGACCAT 1176
AY055214.1   GAAGAACTCGAACAATTCACAGAGGCCTTTCGAAGATGGGATGTGAGTTCAATCGACCAA 1232
cumin        AGCAATCTCGCTGGCGAAATTGTTAAGGGACAGAAAACATAATTTCATGTAGTGTGGAAG 401
            * ****       *  *       *  * *    *  *      * *     *

L13459.1     CTTCCCGATTACATGCAACTGTGCTTTCTTGCACTCAACAACTTCGTCGATGATACAT-C 1230
AF175323.1   CTTCCCGATTACATGCAACTGTGCTTTCTTGCACTCAACAACTTCGTCGATGATACAT-C 1239
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Figure 1. (A) Partial sequence of nucleotide and the deduced amino acid of limonene synthase gene of *Cuminum cyminum* (492 bp). (B) Alignment of nucleotide sequence of *Cuminum cyminum* limonene synthase. Accession numbers: L13459.1, *Mentha spicata*; AF175323.1, *Mentha longifolia*; AF233894.1, *Perilla citriodora*; D49368.1, *perilla frutescens*; AY055214.1: *Agastache rugosa*; cumin, *Cuminum cyminum*.

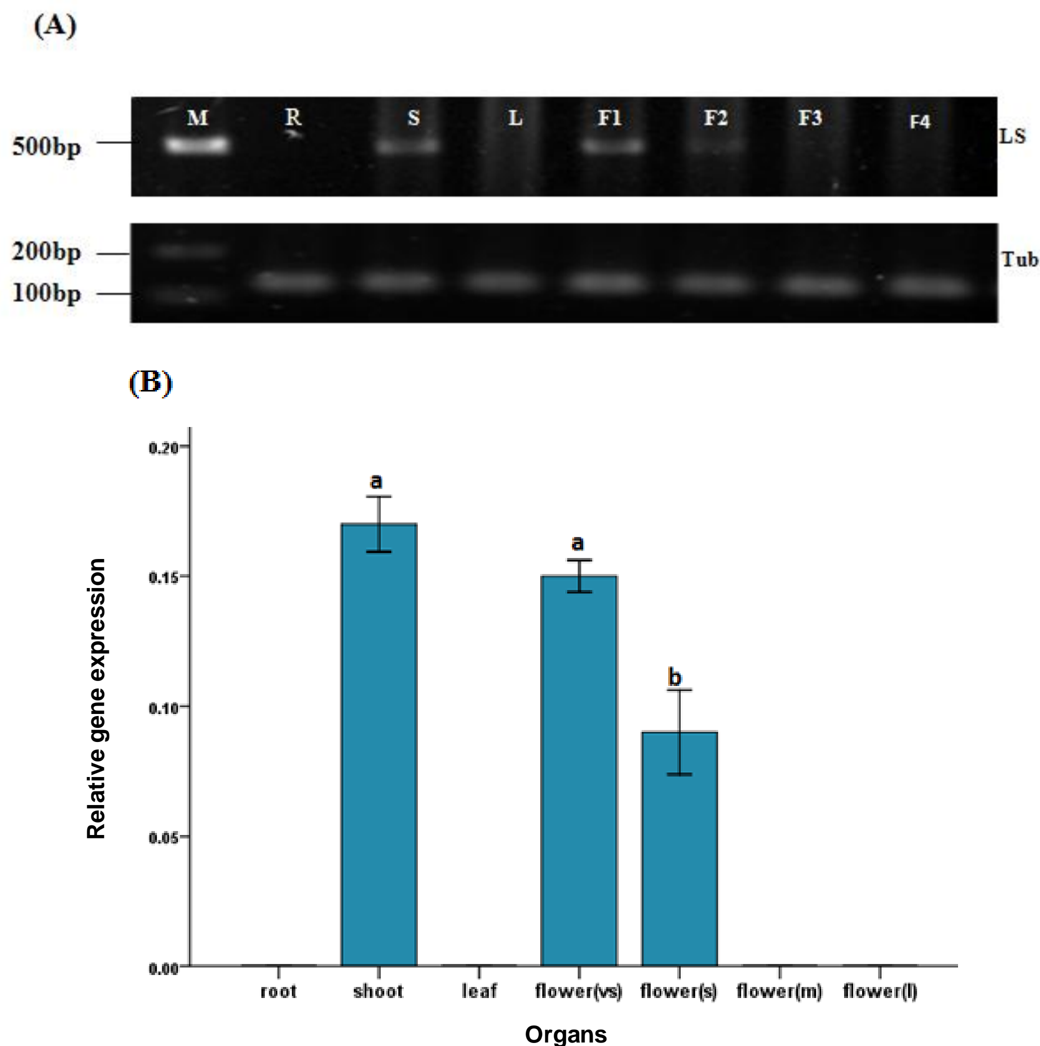

Figure 2. Semi-quantitative RT-PCR show differential expression of limonene synthase gene in different organs of *C. cyminum.* LS, limonene synthase (492 bp); Tub, tubulin (119 bp); M, 100 bp DNA ladder; R, root; S, shoot; L, leaf; F_1, flower < 2 mm; F_2, flower 3-4 mm; F_3, flower 4-5 mm; F_4, flower > 5 mm. The tubulin gene was used as housekeeping. (A) Agarose gel electrophoresis of SQ-RT-PCR. (B) Densitometric analysis of gel bands.

this gene were investigated under various treatments. A semi–quantitative RT–PCR analysis was performed on total RNA from F_2 flowers (3 to 4 mm) under manganese treatments (0, 40, 80 and 160 ppm) as spray. Twice spray of 40, 80 and 160 ppm of manganese at PBAF40, PBAF80 and PBAF160 treatments increased the concentration of Mn approximately 1.2 to 1.3 fold in comparison with one spray only at blooming in PAF40, PAF80 and PAF160 treatments, respectively (Figure 3). The results revealed that exposure to 40 ppm of Mn decreased considerably limonene synthase gene expression both on T_1 (PBAF40) and on T_2 (PAF40) compared to controls (0 ppm of Mn) (Figure 4). The expression of LS gene was found to be up-regulated by 80 ppm of Mn especially at blooming. The highest concentration of Mn (160 ppm) is significantly repressed expression of LS gene. Such concentration of Mn caused the weakest expression of LS gene on both of late vegetative stages and blooming (PBAF160). Applying 160 ppm of Mn at blooming (PAF160) slightly enhanced the limonene synthase gene expression.

Existence of oil ducts in *C. cyminum* shoot

The results of this study confirmed the oil ducts location in flowers (fruits) of cumin (Figure 5A) as storage organs. Thus, the existence of oil ducts in cumin shoot was an

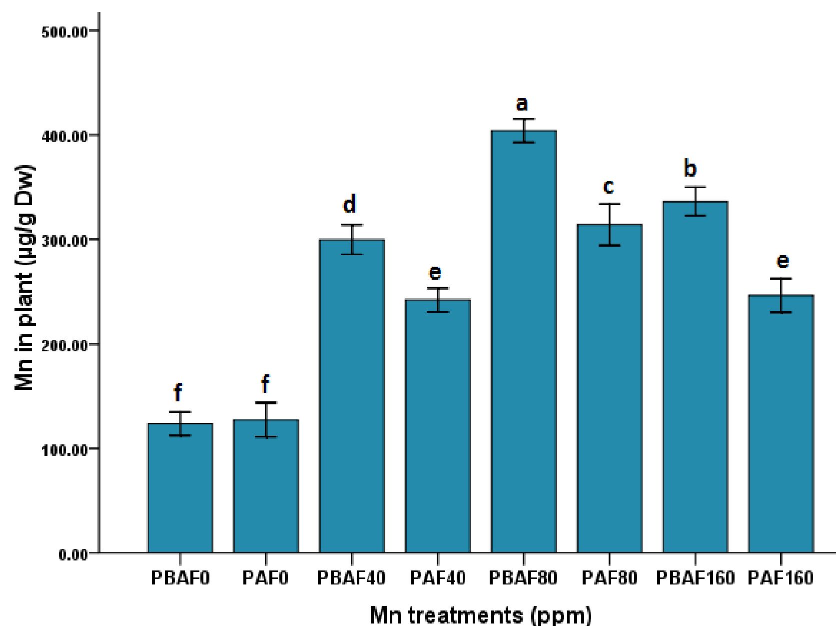

Figure 3. Effect of different tratments on Mn content of aerial parts of *C. cyminum*. Treatments: PBAF0, PBAF40, PBAF80 and PBAF160 respectively spray with distilled water (0), 40, 80 and 160 ppm of manganese at late stages of vegetative phase and blooming; PAF0, PAF40, PAF80 and PAF160 respectively spray with distilled water (0), 40, 80 and 160 ppm of manganese only at blooming.

unexpected and notable result (Figure 5B).

DISCUSSION

Expression of limonene synthase gene in different organs of *C. cyminum* L.

We reported here a partial cDNA sequence from LS gene in *C. cyminum.* According to our knowledge, this is the first evidence for existence of limonene synthase gene in cumin. Biochemical studies had indicated the existence of limonene in fruits of cumin (Kan et al., 2007). In this study, we confirmed this assignment at the molecular level by studying the expression of LS gene in different organs of this plant. Results of this study revealed that LS gene expression is restricted to a short interval during fruit ontogeny in *C. cyminum*. The highest expression of the gene is in the youngest flowers (< 2 mm in size). These results are consistent with those of several previous investigations on plant terpene formation which revealed that high level of monoterpene biosynthesis in immature tissue. For example the biosynthesis of monoterpene in *S. officinalis* leaves (Croteau et al., 1981), *Majorana hortensis* leaves (Croteau, 1977), *Carum carvi* fruits (Boumeester et al., 1998), *C. flexuosus* blades (Singh et al., 1989) and maritime pine (*Pinus pinaster*) foliage (Bernard-Dagan et al., 1982) is restricted to a short interval during organ ontogeny. Accumulation of monoterpenes is confined to the early stages of fruit development so that young fruits contain high concentrations of limonene in *C. carvi* L. (Boumeester et al., 1998). Similar to caraway, it appears possible that accumulation of limonene in fruits of cumin is a developmentally regulated process. During the early stages of caraway fruit development there is an abundant accumulation of limonene and limonene synthase enzyme exhibits high levels of activity (Boumeester et al., 1998). Similar changes in terpenoid accumulation patterns occur during plant development in some other plant species including dill (Porter et al., 1983) and peppermint (Voirin and Bayet, 1996). Monoterpenes are believed to function principally in ecological roles, serving as herbivore-feeding deterrents, antifungal defense and attractants for pollinators (Langenheim, 1994). It seems likely that a sufficient amount of free substrate is available or can be made available, particularly in young flowers. In flowers, the GPP pool is large and is not limiting terpene production (Lucker et al., 2004). This pool of GPP in young tissues may benefit the plant by allowing the rapid production of monoterpenes to repel herbivores or attract predators of the herbivores and thus protect new growth. Results of this study revealed that limonene synthase gene is not expressed in larger flowers (>4 mm). Boumeester et al. (1998) showed that, amount of limonene and limonene synthase activity declined to low levels at later stages of caraway fruit development. Also, it has been demonstrated that limonene serves as the

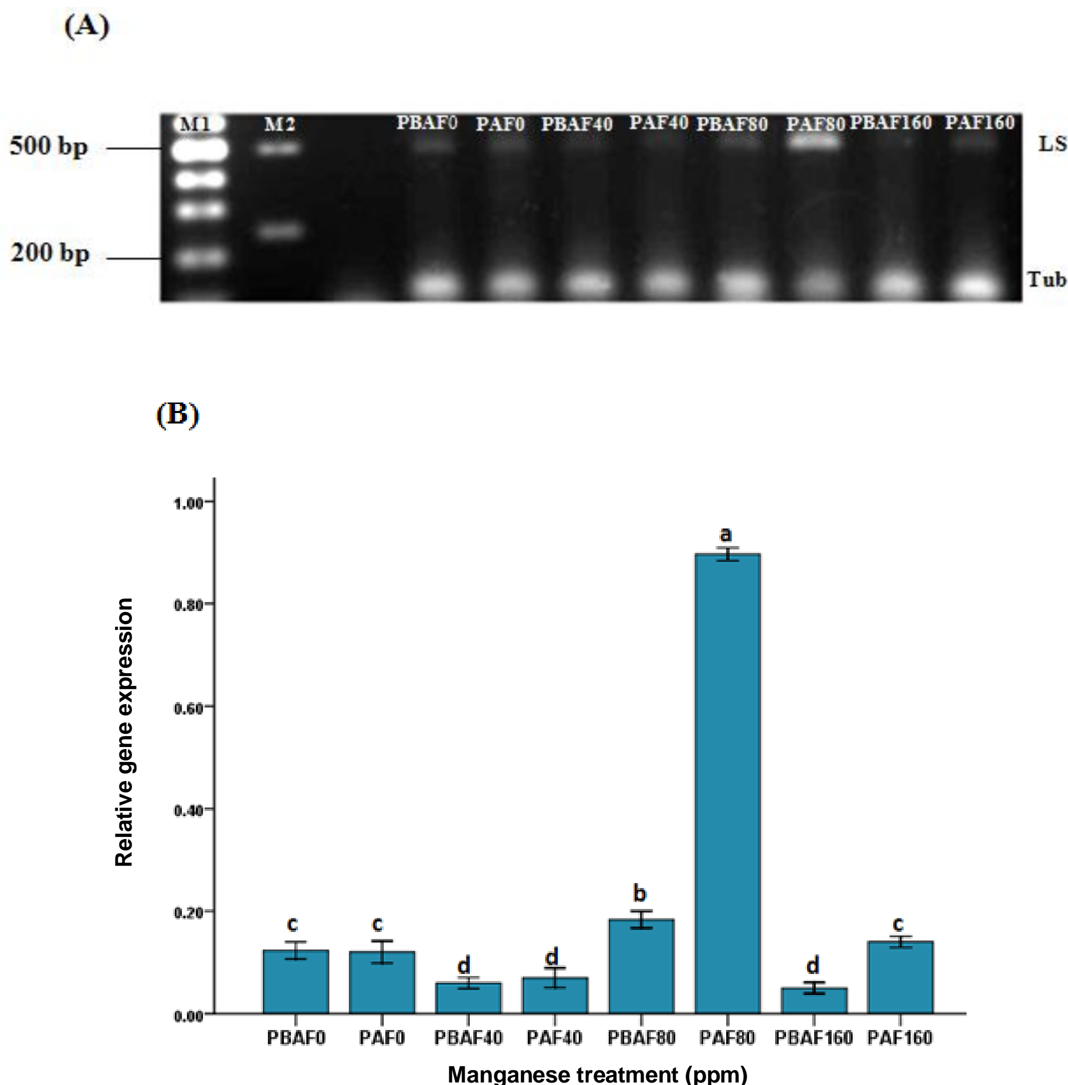

Figure 4. Effect of different concentration of manganese on the limonene synthase gene expression of *C. cyminum*. LS, limonene synthase (492 bp); Tub, tubulin (119 bp); M_1, 100 bp DNA ladder; M_2, 1000 bp DNA ladder; treatments: PBAF0, PBAF40, PBAF80 and PBAF160 respectively spray with distilled water (0), 40, 80 and 160 ppm of manganese at late stages of vegetative phase and blooming; PAF0, PAF40, PAF80 and PAF160 respectively spray with distilled water (0), 40, 80 and 160 ppm of manganese only at blooming. (A) Agarose gel electrophoresis of SQ-RT-PCR. (B) Densitometric analysis of gel bands.

common olefinic precursor of the essential oil terpenes such as peppermint and spearmint (Kjonas and Croteau, 1983) through a series of secondary, largely redox, transformations (Croteau and Gershenzon, 1994). In peppermint, as leaves mature the percentage of limonene drops and the percentage of oxygenated products menthone and menthol increases (Brun et al., 1991; Voirin and Bayet, 1996). Termination of the expression of LS gene in larger flowers is probably accompanied by the expression of other genes such as limonene-6-hydroxylase for conversion of limonene as precursor to storage derivative compounds in ripened fruits (Boumeester et al., 1998). Accordingly, expression of genes participating in such metabolic pathway should be considered. It seems that terpenoids such as limonene play protective role in this organ or participate for production and transformation of such material onto flowers (fruits).

Manganese changes limonene synthase gene expression in *C. cyminum*

Manganese-dependent gene expression is not fully

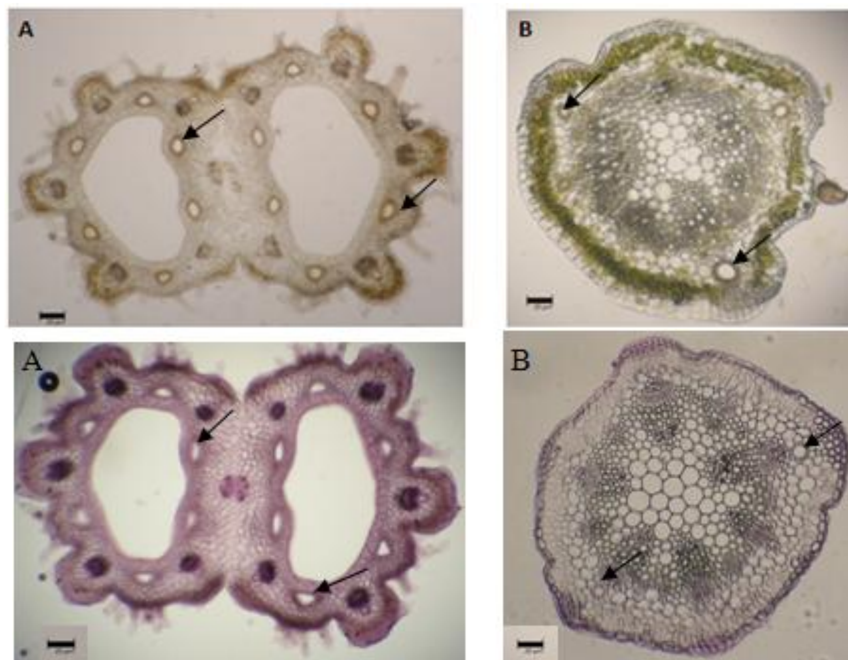

Figure 5. Cross sections from flower 3 to 4 mm in size, (**A**) and shoot (**B**) of *Cuminum cyminum*. Arrows indicate oil ducts (Scale bar 20 µm).

understood in eukaryotic organisms. In turn, the homeostasis of this metal appears to involve a complex network of proteins (Gutiérrez et al., 2008). It has been suggested that Mn^{+2} might induce the expression of manganese–dependent peroxidase through metal-responsive elements (MREs) located in the promoters of the corresponding genes (Brown et al., 1991). MREs have been identified in animals and plants as the target site of transcription factors responding to cadmium, zinc and copper (Thiele, 1992). Mn^{+2} have a transcriptional role which would likely imply a putative transcription factor (Ma et al., 2004). Previous results have reported that application of micronutrients, as supplements to macroelements, leads to significant effects on herb yield and oil contents of many plants (El-Sawi and Mohamed, 2002; Sharma et al., 1980; Wahab and Hornok, 1983).

It is well known that terpenoids are the major components of most plants essential oils (Guenther, 1950) and limonene is precursor for most of these compounds and their derivatives. In the isoprenoid biosynthetic pathway there are several enzymatic reactions that require or can use Mn as a cofactor (Wilkinson and Ohki, 1988). Probably the effects of Mn on LS gene expression might be due to enzyme cofactor (Coates et al., 1997) or transcription factor (Ma et al., 2004) roles for this element. Enzyme activity usually requires a specific amount of a metal (Mn) that any change in its concentration can cause repression (Wilkinson and Ohki, 1988). It can thus be assumed that 80 ppm concentration of manganese can be considered as optimum for limonene synthase gene expression of *C. cyminum* specially at blooming.

Flowers (fruits) are the organs of production and storage of secondary metabolites containing terpenoids in cumin. Accordingly, the effect of Mn (80 ppm) on blooming is reasonable. Results of the present research project revealed that not only concentration of 40 ppm of Mn at both methods but also 160 ppm at T_1 method decreased limonene synthase gene expression. Decrease in transcription levels may result from a decreased stability of transcripts (Fitzgerald et al., 2008). Usually, biphasic responses of plants to increasing Mn concentration were observed; efficient adaptation and toxic heavy metals accumulation lead to oxidative stress (Lidon and Teixeira, 2000). On this basis, 40 ppm concentration can be considered as deficiency concentration for LS gene expression and on the other hand, 'efficient adaptation' leads to oxidative stress. 160 ppm of Mn repressed LS gene probably due to effects on LS enzyme with negative feedback or oxidative stress. Oxidative stress caused by Mn can result in damage to proteins (Demirevska-Kepova et al., 2004) such as enzymes (limonene synthase) and transcription factors and so can cause repression of related gene expression. Peroxidase induction has been observed as a general response of higher plants to toxic amounts of heavy metals (Van and Clijsters, 1990). Due to the fact that shoot is not a storage organ for secondary metabolites such as terpenoids and their derivatives in cumin, therefore limonene synthase gene expression and

existence of oil ducts in cumin shoot are remarkable results for more studies.

In future, further work such as measuring LS enzyme activity, expression of other related genes and produced materials could be accounted in order to elucidate the correlation between limonene synthase gene expression in different organs, developmental stages and their relation with manganese concentration. Moreover, analysis of the promoter sequence of LS gene can yield information of consensus motifs which might identify common functional regulatory elements to metals such as Mn that have not yet been defined.

REFERENCES

Amin G (2000). Handbook of Herbs and Spices. Woodhead Publishing Cambridge. UK, pp. 164-167.

Aruna K, Rukkumani R, Suresh VP, Venugopal MP (2005). Therapeutic role of *Cuminum cyminum* on ethanol and thermally oxidized sunflower oil induced toxicity. Phytother. Res., 5: 416-421.

Aslam M, Jafri MA, Javed K, Singh S (2003). Evaluation of antidiabetic drugs from plant sources. Phytochem. Pharmacol., 2: 105.

Berna A, Bernier F (1999). Regulation by biotc and abiotic stress of a wheat germin gene encoding oxalate oxidase, a H_2O_2 producing enzyme. Plant Mol. Biol., 39: 539-549.

Bernard-Dagan C, Pauly G, Marpeau A, Gleizes M, Carde JP, Baradat P (1982). Control and compartmentation of terpene biosynthesis in leaves of pinus pinaster. Physiol. Veg., 20: 775-795.

Boumeester HJ, Gershenzon J, Konings MCJM, Croteau R (1998). Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway: I. Demonestration of enzyme activities and their changes with development. Plant Physiol., 117: 901-912.

Brown JA, Alic M, Gold MH (1991). Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: activation by manganese. J. Bacteriol., 173: 4101-4106.

Brun N, Colson M, Perrin A, Voirin B (1991). Chemical and morphological studies of the effects of aging on monoterpene composition in *menthe* × *piperita* leaves. Can J. Bot., 69: 2271-2278.

Buckingham J (1998). Dictionary of natural products on CD-ROM, version 6.1. Chapman and Hall, London.

Coates RM, Elmore CS, Croteau RB, Williams DC, Morimoto H, Williams PG (1997). Stereochemistry of methyl methylene elimination in the enzyme-catalysed cyclization of geranyl diphosphate to (4S)-limonene. Chem. Commun., 21: 2079-2080.

Croteau R, Felton M, Karp F, Kjonaas R (1981). Relationship of camphor biosynthesis to leaf development in sage (*Salvia officinalis*). Plant Physiol., 67: 820-824.

Croteau R, Gershenzon J (1994). Genetic control of monoterpene biosynthesis in mints (Mentha: Lamiaceae). Phytochemistry, 28: 193-229.

Croteau R (1977). Site of monoterpene biosynthesis in *Majorana hortensis* leaves. Plant Physiol., 59: 519-520.

Crowley JD, Traynor DA, Weatherburn DC (2000). Enzymes and proteins containing manganese. Biol. Syst., 37: 209-278.

Culotta VC, Yang M, Hall MD (2005). Manganese transport and trafficking: lessons learned from *Saccharomyces cerevisiae*. Eukaryot. Cell, 4: 1159-1165.

Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Hlzer R, Feller U (2004). Biochemical changes in barley plants after excessive supply of copper and manganese. Environ. Exp. Bot., 52: 253-266.

El-Ghadban EAE (1994). The effect of some trace elements on growth and oil yield of spearmint (*Menth viridis* L.). M. Sc. Thesis, Fac. Agric. Cario Univ. Egypt.

El-Khateeb MA, Farahat M, Boselah NAE (1994). The effect of trace elements on growth, yield and chemical constituents of rue plants (Ruta *graveolens* L.). Egypt. J. Appl. Sci., 7: 75-95.

El-Sawi SA, Mohamed MA (2002). Cumin herb as a new source of essential oils and its response to foliar spray with some micro-elements. Food Chem., 77: 75-80.

Fitzgerald TL, Waters DLE, Henry RJ (2008). The effect of salt on betaine aldehyde dehydrogenase transcript levels and 2-acetyl-1-pyrroline concentration in fragrant and non-fragrant rice (*Oryza sativa*). Plant Sci., 175: 539-546.

Gagandeep S, Dhanalakshmi E, Mendiz A, Rao R, Kale RK (2003). Chemopreventive effects of *Cuminum cyminum* in chemically induced forestomach and uterine cervix tumors in murine model systems. Nutr. Cancer, 2: 171-180.

Gershenzon J, Croteau R (1990). Regulation of monoterpene biosynthesis in higher plants. Recent Adv. Phytochem., 24: 99-160.

Guenther E (1950). The essential oils. D. Van Nostrand Company, Inc., New York.

Gutiérrez M, Rojas LA, Mancilla-Villalobos R, Seelenfreund D, Vicuά R, Lobos S (2008). Analysis of manganese-regulated gene expression in the ligninolytic basidiomycete *Ceriporiopsis subvermispora*. Curr Genet., 54: 163-173.

Hagen G, Uhrhammer N, Guilfoyle TJ (1988). Regulation of expression of an auxin –induced soybean sequence by cadmium. J. Biol. Chem., 263: 6442-6446.

Janahmadi M, Niazi F, Danyali S, Kamalinejad M (2006). Effects of the fruit essential oil of *Cuminum cyminum* Linn. (Apiaceae) on pentylenetetrazol-induced epileptiform activity in F1 neurones of Helix aspersa. J. Ethnopharmacol., 1-2: 278-282.

Kan Y, Kartal M, Zzek T, Aslan S, Baser KH (2007). Composition of essential oil of *cuminum cyminum* L. according to harvesting times. Turkish J. Pharm. Sci., 1: 25 -29.

Kassem AH (2002). Effect of planting distances and some trace elements on rosemary plant. Ph. D. Thesis, Fac. Agric. Cario Univ. Egypt.

Keen CL, Ensunsa JL, Clegg MS (2000). Manganese metabolism in animals and humans including the toxicity of manganese. Met. Ions Biol. Syst., 37: 89-121.

Kjonas R, Croteau R (1983). Demonstration that limonene is the first cyclic intermediate in the biosynthesis of oxygenated p-menthane monoterpenes in *Mentha piperita* and other *Mentha* species. Arch. Biochem. Biophys., 220: 79-89.

Langenheim JH (1994). Higher plant terpenoid a photometric overview of their ecological roles. J. Chem. Ecol., 20: 1223-1280.

Lescure AM, Proudhon D, Pesey H, Raglnd M, Theil EC, Briat JF (1991). Ferritin gene transcription is regulated by iron in soybean cell cultures. Proc. Natl. Acad Sci. U.S.A., 88: 8222-8226.

Lidon FC, Teixeira MG (2000). Oxy radical's production and control in the chloroplast of Mn-treated rice. Plant Sci., 152: 7-15.

Lucker J, Schwab W, Hautum BV, Blaas J, Plas LHW, Bouwmeester HJ, Verhoeven HA (2004). Increased and Altered Fragrance of Tobacco Plants after Metabolic Engineering Using Three Monoterpene Syntheses from Lemon. Plant Physiol., 134: 510-519.

Ma B, Mayfield MB, Godfrey BJ, Gold MH (2004). Novel promoter sequence required for manganese regulation of manganese peroxidase isozyme 1 gene expression in *Phanerochaete chrysosporium*. Eukaryot. Cell, 3: 579-588.

Mahmoud SS, Croteau RB (2002). Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends Plant Sci., 7: 366-373.

Marschner H (1995). Mineral nutrition of higher plants. Academic Press. London, UK, p. 889.

Munoz-Bertomeu J, Ros R, Arrillaga I, Segura J (2008). Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves. Metab. Eng., 10: 166-177.

Nandi RP, Chatterjee SK (1991). Improved cultivation and distillation methods, followed by citronella plantations of Darjeeling hills. Indian Perfumer, 35: 80-85.

Porter NG, Shaw ML, Shaw GJ, Ellingham PJ (1983). Content and composition of dill herb oil in the whole plant and the different plant parts during crop development. New Zealand J. Agric. Res., 26: 119-127.

Sharma SN, Singh R, Tripathi S (1980). Effect of NPK and micronutrients on herb, oil and menthol yield of Japanese mint. Indian J. Agron., 2: 279-281.

Singh N, Luthra R, Sangwan RS (1989). Effect of leaf position and age

on the essential oil quantity and quality in lemongrass (*Cymbopogon flexuosus*). Planta Med., 55: 254-256.
Spices Board Stastistics (2006). Spices Board, Kochi, India.
Swaefy HM (2002). Physiological studies on *trachyspermum ammi* L. (*Carum copticum* Benth) plant. Ph. D. Thesis. Cario Univ. Egypt.
Thiele DJ (1992). Metal-regulated transcription in eukaryotes. Nucl. Acids Res., 20: 1183-1191.
Van AF, Clijsters H (1990). Effects of the metals on enzyme activity in plants. Plant Cell Environ., 3: 195-206.
Voirin B, Bayet C (1996). Developmental changes in the monoterpene composition of *menthe piperita* leaves from individual peltate trichomes. Phytochemistry, 43: 537-580.
Wahab ASA, Hornok L (1983). Effect of NPK fertilization on yield and oil content of *Ocimum basilicum*. Kerteszeti Egypten Kozlemenye, 13: 66.
Weiss EA (2002). Umbelliferae in Spice Crops. CAB International, Wallingford, UK, pp. 261-268.
Wilkinson RE, Ohki K (1988). Influence of Manganese Deficiency and Toxicity on Isoprenoid Syntheses. Plant Physiol., 87: 841-846.

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Full Length Research Paper

Differences in essential oil content and chemical composition of new *Ocimum basilicum* genotyps in relation to some quantitative characters

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The quantitative characters, content and chemical composition of the essential oil have been evaluated in eight genotypes by hybrid origin of *Ocimum basilicum* L. that differ also by the color of leaves and flowers. The content of the essential oil in the assessed genotypes vary from 0.403 to 1.405% (dry matter). The values of the essential oil content are more elevated in the majority of the genotypes with red flowers. A gas chromatographic analysis has demonstrated considerable differences in the chemical composition of essential oil and the concentration of each component depending on the genotype. The number of the identified components varies from 30 to 52 and does not depend on the values of the qualitative characters. The concentration of the major components in essential oil varies in the following way: two genotypes have citral as a primary major component (47.16 to 62.29%), six genotypes have the highest linalool concentration (36.93 to 50.85%), while linalool (10.85%) is the second major component in a genotype and citral is in the other genotype (12.59%), while methylchavicol (9.97 to 24.34%) is in the other three genotypes. The assessed basil genotypes fall into the following five chemotypes: "citral/linalool"; "citral"; "linalool"; "linalool/citral" and "linalool/methylchavicol".

Key words: *Ocimum basilicum*, essential oil, chemical composition, genotype, chemotype.

INTRODUCTION

Ocimum basilicum L., a common basil that belongs to the family *Lamiaceae (Labiatae)*, an annual herb with a fragrant, characteristic scent, is one of the most well-known and appreciated aromatic plants. The species is also used as a medicinal plant in the treatment of a wide range of affections (Akhtar et al., 1992). The flavour as well as vitamin C and carothene make it possible to use basil fresh-cut and dried as a spice (Gonceariuc, 2008; Gonceariuc et al., 2008). Basil synthesizes and accumulates terpenic and phenolic compounds, the antioxidant action of which is apprecited in the pharmacy industry (Grayer et al., 1996; Hancianu et al., 2006; Gonceariuc, 2008). Our earlier investigation has established that the content of polyphenolic and flavonic derivatives is variable and does not strictly correlate with leaf and flower color, though the content of these compounds is high in the genotypes with green leaves and red flowers (Gonceariuc et al., 2008). A certain correlation between the flavon concentration and morphological traits of the assessed samples has not been attested by other investigators, as well (Grayer et al., 1996).

Basil accumulates essential oil (1.5 to 2.0%) (Elementi et al., 2006; Wagner et al., 2007; Gonceariuc, 2008; Valtcho et al., 2008) with a fine balsamic scent

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Table 1. The values of some morphological characters in the *O. basilicum* L. genotypes.

Genotype and distinctive characters	Plant height (cm)		Number of ramifications			
			I-st degree		II-nd degree	
	X	sX	X	sX	X	sX
1-GT(1) green leaves and red flowers	90.4	3.9	10.8	1.3	22.0	4.3
2-GT(2) green leaves and white flowers	55.2	5.2	7.5	2.7	18.9	3.5
3-GT(2) violet leaves and red flowers	43.4	8.9	8.2	3.1	13.9	4.8
4-GT(3) green leaves and white flowers	55.5	4.2	7.3	0.9	29.2	5.5
5-GT(3) reddish-green leaves and red flowers	55.3	4.6	8.2	1.8	29.1	6.3
6-GT(4) green leaves and white flowers	54.1	4.2	8.6	1.4	34.1	7.5
8-GT(5) green leaves and red flowers	59.6	3.2	9.0	1.2	26.2	5.7
9-GTrom green leaves, white flowers and standard	52.4	5.2	10.5	2.3	31.6	6.3

appreciated in perfumery in oleiferous glands situated on leaves and flowers (Grayer et al., 1996; Wagner et al., 2007; Goncealiuc, 2008). The therapeutic properties of *O. basilicum* essential oil are certified as analgetic, antidepressant, antispasmodic, antivenin, carminative, cephalic, digestive, emanogog, expectorant, febrifuge, insecticidal, stomahic, sudorific, tonic and stimulant. Basil essential oil is known to have a pronounced antimicrobial, antiviral and antifungal (Werner and Braunschweig, 2006; Almeida et al., 2007; Wagner et al., 2007; Hussain et al., 2008; Zeljazkov et al., 2008; Banchio et al., 2009) action that varies significantly depending on the time of harvesting and extraction of essential oil (Werner and Braunschweig, 2006; Hussain et al., 2008; Valtcho et al., 2008). This work aims at evaluating the content and chemical composition of essential oil, the concentration of component in the essential oil of new *O. basilicum* genotypes and their correlation with the distinct morphological characters of the new genotypes. However, the chemotype of each genotype will determine the scope of use of genotypes.

MATERIALS AND METHODS

Eight new *O. basilicum* L. genotypes of hybrid origin were used. A number of origins from the Republic of Moldova, Germany, France and Romania were included as parental forms in the hybridization program. The genotypes (hybrids) with a relatively higher *herba* production due to the plants with taller stems, an increased number of ramifications and those that retain leaves until harvesting were included into the study. A genotype from Romania that realized an elevated *herba* production during the precedent years was used as a witness. Essential oil was derived from the aerial part of the plants at the flowering stage through hydrodistillation in the Ginsberg apparatus, while the oil content was recalculated for dry matter. After distillation, the essential oil was dried over anhydrous sodium sulphate.

The chemical composition of the essential oil and the concentration of the components was determined using a gas chromatographic analysis coupled with mass spectrometry (GC-MS). The conditions of the gas chromatographic analysis were as follows: Gas Chromatographer Agilent Technologies type 7890 A GC system, MS agilent Technolies, type 5975 C Mass Selective Detector; Column HP 5 MS 30 m × 0.25 mm × 0.25 µm (5% Phetylmethylsiloxane); injector temperature 2,500℃, detector temperature 2,800℃; the temperature regime of 250℃ (10 degrees/min) up to 280 degrees (const. 5.5 min); mobile phase – helium 1 ml/min; injected volume – 0.1 µl of essential oil; splitting rate of 1:100.

RESULTS AND DISCUSSION

The new basil genotypes of hybrid origin represent an obvious biodiversity confirmed by the leaf color and flower color, as well as the value of the quantitative characters that influence directly *herba* production. For example, plant height varies from 52.4 cm in the genotype 9-GT ROM that originates from Romania and to 90.4 cm in the genotype 1-GT(1) with green leaves and reddish flowers (Table 1). The other two genotypes with red flowers, 5-GT(3) in which the leaves have a reddish nuance and 3-GT(2) with violet leaves and red flowers have a height of 55.3 cm and 43.4 cm, respectively. Thus, no correlation between the flower and leaf color and plant height is observed. The indices of other quantitative characters, such as the ramification number vary considerably. The highest number of the 1st degree ramification was attested in two genotypes: 1-GT(1) and 9-GT (standard), they constituting 10.8 and 10.5, respectively (Table 1). Four genotypes with a high number of the 2nd degree ramifications were recorded, varying from 29.2 and 29.1 in the genotypes 4-GT(3) and 5- GT(3) to 34.1 in 6-GT(4) and 31.6 in the genotype 9-Gtrom (Table 1).

Inflorescence length varies from 12.8 cm to genotype 3-GT(2) to 23.2 cm to 5-GT(3) genotype. Thus, the inflorescences have long been proven to genotype 1-GT(1) and 5-GT(3): 22.8 and 23.2 cm, respectively. In

Table 2. The value of quantitative characters indices of the inflorescence in *O. basilicum* L. genotypes, 2010.

Genotype and distinctive characters	Inflorescence length, cm		Whorls/ central spike of inflorescence		Essential oil content, % (dry matter)
	X	sX	X	sX	
1-GT(1) green leaves and red flowers	22.8	4.1	16.2	2.5	1.191
2-GT(2) green leaves and white flowers	17.2	3.5	14.3	2.2	0.443
3-GT(2) violet leaves and red flowers	12.8	1.9	10.2	1.5	1.405
4-GT(3) green leaves and white flowers	19.7	1.1	11.6	2.0	0.867
5-GT(3) reddish-green leaves and red flowers	23.2	4.6	14.8	1.5	0.403
6-GT(4) green leaves and white flowers	17.0	2.4	13.0	1.1	0.736
8-GT(5) green leaves and red flowers	19.9	1.7	14.0	1.6	1.168
9-GTrom green leaves, white flowers and standard	16.9	2.0	12.0	0.9	0.777

genotype 1-GT(1) central spike inflorescence is compact and includes verticiles 16.2 (Table 2). A definite correlation in both the number of branches, inflorescence length and number of verticiles central spike inflorescence with flower colour and leaf colour was not proven. A comparison of the results obtained with Gonceariuc et al. (2008) shows that they vary considerably depending on the cultivation conditions. The enviromental conditions were draughty with very elevated temperatures. In these conditions, the plant height, the number of ramification and inflorescence length had indices with lower values in all the genotypes than in this study. However, significant differences were not observed as regards the verticil number per inflorescence central spike, the characters having similar values. The evaluated *basil* genotypes vary by the content of essential oil from 0.403% (dry matter) in the genotype 5-GT(3) up to 1.405% in the genotype 3-GT(2) (Table 2). The content of essential oil in three of the genotypes is the highest making 1.191% (dry matter) in the genotype 1-GT(1); 1.405% (dry matter) in the genotype 3-GT(2); and 1.168% (dry matter) in the genotype 8-GT(5). The concentration of essential oil is relatively reduced in the genotype 2-GT(2) making 0.443% (dry matter) and in the genotype 4-GT(3) making 0.403% (dry matter).

It is noteworthy; three of four genotypes with red flowers have an increased content of essential oil (Table 3). Thus, the content of essential oil, as well as the values of morphological characters in the evaluated genotypes varies within quite a wide range. Similar results regarding the variation of essential oil and the chemical composition in relation to the variety, genotype, geographical zone or the stage of basil harvesting are frequently recorded in special literature (Grayer et al., 1996; Lachowicz et al., 1996; Werner et al., 2006; Gille et al., 2008). Gas chromatography analysis of the essential oil extracted from the hybrid genotypes *O. basilicum* demonstrated their biodiversity based on the chemical composition of essential oil. Two major components have been identified in the genotypes 1-GT(1) with reddish flowers and the highest height: E-citral making 27.03% and Z-citral making 20.13% (Table 3). It can be concluded that citral at a concentration of 47.16% is the major component in essential oil of this genotype since citral is a mixture of two isomers: E-citral (geranial or A citral) and Z-citral (neral or citral B) (Lawrence, 1982, 1988; Lawless, 1995; Pihlasalo et al., 2007).

Linalool is another component of the essential oil extracted from the genotype 1-GT (1); its concentration is making 10.85%. Essential oil also contains minor components, such as estragol (methylchavicol), 4.98%; nerol, 4.2% and germacren D, 3.36%. The other 24 components occur in lower concentrations. Citral is also a predominant component of the essential oil extracted from the genotype 2-GT (2) that has green leaves and white flowers (Table 3). The citral concentration in the essential oil of this genotype is increased and constitutes 62.29% (E-citral, 34.65%; Z-citral, 27.64%), followed by nerol, 6.44%, geraniol, 6.53%, linalool at a concentration of only 1.11%. Linalool the concentration of which is the highest and makes 50.85% rather than citral is the predominant component of the essential oil in the genotype 3-GT (2) with violet leaves and red flowers, though derived from the same hybrid combination as the genotype 2-GT(2 (Table 3). Linalool is also the major component (36.93%) in the essential oil extracted from the genotype 4-GT(3) with green leaves and white flowers. This genotype also contains a significant concentration of estragol (methylchavicol) (10.45%). The genotype 5-GT(3) has a composition of the essential oil similar to that of the genotype 4-GT(3) with reddish leaves and red flowers: both genotypes are derived from the same hybrid combination but differ in the colour of leaves and flowers.

Linalool, the major component of the essential oil of the genotype 5-GT(3), has a concentration 44.41%, that is,

Table 3. Chemical composition (%) of the essential oils of eight *O. basilicum* L. genotypes.

Component	Area (%)							
	1-GT(1)	**2-GT(2)**	**3-GT(2)**	**4-GT(3)**	**5-GT(3)**	**6-GT(4)**	**8-GT(5)**	**9-GT,st**
α-pinen	0.08		0.04	0.03			0.02	
β-pinen			0.09	0.09				
Sabinen			0.05	0.04				
Cis-sabinen hidrat			0.20	0.24		0.15	0.11	0.08
Camfen	0.07							
1-octen-3-ol	0.18		0.07	0.10	0.12	0.08	0.09	0.07
2-metil-2-hepten-6-ona				0.15				
β-mircen			0.13	0.12				
6-metil-5-hepten-2-ona		0.19			0.20		0.05	0.03
Mircen					0.04		0.05	0.03
3-octanol				0.03			0.02	0.02
Limonen			0.10	0.10			0.04	0.04
1,8-cineol	0.69	0.12	3.09	3.33	0.32	0.95	0.86	0.39
n-octanol		0.34						
Trans-β-ocimen			0.22	0.46	0.07	0.24	0.16	0.16
Octiciclopropan					0.14			
Cis-sabinen hidrat			0.20	0.24		0.15	0.11	0.08
(+)0-2-caren						0.09		
α-terpinolen			0.16	0.14				
Terpinolen					0.08		0.13	0.22
(+)-fenchona							0.09	
L-linalool	10.85	1.11	50.85	36.93	44.41	37.09	39.41	45.96
L-camfor					1.67			
α-thujone	0.70	0.28						
β-thujone	0.34	0.10	0.31			0.42	0.36	
Mirtenol						0.26		
(+)- linalil acetate							0.06	
Cis-epoxi-ocimen							0.23	0.31
Camfor	0.78		1.18	1.72		1.35	1.76	1.63
Borneol								0.34
L-borneol	0.21	0.06	0.98		0.37			
Endo-borneol						0.48		
Trans-crizantemal	0.34	0.87						
Vinilciclooctan					0.76			
α-terpineol							0.77	
4-terpineol			0.29			0.63		
β-fenchol	0.48	0.13	1.48	1.26	0.58	0.81		0.60
Izoborneol							0.36	
Estragol (methylchavicol)	4.98	0.72	3.78	10.45	2.94	9.97	24.34	3.01
n-octilacetat		0.74	0.24					
Fenchilacetat							0.07	
α-fenchilacetat		0.34						
Nerol	4.26	6.44	0.50	1.47	2.71	1.43	0.40	1.72
Z-citral (neral)	20.13	27.64	0.74	3.57	6.23	1.14	1.36	0.68
Geraniol	6.98	6.53		4.22	7.78	3.98	2.09	4.60
Geraniolformat			1.19					
E-citral (geranial)	27.03	34.65	1.06		6.36	1.41	1.68	0.86

Table 3. Contd.

Bornilacetat	0.47		1.88	0.61	0.87	1.09	0.56	1.24
Metilgeranat					0.19			
α-terpinilpropionat			0.05					
Cis-3-hexenil tiglat							0.06	
α-cubeben		0.08		0.06	0.12		0.05	0.03
Nerilacetat								0.18
Nerilpropionat					0.20			
Geranilizobutirat		0.37						
Eugenol			5.62	2.00		0.97	0.44	
Geranilpropionat	0.30	0.28					0.18	
Calaren	0.55							
β-cariofilen	2.95	3.59	0.41	1.69	1.51	0.71	0.95	0.58
γ--himachalen				0.06				0.06
α-bergamoten	1.31			0.89	0.99			
α-copaen		0.51	0.23		0.53	0.30	0.25	0.40
α-bourbonen						0.19		
β-bourbonen		0.09	0.08	0.06				0.10
β-elemen			1.17	2.22	1.45	2.31	1.92	2.35
Metileugenol			0.51	0.18			2.04	
Trans-α-bergamoten	0.32	0.99	5.31			3.13	1.52	1.32
α-guaien			0.65	1.16	0.57	0.98	0.74	0.98
(E,Z)-α-farnesen				0.09				0.05
(+)-β-funebren			0.91					
Trans-β-farnesen		0.39					0.88	
α-humulen	1.03	0.59	0.67	0.90	0.86	1.37	0.83	1.36
β-cubeben	0.15		0.17			0.19		0.25
Nerolidol								0.68
d-nerolidol			0.10		0.24		0.19	
Nerolidol B						0.52		
(-)-α-selinen			0.22					
β-selinen							0.19	0.11
Epi-biciclosescvifelandren			0.60	0.63	0.54	0.88	0.49	1.53
β-paciulen								0.29
Germacren D	3.36	3.28	3.25	4.39	4.91	7.72	4.56	7.44
Zingiberen	0.18	0.13				0.17		0.05
Elemol								0.08
β-bisabolen		0.18		0.12				0.23
Aromadendren				0.48			0.24	0.35
Biciclogermacren	0.33		0.95	1.18	0.78	1.76	1.21	1.19
Delta-guaien			1.00	1.64	1.00	1.79	1.41	1.81
α-amorfen	0.46		0.17	1.94	0.91	2.65	1.58	2.85
α-muurolen			0.30					0.22
γ-muurolen			4.57	5.21	3.43			0.15
Delta-muurolen	1.44	0.18						
γ-cadinen			1.74	0.46	1.54		3.98	
Delta-cadinen		0.13		0.25	0.27	0.96	0.24	0.35
β-sescvifelandren			0.46					
Cadina-1,4-diena			0.65					0.13
(-)-izoleden					0.34			

Table 3. Contd.

Cis-α-bisabolen	2.55	3.19		0.39	0.55	0.40	0.18	
Farnesol				0.28				
Epizonaren				0.12				
α-selinen				0.11	0.19			0.57
T-cadinol						6.68	0.15	8.69
Cis-3-hexenil benzoat							0.03	0.05
γ-gurjunen					0.48		0.38	
α-elemen						0.61		
Total	93.5	94.24	98.62	91.81	97.25	96.01	99.87	96.50
Total components identified	30	30	47	45	39	39	52	51

more elevated than in the essential oil of the genotype 4-GT(3). Citral, 12.59% (Z-citral, 6.23%; E-citral, 6.36%) and geraniol, 7.78% are among minor components but with significant concentrations in the essential oil of the genotype 5-GT(3). A gas chromatogram of the essential oil extracted from the *herba* of the genotype 6-GT(4) with green leaves and white flowers has identified 39 components, the major component being linalool at a concentration of 37.09% but which also contains concentrations relatively elevated for the minor components of estragol (methylchavicol), 9.97%; germacren D, 7.72%, and T-cadinol, 6.68% (Table 3). Two major components, linalool – 39.41% and estragol (methylchavicol) – 24.34% have been identified in the essential oil of the genotype 8-GT (5) with green leaves and red flowers. Two components, germacren-D (4.56%) and γ-cadinen (3.98%) can be mentioned among the minor constituents with relatively higher concentrations (Table 3).

The genotype 9-GTrom, a standard with green leaves and white flowers is distinguished by the fact that it has a single major component in the essential oil, linalool – 45.96%, followed by three minor constituents that are more important for the concentration: geraniol (4 to 6%), germacren D (7.44%), and T-cadinol (8.69%). A gas chromatographic analysis of basil essential oil has demonstrated the biodiversity of the evaluated genotypes. The number of the components identified in the essential oils and their concentrations are different in the genotypes, the number varying from 30 in the genotypes 1-GT(1) and 2-GT(2) to 52 in the genotype 8-GT(5) (Table 3). It should be mentioned that all the genotypes contain the following eight components in the essential oil: 1.8-cineol, linalool, estragol (methylchavicol), nerol, citral, β-cariofilen, α-humulen and germacren D. Their concentrations vary considerably in all the genotypes. Thus, the linalool concentration varies from 1.11% in the genotype 2-GT(2) to 50.85% in the genotype 3-GT(2), while the concentration in Z-citral (neral) varies from 0.68% in the essential oil of the standard genotype 9-Gtrom to 27.64% in the oil of the genotype 2-GT(2). The other 31 components have been identified in the essential oil of only one of the eight assessed genotypes. Some components are present in the oil of two genotypes.

As a cross-fertilized species, *O. basilicum* is represented by many genotypes and varieties that are distinguished for plant habitat, the color and sizes of leaves, the color of flowers, the content and chemical composition of essential oil (Lachowicz et al., 1996; Voitkevici, 1999; Hancianu et al., 2006; Teli et al., 2006; Gille et al., 2008), as well as for geographic origin, pedoclimatic cultivation conditions. *O. basilicum* is known to synthesize two types of essential oil: 1- the most cultivated in Europe and America with major components of essential oil, methylchavicol, linalool is of a high quality with a tender aroma and that does not contain camphor; 2- common basil from Africa with the major component, methylchavicol and an appreciated content of camphor (Viturro et al., 1999). Camphor is contained in the essential oil extracted from six assessed genotypes at concentrations varying from 0.78 to 1.76% (Table 3). The chemical composition, the concentration of essential oil components in basil are variable characters that are controlled genetically and may be modified, including through hybridization. A study on the heritability of the major constituents of the essential oil of the *Ocimum* genus has demonstrated that the development of four chemotypes of *O. basilicum* that would synthesize and accumulate essential oil at the following concentrations of the major component – 1) linalool; 2) methylchavicol; 3) methyleugenol; 4) methylcinnamate can be predicted (Lawrence, 1982, 1988; Voitkevici, 1999).

Seven chemotypes, including linalool, methylcinnamate, methylcinnamate/linalool, methyleugenol, citral, methylchavicol (estragol) and methylchavicol/citral have been identified in the experiments with *O. basilicum* (Wagner et al., 2007). Other researchers have found that

the major components of the essential oil extracted from some varieties *O. basilicum* of the USA are linalool (30 to 40%) and eugenol (8 to 30%) (Amparo and Elizabeth, 2003; Zheljazkov et al., 2008), or linalool and methylchavicol (Marotti et al., 1996). Our evaluation of the basil genotypes for the major components has shown that their concentration in essential oil differs from those predicted and described by other authors. Thus, two genotypes have citral as a primary major component (47.16 to 62.29%), while the other six genotypes have the highest linalool concentration (36.93 to 50.85%). Linalool (10.85%) is the second major component in a genotype; citral is in the other genotype (12.59%), while methylchavicol (9.97 to 24.34%) is in the other three genotypes. Actually, each genotype has a unique chemotype. Even the genotypes that have the same two major components differ by the third and/or fourth component.

The different major components of the essential oil have the genotypes that originate from the same hybrid combination. Thus, the major components are different in all the genotypes of hybrid origin and all these genotypes differ from the standard genotype and have no correlation with the quantitative characters, leaf and flower colour. The results of the chromatographic analysis allow a statement that the assessed genotypes form five chemotypes including: “citral/linalool”, “citral”, “linalool”, and “linalool/citral and linalool/methylchavicol” chemotypes. The number of the minor components that are more important for their concentration is six (eugenol, geraniol, nerol, trans α-bergamoten, germacren D, and T-cadinol). No correlation has been found between the quantitative characters that influence *herba* production, the content of essential oil and the number of the identified components, their concentration in essential oil, basil chemotype. In our opinion, this variability results from hybridization in which the genotypes of different genetic and geographical origin have taken part and has a genetic nature.

Conclusion

The quantitative characters, the content and chemical composition of the essential oil have been evaluated in eight genotypes *O. basilicum* L. of hybrid origin that differ also by the colour of leaves and flowers. The indices of the quantitative character values in the assessed basil genotypes vary considerably depending on the genotype and cultivation conditions, but no correlation has been attested between these characters and the colour of leaves and flowers. The content of the essential oil in the assessed genotypes vary from 0.403 to 1.405% (dry matter). The values of the essential oil content are more elevated (1.168 to 1.405% (dry matter) in the majority of the genotypes with red flowers. A gas chromatographic analysis has demonstrated considerable differences in the chemical composition of essential oil and the concentration of each component depending on the genotype. The number of the identified components varies from 30 to 52 and does not depend on the values of the qualitative characters, the colour of leaves and flowers. The concentration of the major components in essential oil varies in the following way: two genotypes have citral as a primary major component (47.16 to 62.29%), six genotypes have the highest linalool concentration (36.93 to 50.85%), while linalool (10.85%) is the second major component in a genotype and citral is in the other genotype (12.59%), while methylchavicol (9.97 to 24.34%) is in the other three genotypes. The assessed genotypes *O. basilicum* fall into the following five chemotypes: “citral/linalool”, “citral”; “linalool”, “linalool/citral” and “linalool/methylchavicol”.

REFERENCES

Akhtar MS, Akhtar AH, Khan A (1992). Antiulcerogenic Effects of *Ocimum basilicum* Extracts, Volatile Oils and Flavonoid Glycosides in Albino Rats. J. Pharmacogn., 3(2): 97-104.

Almeida I, Alviano DS, Vieira DP, Alves PB, Blank AF, Lopes AH, Alviano CS, Rosa MS (2007). Antigiardial activity of *Ocimum basilicum* essential oil. J. Parasitol. Res., 101(2): 443-452.

Amparo V, Elizabeth M (2003). Essential oil composition from twelve varieties of basil (Ocimum spp) grown in Colombia. J. Braz. Chem. Soc., 14(5): 121-128.

Banchio E, Xie X, Zhang H, Pare PW (2009). Soil Bacteria Elevate Essential Oil Accumulation and Emissions in Sweet Basil. J. Agric. Food Chem., 57(2): 653-657.

Elementi S, Neri R, D'Antuono LF(2006). Biodiversity and selection of “European” basil (*Ocimum basilicum* L.) types. J. Acta Hortic., 2: 723.

Gille E, Danila D, Hancianu M, Aprotosoae C, Spac A, Gonceariuc M, Stănescu U (2008). Contribution to the study of secondary metabolites bioaccumulation in the aerial blossomed parts of *Ocimum basilicum* L. Rev. Med. Chir. Soc. Med. Nat., 112(2): 254-261.

Gonceariuc M (2008). *Ocimum basilicum* L. In: Cultivated medicinal and aromatic plants. Edit. Center UASM, Chisinau, pp. 18-25.

Gonceariuc M, Gille E, Florea C, Brânzilă I, Danila D (2008). Creating and evaluating the *Ocimum basilicum* L. genotypes. A.S.M. Bull. Life Sci., 1(304): 94-100.

Grayer RJ, Bryan SE, Veitch NC, Goldstone FJ, Paton A, Wollenweber E (1996). External flavones in sweet basil, *Ocimum basilicum*, and related taxa. J. Phitochem., 43(5): 1041-1047.

Hancianu M, Gille E, Constantin L, Aprotosoae C, Miron A, Cioanca O, Curca D, Stanescu U (2006). Phytochemical and pharmacological study of a new extract obtained from *Ocimum basilicum* L. and *Ocimum sanctum* L. J. Rom. Biol. Sci., 4(3-4): 33-38.

Hussain AI, Anwar F, Hussain SST, Przybylski R (2008). Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. J. Food Chem., 108(3): 986-995.

Lachowicz KJ, Gwyn PJ, Briggs D, Bienvenu F, Palmer M, Ting S, Hunter M (1996). Characteristics of Essential Oil from (*Ocimum basilicum* L.) Grown in Australia. J. Agric. Food Chem., 44(3): 877-881.

Lawless J (1995). The Illustrated Encyclopedia of Essential Oils, p. 242.

Lawrence VM (1982). Materials VIII[th] International Congress of Essential oils, p. 111.

Lawrence VM (1988). Proceedings of the Xth International Congress of Essential oils. Fragr. Flav., p. 161.
Marotti M, Piccaglia R, Giovanelli E (1996). Differences in Essential Oil Composition of Basil (*Ocimum basilicum* L.) Italian Cultivars Related to Morphological Characteristics. J. Agric. Food Chem., 44(12): 3926-3929.
Pihlasalo J, Klika KD, Murzin DY, Nieminen V (2007). Conformational equilibria of citral. Journal of Molecular Structure: THEOCHEM., 814(1-3): 33-41.
Teli I, Bayram E, Yilmaz G, Avci B (2006). Variability in essential oil composition of Turkish basils (*Ocimum basilicum*). J. Biochem. Syst. Ecol., 34(6): 489-497.
Valtcho DZ, Cantrell C, Tekwani B, Shabana I K (2008). Content, Composition and Bioactivity of the Essential Oils of Three Basil Genotypes as a Function of harvesting. J. Agric. Food Chem., 56(2): 380-385.
Viturro CI, Molina AC, Villa WC, Villa WC, Saavedra ON (1999). Preliminary assays of adaptation in Jujuy (Argentina) of *Satureja hortensis* L., *Ocimum basilicum* L. and *Coriandrum sativum* L. J. Acta Horticulturae 500: II WOCMAP Congress Med. and Aromatic Plants.
Voitkevici SA (1999). Essential oils for perfumes and aromatherapy, Moskva, p. 38.
Wagner H, Vollmar A, Bechthold A (2007). Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart. J. Pharm. Biol., 2(7): 396-401.
Werner M, Braunschweig R (2006). Praxis Aromatherapy.
Zheljazkov V, Cantell CL, Tekwani B, Khan S (2008). Content, composition, and bioactivity of essential oils of the three Basil genotypes as a function of harvesting. J. Agric. Food Chem., 56(2): 380-385.

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Full Length Research Paper

Morphology and histochemistry of the glandular trichomes of *Isodon rubescens* (Hemsley) H. Hara [Lamiaceae]: A promising medicinal plant of China

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***Isodon rubescens*, a perennial herb indigenous to China, with medicinal application, potentially has economic value. The morphology of the glandular trichomes was investigated with light microscopy. At the same time the chemical content was analyzed by applying chemical reagents and fluorescence microscopy. This morphoanatomical and histochemical study revealed that leaves of *I. rubescens* possess one type of non-glandular and two types of glandular trichomes, with the latter differing both anatomically and in the composition of their secondary metabolites. Non-glandular trichomes were uniseriate with an ornamented surface. Peltate and capitate glandular trichomes comprised one basal cell, one stalk cell and one head. The head of mature peltate glandular trichomes consisted of four-twelve secretory cells while that of the capitate glandular hairs was comprised of two cells. Peltate glandular trichomes containing compounds of terpenoid nature are probably the main site of oridonin and ponicidin accumulation. The fluorescent stain of peltate and capitate glandular trichomes indicated the possible presence of phenolic compounds.**

Key words: *Isodon rubescens*, Lamiaceae, glandular trichomes, microscopy, histochemistry, terpenoids, phenolic.

INTRODUCTION

The genus *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae) contains ca. 100 species distributed predominantly in tropical and subtropical Asia, with the center of diversity in southwestern China with outliers in tropical Africa (Wu and Li 1977; Codd 1984; Li 1988; Li and Hedge, 1994). The use of *Isodon* species in Chinese popular folk medicine has a long tradition, among which, *Isodon rubescens* (Hemsley) H. Hara, a perennial herb of the *Isodon* genus, is native to the Yellow River valley of China. The leaves of *I. rubescens*, which is the most studied species and is known in China by the name, "*donglingcao*", are still used by the local people in Henan province for the treatment of respiratory and gastrointestinal bacterial infections, inflammation, and cancer (Sun et al., 2006). Most Lamiaceae species have glandular trichomes that emerge from the epidermal surface of their leaves, stems and reproductive structures. Generally, trichomes are divided into two subcategories, glandular and non-glandular (Wagner et al., 2004). Glandular trichomes secrete various types of compounds. A growing body of experimental evidence shows that terpene biosynthesis takes place within these trichomes (Croteau and Johnson, 1984; Hay and Svoboda, 1993; Duke et al., 2000; Hallahan, 2000; Siebert, 2004). Terpenes usually constitute the major lipophilic components of these secretions. Pharmacological study has shown that the major constituents of *I. rubescens* are diterpenoids, especially oridonin and ponicidin, which have significant antiangiogenic activity (Meade-Tollin et al., 2004).

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Other types of compounds found in *I. rubescens* include triterpenoids and flavonids. However, to date, knowledge of the morphology and functioning of *I. rubescens* trichomes has been absent, therefore, we carried out an investigation of the morphology, distribution, and histochemistry of the glandular trichomes present on leaves of *I. rubescens*, with the aim of clarifying their role. The investigations described here are motivated by an interest in the pharmacological properties of the species. The identification of structures responsible for oridonin and ponicidin production and accumulation is useful from an economic standpoint because it can be used to develop strategies for maximizing yields of useful compounds. This information is also meaningful from an ecological perspective because it can help us better understand what role these compounds may have in the plant's ecology, and may lead to a better understanding of natural plant protection.

MATERIALS AND METHODS

Plant material

Fresh aerial plant parts of *I. rubescens* were harvested in August (2010) from the Chinese Herb Garden, Henan College of Traditional Chinese Medicine. For the purpose of these investigations, the term 'mature leaves' refers to vegetative leaves harvested from the fourth and fifth nodes of the plant, whilst 'young leaves' describes leaves that had recently emerged from the first node. Light microscopy and histochemical investigations were performed on fresh, as well as air-dried material.

Light microscopy

Leaf material was cut into small pieces (5 mm^2) in the laboratory and fixed in FAA (formalin–glacial acetic acid–70% ethanol, 5: 5 : 90, v/v) for 48 h. The sample was dehydrated in an ethanol dilution series (30 50, 70, 85, 95 and 100 % ethanol, followed by 2 × 100% xylene). The sample was then embedded in wax before sectioning to 10 to 15 μm thick using a microtome dissector. The sections were dewaxed using two changes of xylene, and then rehydrated using descending grades of ethanol (up to 50%) and finally water. The Sections were stained with 0.5% toluidine blue-O for approximately 2 min, and briefly dehydrated in ascending grades of alcohol. The alcohol was washed off (2 washes) in xylene before mounting using neutral balsam. Slides were viewed and photographed with a Leica DM3000 light microscope. Leaf clearing was done with 10% NaOH solution followed by water and further clearing with 25% Sodium hypochlorite. Chloral hydrate 250% solution was used as mordant, specimens were mounted on slides with neutral balsam and photographed with a Leica DM3000 light microscope.

Histochemical investigations

A Natural Product reagent was prepared by mixing aqueous AlCl3 solution 5 and 0.05% diphenylboric acid-b-ethylaminoester in 10% methanol as described by Heinrich et al. (2002) for the detection of flavonoids. Entire leaves were soaked in the solution for 10 min, after which the plant material was dried on absorbent paper and mounted on a glass slide. Vanillin-HCl was prepared by dissolving 0.1 g vanillin in 100 ml concentrated HCl (Nikolakaki and Christodoulakis, 2004). This reagent is a universal indicator of various compounds, enabling differentiation between different classes of secondary metabolites such as flavonoids, terpenoids and phenyl carboxylic acids based on the colouration obtained (Wagner and Bladt, 1996). Leaves were soaked for 2 min and then dried on absorbent paper before being mounted on a glass slide. Hand-cut sections of fresh leaf tissue were stained with vanillin–HCl for terpenoids. Slides were viewed directly under UV and GF (Green fluorescence) light with a Leica DM5500 fluorescence microscope.

RESULTS

The indumentum of *I. rubescens* leaves consisted of glandular and non-glandular trichomes distributed on both the adaxial and abaxial surfaces (Figure 1A and B). The non-glandular trichomes were distributed mainly on the veins and leaf margins and appeared more abundant on the abaxial leaf surface. In young leaves, the non-glandular trichomes partially obscured the glandular trichomes and it appeared that they matured at an early stage of leaf development (Figure 1A). With leaf expansion, both non-glandular and glandular trichomes density decreased progressively as shown in Figure 1. Morphologically, non-glandular trichomes were uniseriate, and sharply pointed with warts on the surface (Figure 1C). The glandular trichomes were classified into two types: peltate and capitate. (Figure 1B and D). The peltate glandular trichomes consisted of a basal cell embedded in the epidermis, a unicellular stalk and a large spherical or slightly flattened multicellular secretory head (Figure 1 I). The heads of mature peltate glandular trichomes generally consisted of four cells that were enclosed in a smooth cuticle, but twelve-celled heads were also found (Figure 1E and F). The head cells were grouped together above the stalk cell, surrounding its central axis (Figure 1E). The fully developed head was quadrilobate and 40 ± 2 μm in diameter. The elevated cuticle of the head possibly accumulated secretory material (Figure 1 I). The secretions caused the cuticle to lift and expand, giving it a tumescent, somewhat globular appearance (Figure 1D and F). The peltate glandular trichomes had a single basal cell, a unicellular stalk and a bicellular secretory head (Figure 1G and H) The capitate glandular trichomes were typically 18 ± 2 μm tall, with a head that is 18 ± 2 μm in diameter. Peltate and capitate glandular trichomes were also observed on the stems, cotyledons, rachises, pedicles, bracts and calyces.

Histochemistry of glandular trichomes

On the young leaf surface, numerous capitate glandular trichomes had a blue fluorescence (Figure 2A and 2B) under UV light when stained with the Natural Product reagent, indicating the presence of flavonoids. Peltate

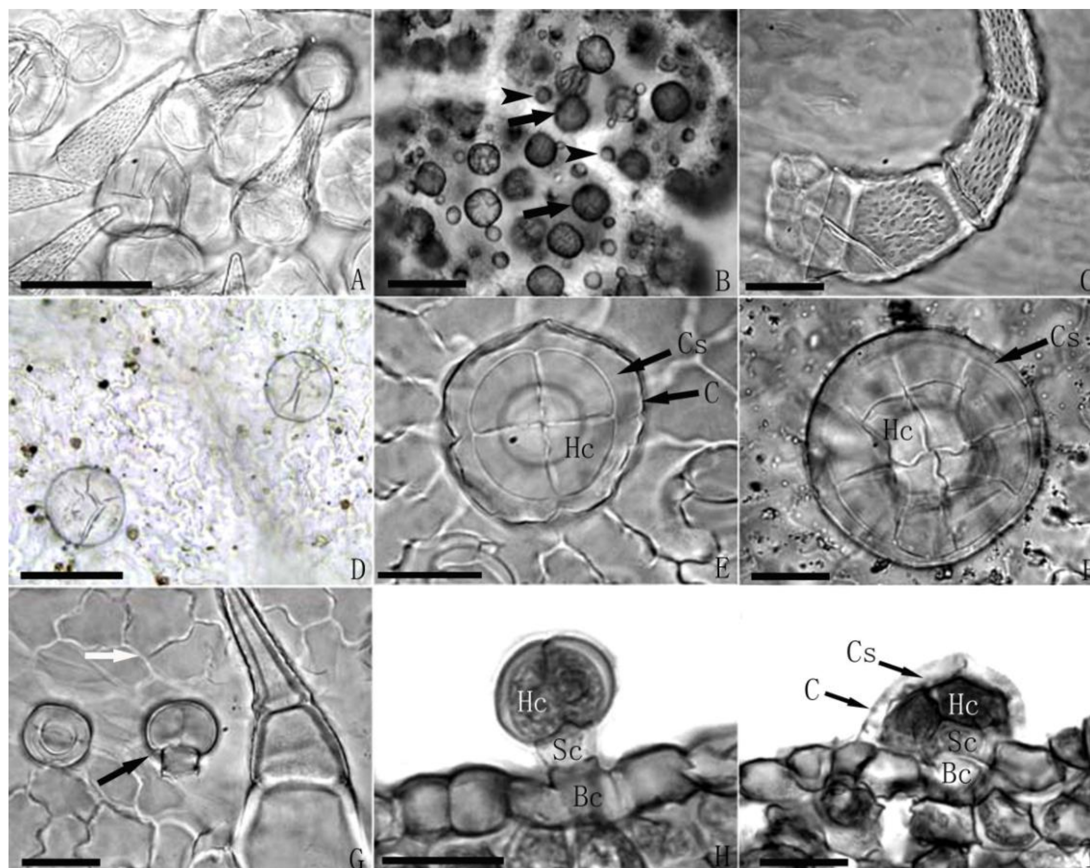

Figure 1. Trichomes of *I. rubescens*. A. Glandular and non-glandular trichomes on the abaxial leaf surface of a young leaf. Bar = 50 μm. B. Capitate (arrowheads) and peltate (arrows) glandular trichomes on the abaxial leaf surface. Bar = 100 μm. C. Non-glandular trichome with warts on leaf surface. Bar = 50 μm. D. Mature peltate glandular trichome. Bar = 50 μm. E. Mature peltate glandular trichome with 4-celled secretory head (H) assembly. The circle in the center indicates the anticlinal wall of the stalk cell. Arrows indicate cuticle(C) and subcuticular space (Cs). Bar = 20 μm. F. Mature peltate glandular trichome with 12-celled secretory head (H) assembly. The arrow indicates subcuticular space (Cs). Bar = 20 μm. G. The arrow indicates capitate glandular trichome on leaf surface. Bar = 50 μm. H. Capitate glandular trichome in longitudinal section. Bar = 20 μm. I. Peltate glandular trichome in longitudinal section. Arrows indicate cuticle(C) and subcuticular space (Cs). Bar = 20 μm. (H and I). Bc = basal cell; Hc = head cell; Sc = stalk cell.

glandular trichomes of mature leaves showed intense fluorescence after being treated with the natural product reagent (Figure 2D and 2E) or vanillin–HCl (Figure 2F, 2G and 2H), respectively. After 10 days of drying, peltate glandular trichomes still showed intense fluorescence following staining with vanillin–HCl (Figure 2I). However, the rest of the tissue did not fluoresce following staining with vanillin–HCl (Figure 2I). Capitate glandular trichomes didn't fluoresce on mature or dried leaves as shown in Figure 2.

DISCUSSION

Like other members of the Lamiaceae, *I. rubescens* leaves possess one type of non-glandular and two types of glandular trichomes (called peltate and capitate), on both adaxial and abaxial surfaces. The density of trichomes, including non-glandular and glandular, gradually decreases with leaf maturity. The observation that non-glandular trichomes did not give any positive reaction supports the argument that their role is protection from water loss, the regulation of temperature through their reflective capacity and mechanical protection from herbivores (Ascensão et al., 1995, 1999; Yashodhara et al., 2001).The density of glandular trichomes decreases with leaf growth (young leaves have a denser pubescence). This might be an adaptive mechanism, whereby the young leaves, most tender and appetizing to herbivores, are given the highest protection (many secretions of glandular trichomes are deterrent or toxic to insects). The density of glandular trichomes is further considered to be associated with transpiration, leaf overheating, UV-B radiation, etc. (Wagner et al.,

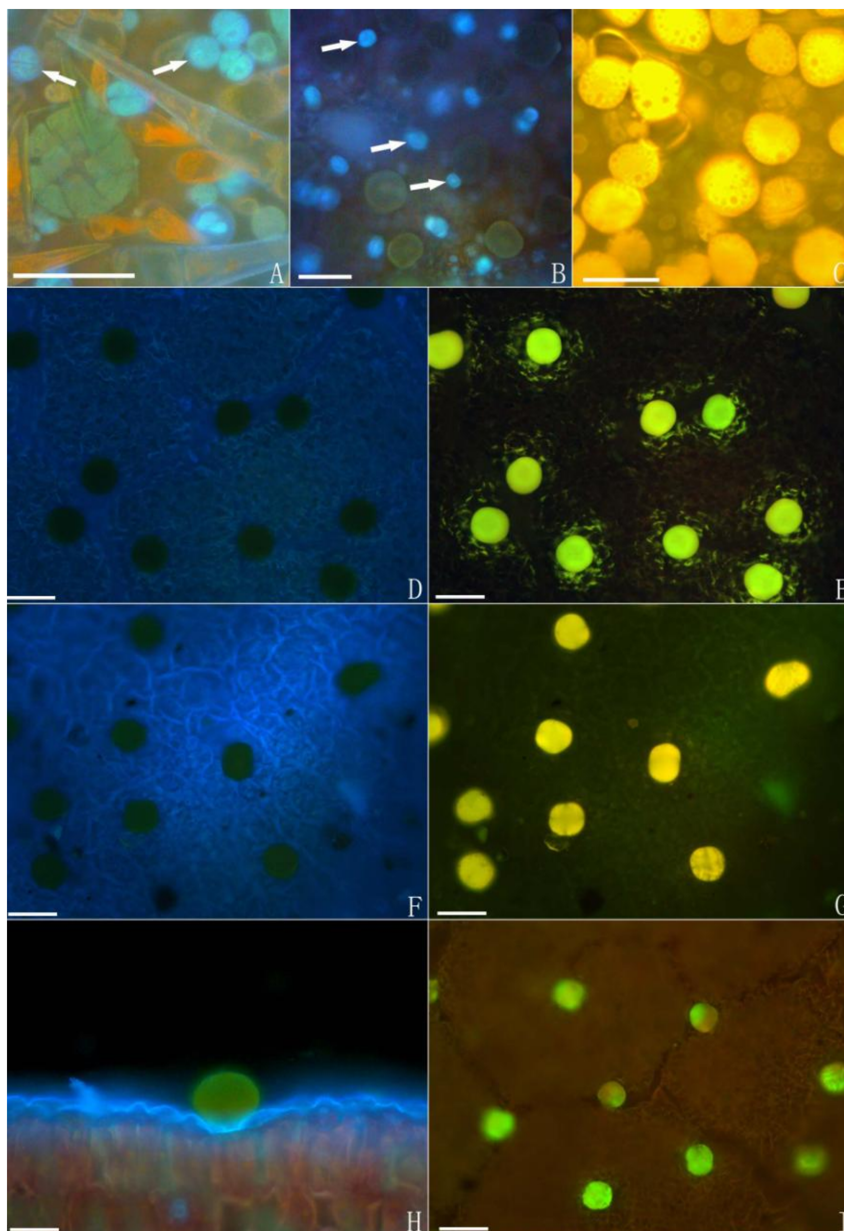

Figure 2A and B. Arrows indicate capitate glandular trichomes of a young leaf following staining with the Natural Product reagent. C. Peltate glandular trichomes on the adaxial surface of a young leaf following staining with vanillin–HCl. D and E. Paired micrographs of peltate glandular trichomes on a mature leaf surface fluoresce after staining with the Natural Product reagent. F and G. Paired micrographs of peltate glandular trichomes on a mature leaf surface after staining with vanillin–HCl. H. Fluorescence micrograph of free-hand section using mature leaves. Note the bright fluorescence of peltate trichome after staining with vanillin–HCl. I. Peltate glandular trichomes on a dried leaf surface fluoresce after staining with vanillin–HCl. A, B, D, F and H. Indicate glandular trichomes that exhibit fluorescence when exposed to UV light, while C, E, G and I. indicate fluorescence when exposed to green fluorescence (GF) light. Scale bars: H = 100 μm, others = 50 μm.

2004). Most studies on glandular trichomes use histochemical methods (Ascensão et al., 1999; Bisio et al., 1999; Corsi and Bottega 1999; Bottega and Corsi 2000; Nikolakaki and Christodoulakis 2004, 2007) because they are considered useful for an initial investigation of the presence of some substances. In this

work, histochemical tests are applied for the first time to the leaves of *I. rubescens*. Autofluorescence is diagnostic of flavonoids, which, depending on the structural type, show dark yellow, green or blue fluorescence under UV light (Wagner and Bladt, 1996). Natural product reagent was used to enhance the natural autofluorescence of phenolic compounds (Andary et al., 1984). The fact that capitate glandular trichomes exhibited light blue fluorescence when GF was used indicated presence of flavonoids (Figure 2B). However, in mature leaves capitate glandular trichomes showed no fluorescence. Observations made during this study suggest that the content of capitate glandular trichomes are metabolically altered during leaf maturation and result in disappearance of fluorescence. These short-term capitate glandular trichomes are assumed to function for a very short period during the early development of young organs (Fahn, 1988; Duke and Paul, 1993). As compared to the capitate glandular trichomes, peltate glandular trichomes of *I. rubescens* have a short one-celled stalk and a large round or slightly flattened head. During the secretion phase, peltate glandular trichomes of *I. rubescens* have a characteristic spherically shaped head due to the development of a large sub-cuticular space where possibly secretory products accumulate. Terpenoid secretion was found to be restricted to peltate glandular trichomes. The remarkable colour, typifying terpenoids, obtained with vanillin–HCl (Figure 2F and 2G) was due to the mixed colour reactions of individual terpenoids. However, this reaction is not specific for diterpenoids. So, the reaction does not necessarily indicate that the peltate glandular trichomes contain diterpenoids. More telling is the lack of a reaction in the rest of the tissue. The vanillin–HCl is quite sensitive to terpenoids, so a lack of a reaction is a clear indication that diterpenoids are absent in these tissues. The presence of phenolic compounds in peltate trichomes was also indicated by colour reactions. The fact that peltate trichomes exhibited yellow fluorescence when GF was used indicated presence of flavonoids. The different colour reactions between peltate trichomes and capitate trichomes using the natural product reagent indicating structural difference of flavonoids. The peltate glandular trichomes of *I. rubescens* are regarded as long-term glandular trichomes in which the secretory material appears to accumulate gradually and consistently under elevated cuticular sacs during the development and growth of the aerial parts of *I. rubescens*. These glandular trichomes are believed to play a vital role in defensive mechanisms against the pathogens and herbivores (Werker, 1993). Present observations suggest that oridonin and ponicidin are possibly only present in peltate glandular trichomes. The number of glandular trichomes on the leaves is linearly associated with the yield in terpenes. Thus, the greater the number of glandular trichomes on the leaves, the higher the amount of terpene substances derived from them by distillation (Bosabalidis and Kokkini, 1997). This is due to the fact that the glandular trichomes are the main leaf sites of terpene biosynthesis and possess a complete enzymatic equipment (McCaskill and Croteau, 1995). Oridonin yields from leaves are up to 20 times as high as from stems (Lu et al., 2000). This dramatic differentiation is primarily due to the fact that the stems are largely composed of woody vascular tissue and pith, which do not contain oridonin. Stems typically weigh about ten times as much as leaves when dry sections of both organs with equal adaxial surface areas are compared. The density of peltate glandular trichomes on the abaxial stem surface is about the same as that on the abaxial leaf surface (Siebert, 2004). The concentration of these compounds in whole stems and whole leaves differs in a manner that roughly corresponds to their different ratios of weight to abaxial surface area. Oridonin yield is directly related to the distribution and density of terpenoid-bearing glands. In the case of *I. rubenscens*, whole plants would have to be utilized for any industrial production of oridonin since stems are difficult to separate from leaves.

However our study does not provide direct proof that these compounds are present inside, or are derived from peltate glandular trichomes. Only further investigation (for example, through shearing off trichomes, isolating them and analyzing their content) will determine which type of trichomes is responsible for each of the various compounds.

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REFERENCES

Andary C, Roussel JL, Rascol JP, Privat G (1984). Micro methode analysis of acid esters. J.Chromatogr., 303: 312–317.
Ascensão L, Marques N, Pais, MS (1995). Glandular trichomes on vegetative and reproductive organs of *Leonotis leonurus* (Lamiaceae). Ann. Bot., 75: 619–626.
Ascensão L, Moat L, Castro MM (1999). Glandular trichomes on the leaves and flowers of *Plectranthus ornatus*. morphology, distribution and histochemistry. Ann. Bot., 84: 437–447.
Bisio A, Corallo A, Gastaldo P, Romussi G, Ciarallo G, Fontana N, De Tommasi N, Profumo P (1999). Glandular hairs and secreted material in *Salvia blepharophylla* Brandegee ex Epling grown in Italy. Ann. Bot., 83: 441-452.
Bosabalidis AM, Kokkini S (1997). Intraspecific variation of leaf anatomy in Origanum vulgare grown wild in Greece, Bot. J. Linn. Soc., 123 (4): 353-362.
Bottega S, Corsi G (2000). Structure, secretion and possible functions of calyx glandular hairs of *Rosmarinus officinalis* L. (Labiatae). Bot. J. Linn. Soc., 132: 325-335.
Codd LE (1984). The genus *Isodon* (Schrad. ex Benth.) Spach in Africa and a new genus *Rabdosiella* Codd (Lamiaceae) . Bothalia, 15: 7-10.

Corsi G, Bottega S (1999) Glandular hairs of *Salvia officinalis*: new data on morphology, localization and histochemistry in relation to function. Ann. Bot., 84: 657-664.

Croteau R, Johnson MA (1984). Biosynthesis of terpenoids in glandular trichomes. In: Rodriguez, E., Healey, P.L., Mehta, I., eds. Biology and chemistry of plant trichomes. Plenum Press, New York, pp. 133-185.

Duke SO, Canel C, Rimando AM, Tellez MR, Duke MV, Paul RN (2000). Current and potential exploitation of plant glandular trichome productivity. In: Hallahan DL, Gray JC, eds. Advances in botanical research. Incorporating advances in plant pathology. Vol. 31. Plant trichomes. Academic Press, London, pp. 121-151.

Duke OS, Paul NR (1993). Development and fine structures of the glandular trichome in *Artemisia Annua L.* Int. J. Plant. Sci., 154(1): 107-118.

Fahn A (1988). Secretory tissues in vascular plants. New Phytol., 108: 229-257.

Hallahan DL (2000). Monoterpenoid biosynthesis in glandular trichomes of labiate plants. In: Hallahan DL, Gray JC, eds. Advances in botanical research. Incorporating advances in plant pathology. Vol. 31. Plant trichomes. Academic Press, London, pp. 77-120.

Hay RKM, Svoboda KP (1993). Botany. In: Hay RKM, Waterman PG, eds. Volatile oil crops: their biology, biochemistry, and production. Longman Scientific and Technical, Harlow, Essex, England, pp. 5-22.

Heinrich G, Pfeifhofer HW, Stabentheiner E, Sawidis T (2002). Glandular hairs of Sigesbeckia jorullensis Kunth (Asteraceae): morphology, histochemistry and composition of essential oil. Ann. Bot., 89: 459-469.

Li HW (1988). Taxonomic review of *Isodon* (Labiatae). J. Arnold Arbor., 69: 289-400.

Li HW, Hedge IC (1994). *Lamiaceae* (Labiatae). In: Flora of China 17, Wu CY, Raven PH, eds. Science Press and Missouri Botanical Garden, Beijing and Saint Louis, pp. 50-299.

Lu YH, Liu GJ, Li MR, Li DC, Yu XL (2000). Determination of oridonin in leaves and stems of *Blushred rabdosia* by HPLC (in Chinese). Chin. J. Pharma. Ana., 20(6): 390-392.

Meade-Tollin LC, Wijeratne EMK, Cooper D, Guild M, Jon E, Fritz A, Zhou GX, Whitesell L, Liang JY, Gunatilaka AAL (2004), Ponicidin and oridonin are responsible for the antiangiogenic activity of *Rabdosia rubescens*, a constituent of the herbal supplement PC SPES. J. Nat. Prod., 67(1): 2-4.

McCaskill D, Croteau R (1995). Monoterpene and sesquiterpene biosynthesis in glandular trichomes of peppermint (*Mentha* × *piperita*) rely exclusively on plastid-derived isopentenyl diphosphate. Planta, 197: 49–56.

Nikolakaki A, Christodoulakis NS (2004). Leaf structure and cytochemical investigation of secretory tissues in Inula viscosa. Bot. J. Linn. Soc., 144: 437-448.

Nikolakaki A, Christodoulakis NS (2007) Histological investigation of the leaf and leaf-originating calli of *Lavandula vera* L. Isr. J. Plant Sci., 54: 281-290.

Siebert DJ (2004). Localization of Salvinorin A and Related Compounds in Glandular Trichomes of the Psychoactive Sage, Salvia divinorum. Ann. Bot., 93: 763-771.

Sun HD, Huang SX, Han QB (2006). Diterpenoids from *Isodon* species and their biological activities. (Natural Product Reports) Nat. Prod. Rep., 23: 673-698.

Wagner H, Bladt S (1996). Plant drug analysis: a thin layer chromatographic atlas, 2nd edn. Springer-Verlag, Heidelberg

Wagner GJ, Wang E, Shepherd RW (2004). New approaches for studying and exploiting on old protuberance, the plant trichomes. Ann. Bot., 93: 3-11.

Werker E (1993). Fuction of essential oil-secreting glandular hairs in aromatic plants of the Lamiaceae. A review. Flavour Frag., J. 8: 249-25.

Wu CY, Li HW (1977). Labiatae (in chinese). In: Flora Reipublicae Popularis Sinicae Science Press, Beijing, 65(2): 66.

Yashodhara K, Shanmukha Rao SR, Subba Rao JV (2001). Structure, distribution and taxonomic importance of trichomes in the tribe Verbeneae (Verbenaceae). Contributions to the biology of plants, 72: 265-285.

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Full Length Research Paper

Evaluation of the antioxidant activities of total flavonoids from tartary buckwheat

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In this study, the antioxidant activities of total flavonoids from tartary buckwheat (FTB) were evaluated. *In vitro*, antioxidant activities of FTB were conducted by determining the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and butylated hydroxyl toluene (BHT) radical scavenging activities. *In vivo*, antioxidant activities of FTB were conducted by determining antioxidant enzymatic activities and malondialdehyde (MDA) contents in serum and liver in mice. FTB were found to be potent scavengers of the DPPH and BHT radicals. Furthermore, FTB significantly increased the activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) and reduced the lipid peroxidation in both serum and liver of mice. Results of this study showed that FTB have potent antioxidant and free radical-scavenging activities.

Key words: Antioxidant, flavonoids, tartary buckwheat.

INTRODUCTION

Buckwheat, also called triangle wheat, belongs to the family Polygonaceae, *genus Fagopyrum Meisn* (Zhang et al., 2008). It is a good source of food with higher protein content than rice, wheat, or sorghum. It also benefits humans in preventing leg edema, hypertension and cardiovascular diseases. In recent years, buckwheat has become much more popular in many countries as a kind of health food (Wang et al., 2004). There are two buckwheat cultivars: Common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum*). In some countries of Asia, European and Latin America, common buckwheat is more commonly grown, while tartary buckwheat is mainly grown in cold regions of loess plateau and Yun-Gui plateau in China (Wang et al., 2006). It has been reported that the general composition of crude protein, crude fiber, crude fat, and crude ash of common buckwheat and tartary buckwheat are essentially the same (Bonafaccia et al., 2003). However, tartary buckwheat may even contain more bioactive components than common buckwheat. For instance, it has been reported that the flavonoids content of tartary buckwheat is higher than that of common buckwheat (Liu et al., 2008). Free radicals are responsible for aging and causing various human diseases (Moskovitz et al., 2002). In recent years, flavonoids have attracted researchers' interest because they are powerful antioxidants which can protect the human body from free radicals. Flavonoids cannot be produced by the human body and have thus to be taken in mainly through the daily diet (Peng et al., 2003). Hence, the present work was to investigate the possible antioxidant activities of the total FTB. The research results will be useful for elucidating the nutritional and health values of tartary buckwheat.

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MATERIALS AND METHODS

Plant material

The air-dried tartary buckwheat grains were purchased from Liangshan Agricultural Institution (Sichuan, China). It was ground in a blender (FW177, Taisite Instrument Company Limited Tianjin, China) for 10 s to produce powder with an approximate size of 1 mm. The powders of tartary buckwheat grains were stored at -20°C before the experiment.

Chemicals and reagents

Authentic standard rutin (>97%), DPPH and BHT were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nicotinamide

adenine dinucleotide (NADH) and nitroblue tetrazolium (NBT) were purchased from Fluka Biochemika AG (Buchs, Switzerland). The kit for the determination of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)were purchased from Nanjing Jiancheng Bioengineering Company (Jiangsu, China). All reagents and chemicals used in the experiments were of analytical grade. Triple-distilled water was used throughout the study.

Preparations of total flavonoids from tartary buckwheat

The powders of tartary buckwheat grains (1.0 g) were accurately weighed and placed in a sealed vessel by adding 40 ml of the ethanol-water (60:40, v/v) solvent, then the sealed vessel was placed into the microwave extraction system (WF-4000C, PreeKem Scientific Instruments Company Limited Shanghai, China). The extraction temperature was 70°C, extraction time was 20 min and microwave power was 600 W. After that, the extract was centrifuged at 3000 rpm for 10 min to remove the insoluble and the supernatant was filtrated through 0.45 mm of filter membrane to obtain a clarified solution. The filtrate was collected and evaporated with a rotary evaporator at 20°C for about 5 h. Thus the total FTB were obtained.

Determination of flavonoids content

The flavonoids content of the FTB was measured using a modified colorimetric method (Jia et al., 1999). To 1.0 ml diluted sample, 0.3 ml $NaNO_3$ solution (5%), 0.6 ml $A1Cl_3$ solution (0.1 g/ml and 2.0 ml NaOH solution (1.0 mol/L) were added. The final volume was adjusted to 10.0 ml with deionised water. The absorption was measured at 507 nm against the same mixture, without the sample as a blank. The amount of the total flavonoids was expressed as rutin quivalents (mg rutin/ g sample). The calibration curve (Y=9.265X-0.0113, where Y is absorbance value, X sample concentration) ranges from 0.75 to 6.0 mg/ml (R^2=0.9984). The content of total flavnoids was 27.97 mg/g.

DPPH radical scavenging activity

The DPPH radical scavenging activity was tested by the method of Chan et al. (2007). Briefly, various concentrations of the FTB solutions were prepared. DPPH solution was also prepared by dissolving 6.0 mg of DPPH in 100 ml methanol. Then, 1.0 ml of FTB from each dilution was added into the test tube containing 2.0 ml of DPPH solution. Control was prepared by adding 1.0 ml of methanol to 2.0 ml of DPPH solution. BHT was used as standard. The mixture was shaken vigorously and was left to stand in the dark for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The scavenging activity of DPPH radical was calculated using the following equation:

$$Scavenging\ activity\ (\%) = \left(1 - \frac{A_{sample\ at\ 517\ nm}}{A_{control\ at\ 517\ nm}}\right) \times 100$$

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity was tested by the method of Liang et al. (2009). Briefly, various concentrations of the FTB solutions were prepared. 1.0 ml of NBT solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1.0 ml of NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4), and 0.1 ml of each of the prepared FTB solutions were mixed. After adding 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture, the reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank samples. BHT was used as standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The scavenging activity of hydroxyl radical was calculated using the following equation:

$$Scavenging\ activity\ (\%) = \left(1 - \frac{A_{sample\ at\ 560\ nm}}{A_{control\ at\ 560\ nm}}\right) \times 100$$

Animals and groups

The study protocol was approved by the Institutional Animal Care and Use Committee of the Xichang College. Male C57BL/6 mice (certificate number: 3869) weighing 18 to 22 g were purchased from the Experimental Animal Center of Sichuan University (Sichuan, China) and acclimated to conditions for 1 week before the experiment. The animals were housed in an air-conditioned room with 12 h light and dark illumination cycles at constant temperature 24±0.5°C and humidity (45 to 50%). Food and drinking water were supplied *ad labium*. Ethical clearance for handling the animals and the procedures used in the study was obtained from the institutional animal ethical committee. 28 mice were randomly divided into following four experimental groups (7 mice per group): Group 1 (control group, CG) was orally administered 10 ml/kg of body weight/day (BW/D) of physiological NaCl-solution (Vehicle); group 2 (low-dose treated group, LTG) was orally administered 200 mg/kg BW/D of FTB; group 3 (middle-dose treated group, MTG) was orally administered 400 mg/kg BW/D of FTB; group 4 (high-dose treated group, HTG) was orally administered 800 mg/kg BW/D of FTB. The dose of 200, 400 and 800 mg/kg was chosen based on estimates from prior studies. FTB were redissolved in the same vehicle and administered orally by a cannula for 30 consecutive days.

Biochemical analyses

At the end of the experiment (30 days), blood was taken from the retroorbital plexus under light ether anesthesia. Collected blood was centrifuged for 15 min at 3500 rpm at 4°C. The blood serum was used for measuring antioxidant enzymes activities and MDA contents. Liver tissues were removed immediately after euthanasia by cervical dislocation and tissues were rinsed and homogenized with saline to be 10% (w/v) homogenates which were centrifuged at 3, 000 g for 10 min and the supernatants were centrifuged at 1000 g at 4°C for 15 min. The resultant supernatants were used for measuring antioxidant enzymes activities and MDA contents. SOD, CAT and GSH-Px activities were estimated using commercially available kits. SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The SOD activities were measured by the degree of inhibition of this reaction (Suttle, 1986). CAT determination was based upon alteration of H_2O_2 optical density, depending on enzymatic decomposition of H_2O_2 (by the effect of CAT in the sample).

CAT of hemoglobin was changed to k/g hemoglobin (Hb) after the ‘k’ value was determined, taken into account suitable absorbance for each analysis according to calculated regression (Wang et al., 2008). GSH-Px determination was based on the following principle: GSH-Px catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione (GSSG) is immediately converted into the reduced form

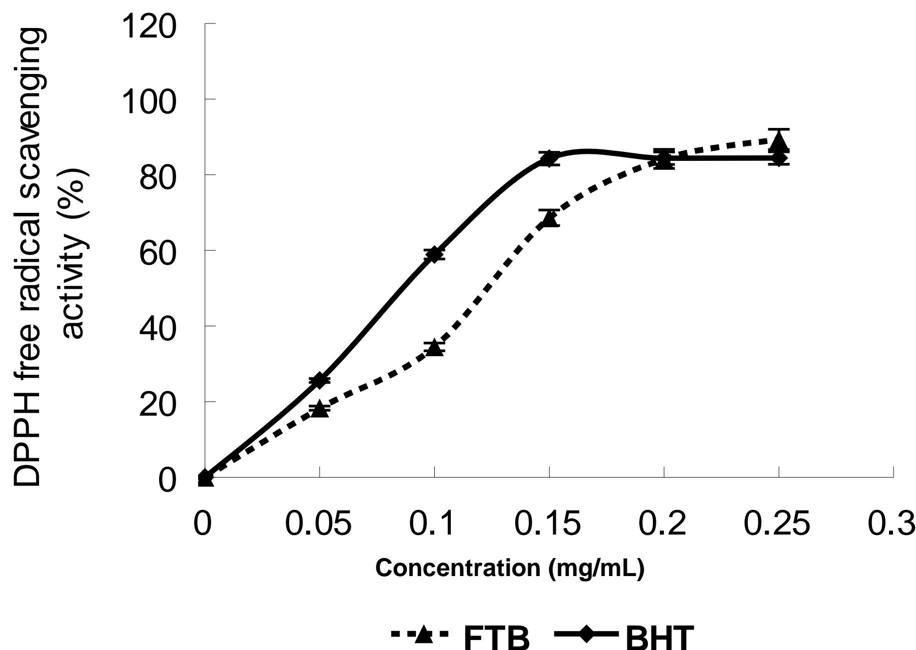

Figure 1. DPPH radical scavenging activity of FTB and BHT. Values were means ± SD (n = 3).

with a contaminant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured by a spectrophotometer (Paglia and Valentine, 1967). MDA contents were estimated by the method of Qi et al. (2008) based on thiobarbituric acid (TBA) reactivity. The degree of lipid peroxide formation was assessed by MDA, which is accepted as an index of lipid peroxide (Jain et al., 1990).

Statistical analysis

All data were reported as mean ± SD of three replicates. The SAS system (SAS for Windows 6.12, SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. Duncan's multiple range tests were used to estimate significant differences among the mean values at the 5% probability level.

RESULTS AND DISCUSSION

DPPH radical scavenging activity assay

DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants (Katerere and Eloff, 2005). The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the nonradical form 1, 1-diphenyl-2-picrylhydrazyl-Hydroiodic (DPPH-H). The extract was able to reduce the stable radical DPPH to the yellow-colored diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (for example, hydroquinone, pyrogallol and gallic acid) and aromatic amines (for example, p-phenylene diamine and paminophenol) are reduced and decolorize into DPPH via their hydrogen donating ability (Li and Zhou, 2007). As shown in Figure 1, DPPH radical scavenging activity of FTB was weaker than that of BHT at the dosage range of 0 to 0.2 mg/ml, but at continually increasing concentrations, the scavenging effect of the FTB rapidly improved and exceeded that of BHT. At a concentration of 0.25 mg/ml, DPPH radical scavenging activity of FTB was approximately 89 and 84% for BHT. The results indicated that FTB have satisfactory scavenging effect on DPPH radical.

Superoxide anion radical scavenging activity assay

Among all these reactive oxygen species, superoxide anion radical is generated. Although it is a relatively weak oxidant, it can decompose to form stronger reactive oxidative species, such as single oxygen and hydroxyl radicals (Wu et al., 2007). Furthermore, superoxide anion radicals are also known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Meyer and Isaksen, 1995). So, scavenging activity of superoxide anion radical is an important index for antioxidants. As shown in Figure 2, superoxide anion radical scavenging activity of FTB and BHT was found to increase with an increase in its concentration, significant between concentration of 0.05 and 0.25 mg/ml. BHT was found to possess a slightly

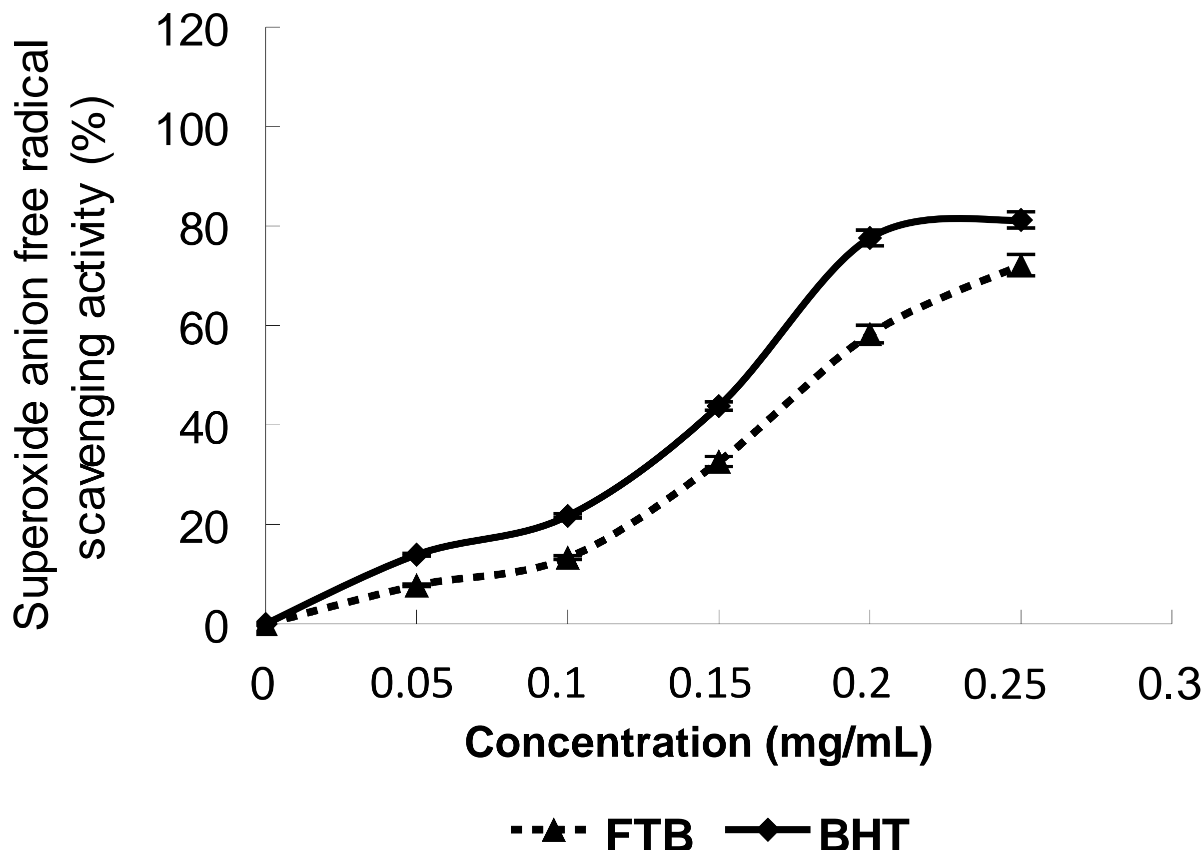

Figure 2. Superoxide anion radical scavenging activity of FTB and BHT. Values were means ± SD.

higher degree of the activity than the FTB at any concentration. At concentration of 0.25 mg/ml, FTB and BHT, respectively showed 87 and 96% superoxide anion radical scavenging activity. The results indicated that FTB have a relatively higher superoxide anion radical scavenging activity.

Antioxidant enzymes activities and MDA contents of mice assay

Antioxidant enzymes are capable of eliminating reactive oxygen species and lipid peroxidation products; thereby protect cells and tissues from oxidative damage. Antioxidant enzymes include SOD, CAT and GSH-Px, of which the first one can mutate the superoxide radicals to form molecular oxygen and H_2O_2, and the other two can decompose H_2O_2 to molecular oxygen and water (Wang et al., 2008). MDA is the major oxidation product of peroxidized poly-unsaturated fatty acids and increased MDA content is an important indicator of lipid peroxidation (Que et al., 2007). As shown in Figure 3, SOD activities in serum and liver were significantly elevated in low temperature generators (LTG), middle temperature generators (MTG) and high temperature generators (HTG) as compared to the control group ($P < 0.05$).

As shown in Figure 4, CAT activities in liver were significantly elevated in the LTG, MTG and HTG as compared to the control group ($P < 0.05$). CAT activities in serum were significantly elevated in the MTG and HTG as compared to the control group ($P < 0.05$). As shown in Figure 5, GSH-Px activities in serum and liver were significantly elevated in the LTG, MTG and HTG as compared to the control group ($P < 0.05$). As shown in Figure 6, MDA contents in serum and liver were decreased in the LTG, MTG and HTG as compared to the normal control group ($P < 0.05$). The results indicated that oral administration with FTB could significantly reduce the lipid peroxidation and enhance the activity of antioxidant enzymes in mice.

Conclusions

The present study demonstrated that FTB has potent antioxidant and free radical-scavenging activities. *In vitro*, FTB has satisfactory scavenging effects on DPPH and superoxide anion radicals. *In vivo*, FTB could significantly reduce the lipid peroxidation and enhance the activity of antioxidant enzymes. Tartary buckwheat is rich in

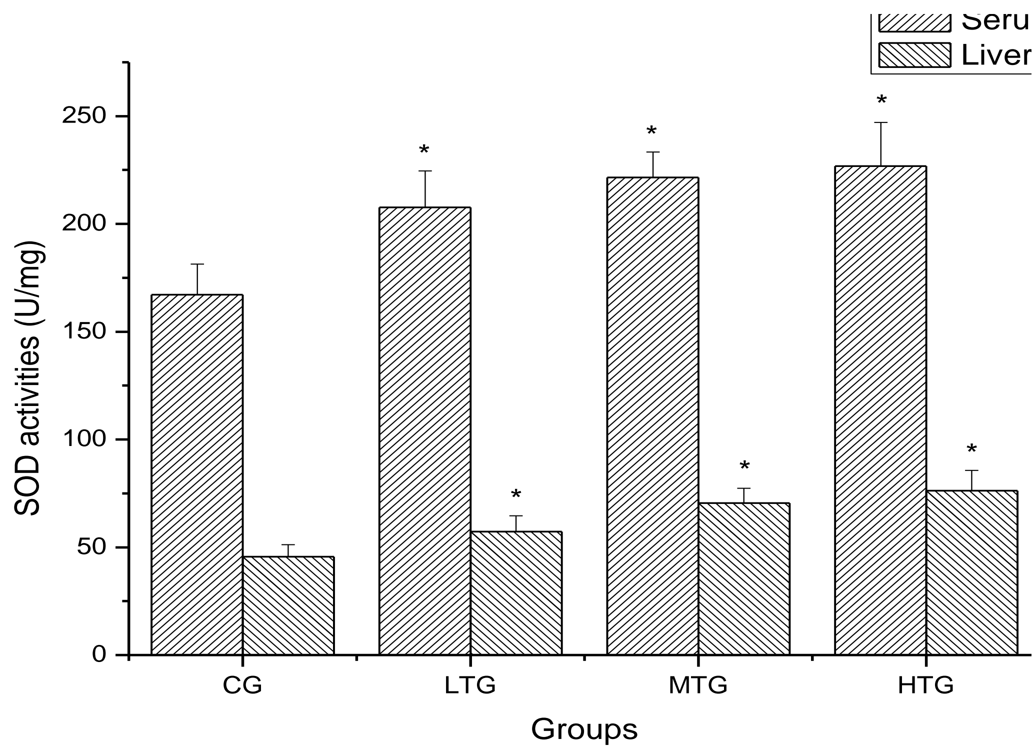

Figure 3. SOD activities in mice serum and liver. Values were expressed as means ± SD. *$P < 0.05$ versus control group.

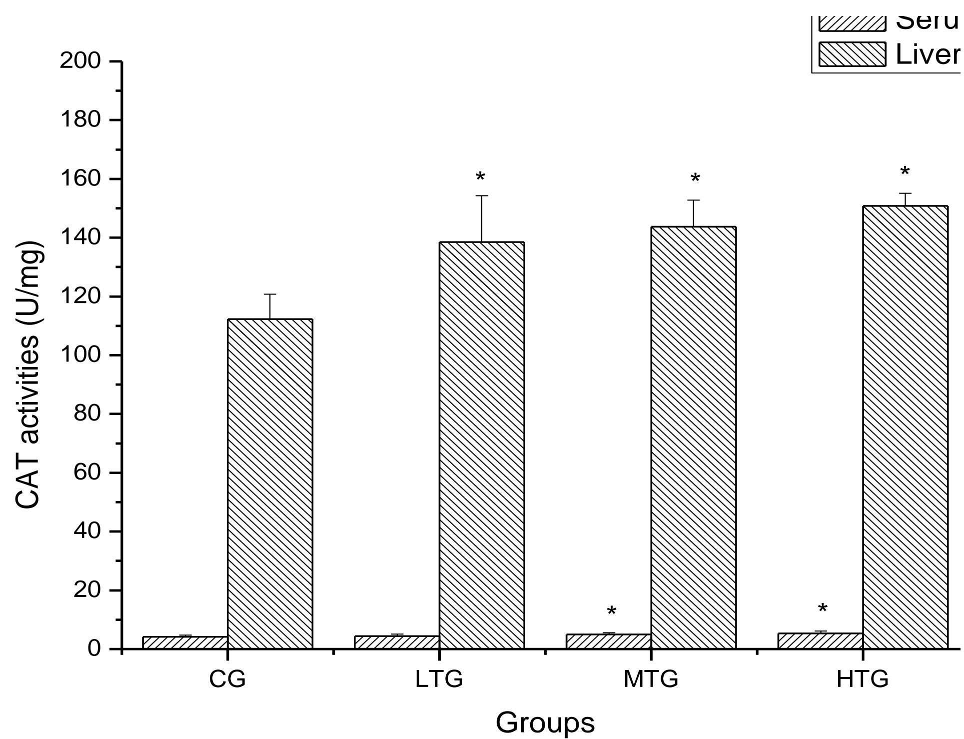

Figure 4. CAT activities in mice serum and liver. Values were expressed as means ± SD. *$P < 0.05$ versus control group.

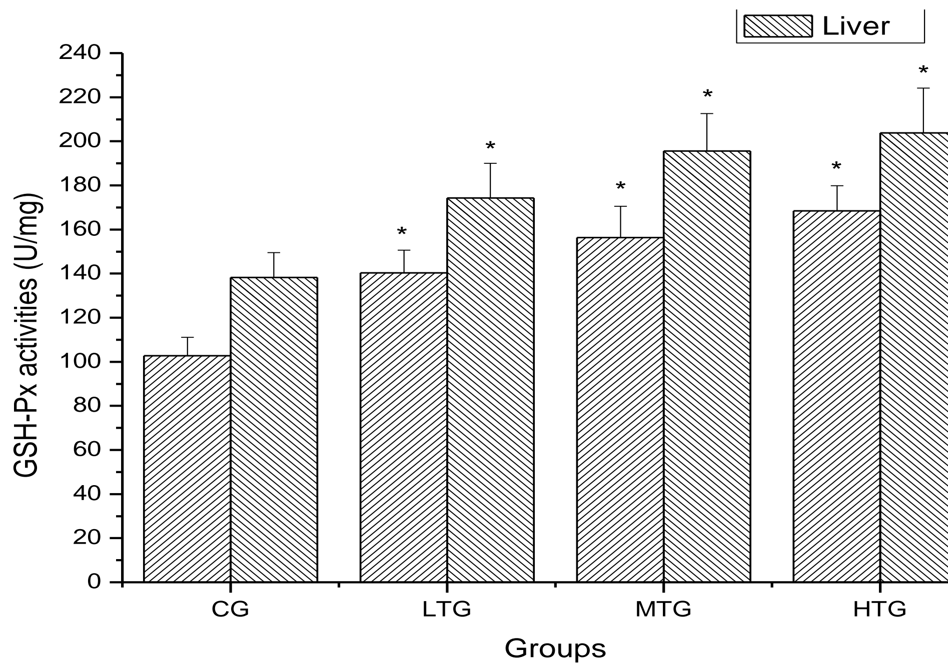

Figure 5. GSH-Px activities in mice serum and liver. Values were expressed means ± SD. *$P < 0.05$ versus control group.

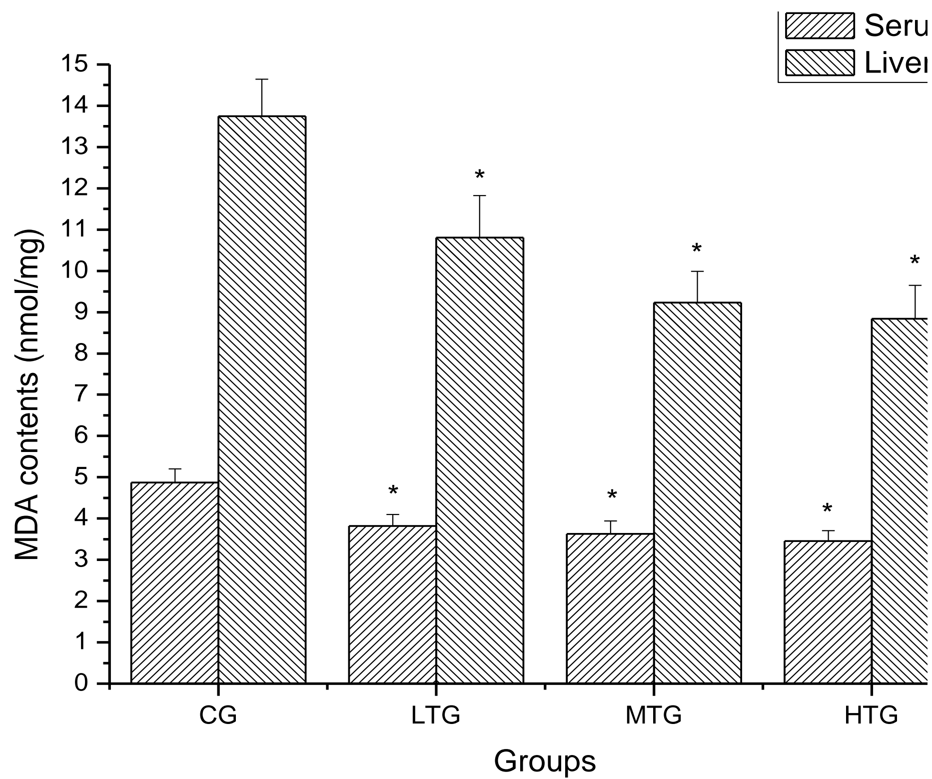

Figure 6. MDA contents in mice serum and liver. Values were means ± SD. *$P < 0.05$ versus control group.

flavonoids compounds and it can serve as the latent source of antioxidant healthy product and deserve further exploitation.

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REFERENCES

Bonafaccia G, Gambelli L, Fabjan N, Kreft I (2003). Trace elements in flour and bran from common and tartary buckwheat. Food Chem., 83: 1-5.

Chan EWC, Lim YY, Omar M (2007). Antioxidant and antibacterial activity of leaves of *Etlingera species* (Zingiberaceae) in Peninsular Malaysia. Food Chem., 104(4): 1586-1593.

Jain SK, Levine SN, Duett J, Hollier B (1990). Elevated lipid peroxidation levels in red blood cells of streptozotocin-treated diabetic rats. Metabolism, 39: 971-975.

Jia ZS, Tang MC, Wu JM (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem., 64(4): 555-599.

Katerere DR, Eloff JN (2005). Antibacterial and antioxidant activity of *Sutherlandia frutescens* (Fabaceae), a reputed Anti-HIV/AIDS phytomedicine. Phytother. Res., 19: 779-781.

Li XL, Zhou AG (2007). Evaluation of the antioxidant effects of polysaccharides extracted from *Lycium barbarum*. Med. Chem. Res., 15: 471-482.

Liang L, Zhang Z, Wang H (2009). Antioxidant activities of extracts and subfractions from *Inonotus Obliquus*. Int. J. Food Sci. Nutr., 2: 175-184.

Liu CL, Chen YS, Yang JH, Chiang BH (2008). Antioxidant activity of tartary (*Fagopyrum tataricum (L.) Gaertn.*) and common (*Fagopyrum esculentum moench*) buckwheat sprouts. J. Agric. Food Chem., 56(1): 173-178.

Meyer AS, Isaksen A (1995). Application of enzymes as food antioxidants. Trends Food Sci. Technol., 6: 300-304.

Moskovitz J, Yim MB, Chock PB (2002). Free radicals and disease. Arch. Biochem. Biophys., 397(2): 354-359.

Paglia DE, Valentine WN (1967). Studies on the quantitative and qualitative characterization of erythrocyte GSH-Px. J. Lab. Clin. Med., 70(1): 158-169.

Peng ZF, Strack D, Baumert A, Subramaniam R, Goh NK, Chia TF, Tan SN, Chia LS (2003). Antioxidant flavonoids from leaves of *Polygonum hydropiper L*. Phytochemistry, 62(2): 219-228.

Qi XY, Chen WJ, Zhang LQ, Xie BJ (2008). Mogrosides extract from *Siraitia grosvenori* scavenges free radicals in vitro and lowers oxidative stress, serum glucose, and lipid levels in alloxan-induced diabetic mice. Nutr. Res., 28(4): 278-284.

Que F, Mao L, Zheng X (2007). *In vitro* and *vivo* antioxidant activities of daylily flowers and the involvement of phenolic compounds. Asia Pac. J. Clin. Nutr., 1: 196-203.

Suttle NF (1986). Copper deficiency in ruminants: recent developments. Vet. Res., 119(21): 519-522.

Wang D, Wang LJ, Zhu FX, Zhu JY, Chen XD, Zou L, Saito M, Li LT (2008). *In vitro* and *in vivo* studies on the antioxidant activities of the aqueous extracts of Douchi (a traditional Chinese salt-fermented soybean food). Food Chem., 107: 1421–1428.

Wang Z, Wang L, Chang W, Li Y, Zhang Z, Wieslander G, Norback D (2006). Cloning, expression, and identification of immunological activity of an allergenic protein in tartary buckwhea. Biosci. Biotechnol. Biochem., 70(5): 1195-1199.

Wang Z, Zhang Z, Zhao Z, Wieslander G, Norbäck D, Kreft I (2004). Purification and characterization of a 24 kDa protein from tartary buckwheat seeds. Biosci. Biotechnol. Biochem., 68(7): 1409-1413.

Wu Q, Zheng C, Ning ZX, Yang B (2007). Modification of Low Molecular Weight Polysaccharides from *Tremella Fuciformis* and Their Antioxidant Activity *in vitro*. Int. J. Mol. Sci., 8: 670-679.

Zhang X, Yuan JM, Cui XD, Wang ZH (2008). Molecular cloning, recombinant expression, and immunological characterization of a novel allergen from tartary buckwheat. J. Agric. Food Chem., 56(22): 10947-10953.

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Full Length Research Paper

Antioxidant activities and reversed phase-high performance liquid chromatography (RP-HPLC) identification of polyphenols in the ethyl acetate extract of Tunisian *Juglans regia* L. treated barks.

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The barks and leaves of *Juglans regia* L. are widely used in Tunisia to maintain good oral hygiene and are known to possess high therapeutic effects. In this paper, and for the first time, we report the reversed phase-high performance liquid chromatography (RP-HPLC) identification of polyphenols in the ethyl acetate extract obtained from the barks of *J. regia* L. and we tested its antioxidant properties. The results showed that the ethyl acetate extract possess high antioxidant activities comparatively to the butylated hydroxytoluene (BHT) (DPPH IC_{50}, 3 µg/ml; reducing power EC_{50}, 99 µg.ml^{-1} and IC_{50} = 280 µg/ml for the β-carotene bleaching test). The total antioxidant activity was estimated at 329 mg GAE.g^{-1} DW, the total phenolic content was 33,833 mg GAE.g^{-1} DW and the total condensed tannin were 16.167 mg EC.g^{-1} DW. The RP-HPLC showed the presence of 69 compounds: 24 with a percentage ranging from 1.37 to 15.02% including the caffeic acid (15,02%), the rutin trihydrate (12.71%), the syringic acid (3.44%), the gallic acid (2.58%) and 55 compounds with a percentage varying from 0.02 to 0.93% including the chlorogenic acid (0.8%), the resorcinol (0.78%), the vanillic acid (0.77%), the naphtho-resorcinol (0.68%), the quercetin dihydrate (0.57%), the *p*-coumaric acid (0.56%), the trans-cinnamic acid (0.53%) and the catechine hydrae (0.18%). In conclusion, we confirm the presence of natural compounds in the bark of Tunisian tree of *J. regia* with great interest, especially the polyphenols which confer to this plant a wide range of biological activities and reinforce the use of walnut bark in the developing countries in order to maintain oral hygiene.

Key words: *Juglans regia*, reversed phase-high performance liquid chromatography (RP-HPLC), polyphenols, antioxidant activities.

INTRODUCTION

The *Juglans* genus comprises 21 species and is widely distributed throughout the world. The species *Juglans regia* L. belongs to the family Juglandaceae. Useful parts of walnut tree are leaf, second shell and fleshy part of green fruit and its wood. Walnut species are not only an agricultural commodity, but its leaves, barks, stems, pericarps, fruits, flowers and ligneous membranes are all

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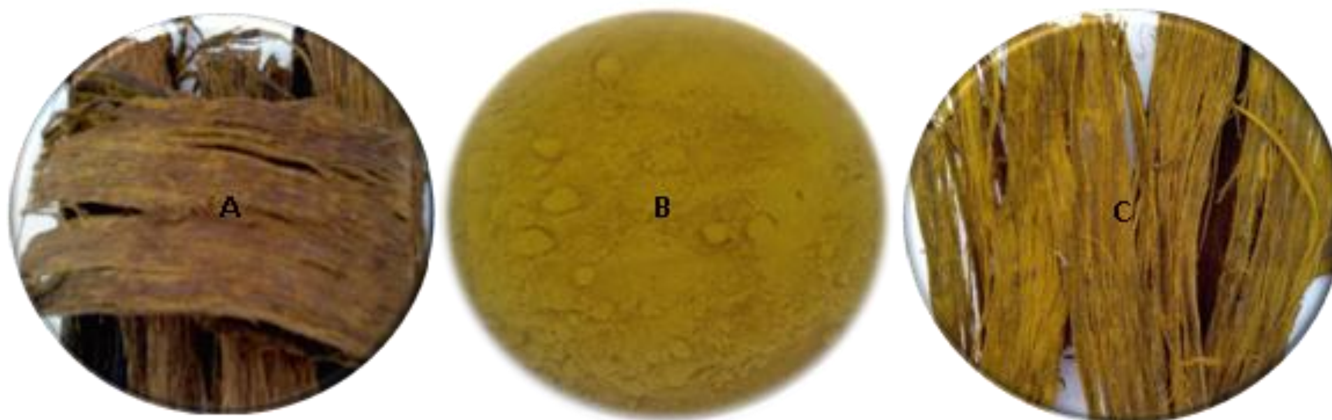

Figure 1. Plant material used in this study. (A): Untreated barks; (B): Yellow dye; (C): Treated barks.

applied for different medicinal uses. In Tunisia, this tree is widely known as the "Swak" tree and its fresh leaves and dried bark used are by women to clean the teeth and to maintain a good oral hygiene. The bark of this tree are collected and treated with yellow dye and dried before use (Noumi et al., 2010).

In fact, walnut leaves are considered a source of healthcare compounds, and have been intensively used in traditional medicine to treat venous insufficiency and due to its antidiarrheic, antifungal, antibacterial and antioxidant properties (Amaral et al., 2003; Pereira et al., 2007; Miraliakbari and Shahidi, 2008). These benefits are usually attributed to the presence of essential fatty acids and tocopherols (Amaral et al., 2005) and especially linoleic, oleic, linolenic, palmitic, and stearic acids (Amaral et al., 2008). Juglone (5-Hydroxy 1,4 naphtoquinone) is one of the most important flavonoides of walnuts green husk (Jaimand et al., 2004; Cosmolescu et al., 2010). Walnut leaf and shell have some medicinal effects, as walnut green husk has antioxidant, antifungal, astringent, wart liquidator effects and uses for skin diseases and anemia cures (Noumi et al., 2010; Amaral et al., 2003).

In addition, walnuts have other components that may be beneficial for health including plant protein, dietary fiber, melatonin (Reiter et al., 2005), plant sterols (Amaral e al., 2005), folate, tannins, and polyphenols (Li et al., 2006; Li et al., 2007). Plant-derived products can also be used as antimicrobial agents, with phenolics and polyphenolic having major interest.

Despite the wide use of *J. regia* L. (walnut) and especially the bark and the leaves in Tunisia, these plants have not received much attention and have not been intensively studied. Therefore, the present work is the first study with aims to identify the polyphenols in the ethyl acetate extract of the barks of *J. regia* by using the RP-HPLC technique and to evaluate its antioxidant properties.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid, sodium nitrite solution ($NaNO_2$), aluminum chloride hexahydrate solution ($AlCl_3$, $6H_2O$), vanillin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), trichloroacetic acid, iron(III)chloride anhydrous ($FeCl_3$), ascorbic acid and ß-carotene were purchased from Fluka (Buchs, Switzerland). Butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich (GmbH, Sternheim, Germany). Sulfuric acid (H_2SO_4) and Kalium-hexacyanoferrat (III); $K_3Fe\ (CN)_6$ were obtained obtained from Merck (Darmstadt, Germany). All solvents used in this study were purchased from Sigma-Aldrich (GmbH, Sternheim, Germany).

Plant material sampling and extraction

500 g of treated dry barks of *J. regia* (Figure 1) were purchased from a local market in the region of Mahdia (Tunisia). Extraction was made with ethyl acetate. The extract was prepared by adding 4 g of small particle dry plant material powder with a commercially available food blender to 40 ml solvent and allowing the mixtures to stand overnight at room temperature, after which the supernatants were filtered and dried/evaporated under a controlled temperature (4°C). They were stored at 4°C until analysis (Trabelsi et al., 2010). To make 30 mg extract-impregnated disks, 1 ml of extract solution in ethyl acetate was applied onto the sterile disks in 10 µl increments with sufficient time in between to allow drying.

Evaluation of total antioxidant capacity

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and sub-sequent formation of a green phosphate/Mo (V) complex at acid pH (Prieto et al. 1999). An aliquot (0.1 ml) of plant extract was combined to 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated in a thermal block at 95°C for 90 min. After, the mixture had cooled to room temperature; the absorbance of each solution was measured at 695 nm (Anthelie Advanced 2, SECOMAN) against a blank. The antioxidant capacity was expressed as mg gallic acid equivalent per gram dry weight (mg GAE/g DW). The calibration curve range was 0 to 500 µg/ml. All

samples were analyzed in triplicate.

Total phenolic content

Phenolic content was assayed using the Folin-Ciocalteu reagent, following Singleton's method slightly modified (Dewanto et al., 2002). An aliquot (0.125 ml) of appropriately diluted sample extract was added to 0.5 ml of distilled water and 0.125 ml of the Folin-Ciocalteu reagent. After 3 min, 1.25 ml of Na_2CO_3 solution (7g/100 ml) were added and the final volume was made up to 3 ml with distilled water. The absorbance was measured at 760 nm, after incubation for 90 min at 23°C in dark. Total phenolic content of leaves was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE.g^{-1} DW) through the calibration curve with gallic acid. The calibration curve range was 0 to 400 µg.ml^{-1}. Triplicate measurements were taken for all samples.

Total condensed tannins

Proanthocyanidins were measured using the modified vanillin assay (Sun et al., 1998). To 50 µl of suitably diluted sample were added 3 ml of methanol vanillin solution and 1.5 ml H_2SO_4, respectively. The mixture was allowed to stand for 15 min at room temperature, and the absorption was measured at 500 nm against solvent as a blank. The amount of total condensed tannins is expressed as mg (+)-catechin g^{-1} DW.

1,1-diphenyl-2-picryl hydrazyl (DPPH) radical–scavenging activity

The antioxidant activity of different solvent extracts was measured in term of hydrogen donating or radical scavenging ability using the stable DPPH method (Hanato et al., 1988). The sample was diluted in pure solvent of extraction at different concentrations (10, 20, 100 and 200 µg.ml^{-1}), then 1 ml of each diluted plant extract was added to 0.25 ml of a 0.2 mmol/l DPPH methanolic solution. The mixture of different extract concentration and DPPH were placed in the dark at room temperature for 30 min. The absorbance of the resulting solution was then read at 517 nm. The antiradical activity was expressed as IC_{50} (µg.ml^{-1}). The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0-A_1)/A_0]*100 \quad (1)$$

Where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

Determination of reducing power

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Oyaizu (1986). Briefly, 1 ml of *Salvadora persica* and *J. regia* extracts was mixed with 2.5 ml of phosphate buffer (0.2 mol/l, pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1 g/100 ml). After incubation at 50°C for 25 min, 2.5 ml of trichloroacetic acid (10 g/100 ml) was added and the mixture was centrifuged at 650x*g* for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of distilled water and 0.5 ml of aqueous $FeCl_3$ (0.1 g/100 ml). The absorbance was measured at 700 nm. The mean of absorbance values were plotted against concentration and a linear regression analysis was carried out. Increased absorbance of the reaction mixture indicated increased reducing power. EC_{50} value (mg.ml^{-1}) is the effective concentration at which the absorbance was 0.5 for reducing power. Ascorbic acid was used as positive control.

β-carotene-linoleic acid model system (B-CLAMS)

The B-CLAMS method by the peroxides generated during the oxidation of linoleic acid at elevated temperature (Koleva et al., 2002). In this study, the B-CLAMS was modified for the 96-well micro-plate reader. In brief, the B-carotene was dissolved in 2ml of $CHCl_3$, to which 20 mg of linoleic acid and 200 mg of tween 40 were added. $CHCl_3$ was removed using rotary evaporator. Oxygenated water (100 ml) was added, and the flask was shaken vigorously until all material dissolved. This test mixture was prepared fresh and using immediately. To each well, 250 µl of the reagent mixture and 35 µl sample or standard solution were added. The plate was incubated at 45°C. Readings were taken at 490 nm using visible/UV microplate kinetics reader (ELx808, Bio-Tek instruments). All samples were prepared and analyzed in triplicate.

Identification of phenolic compounds using reversed phase-high performance liquid chromatography (RP-HPLC)

Phenolic compounds analysis was carried out using an Agilent Technologies 1100 series liquid chromatograph (RP-HPLC) coupled with an UV-Vis multi-wavelength detector using the same protocol previously described by Noumi et al. (2011). The separation was carried out on 250 x 4.6 mm, 4 µm Hypersil ODS C18 reversed phase column at ambient temperature. The mobile phase consisted of acetonitrile (solvent A) and water with 0.2% sulphuric acid (solvent B). The flow rate was kept at 0.5 ml/min. The gradient program was as follows: 15% A/85% B 0 to 12 min, 40% A/60% B 12 to 14 min, 60% A/40% B 14 to 18 min, 80% A/20% B 18 to 20 min, 90% A/10% B 20 to 24 min, 100% A 24 to 28 min. The injection volume was 20 µl and peaks were monitored at 280 nm. Samples were filtered through a 0.45 µm membrane filter before injection. Each experiment was repeated at least two times. Peaks were identified by congruent retention times compared with standards (Table 1).

RESULTS AND DISCUSSION

The results of the polyphenols identification by RP-HPLC showed that the ethyl acetate extract of *J. regia* barks is composed of 69 molecules (Figure 2). Indeed, the extract of "Swak" contains 24 compounds which the percentage varies from 1.37 to 15.02% among them there are: caffeic acid (15,02%), rutin trihydrate (12.71%), syringic acid (3.44%), gallic acid (2.58%) and 55 compounds with a percentage varying from 0,02 to 0,93% among them there are: chlorogenic acid (0.8%), resorcinol (0.78%), vanillic acid (0.77%), naphtho-resorcinol (0.68%), quercetin dihydrate (0.57%), *p*-coumaric acid (0.56%), trans-cinnamic acid (0.53%) and catechine hydrae (0.18%). Our results showed the presence of several compounds with known antioxidant properties and we noted that caffeic acid, rutin trihydrate, syringic acid and gallic acids were the predominant compounds. In fact, walnuts contain several phenolic compounds which are thought to contribute to their biological activities (Table 2).

Table 1. Retention time (R.T) of thirteen standards of phenolic acids and flavonoids.

S/N	Standards	R.T (min)
1	Gallic acid	4.080
2	Gallocatechin	5.465
3	Protocatechic acid	6.680
4	3,4-dihydroxyphenol acetic acid	6.835
5	Epigallocatechin	7.596
6	Catechin	7.785
7	Chlorogenic acid	8.873
8	4-hydroxybenzoïc acid	9.764
9	2,5-dihydroxybenzoïc acid	10.121
10	Vanillic acid	10.897
11	Caffeic acid	11.309
12	3,5 dimethoxy- 4-hydoxybenzoïc acid	11.332
13	Epigallocatechin-3-o-gallate	11.512
14	*P*-coumanic acid	14.771
15	Rutin hydrate	15.679
16	Rutin trihydrate	15.700
17	Sinapic acid	16.493
18	Trans-4-hydroxy-3-methoxycinnamic acid	16.567
19	3,4 dimethoxybenzoïc acid	16.737
20	Trans-2-hydroxycinamic acid	19.384
21	*O*-coumaric acid	20.040
22	Rosmarinic acid	20.231
23	Salicylic acid	21.231
24	Naphtoresorcinol	24.039
25	Trans cinnamic acid	25.999
26	Quercitin dihydrate	26.760
27	Apigenine	27.273
28	4, methoxycinnamic acid	27.693
29	4',5,7 – trihydroxyflavone	30.326
30	Kaempferol	31.840

Previous reports showed that plants can be used in the food industry due to their organoleptic and nutritional qualities, as sources of antioxidants to preserve food quality (Table 2). In fact, tree nuts have long been considered an important component o the Mediterranean diet and according the US Food and Drug Administration, the consumption of 42 g/day of most tree nuts may reduce the risk of heart disease. It is also claimed that the consumption of foods rich in natural antioxidant protect against certain types of cancer and may reduce the risk of cardiovascular and cerebrovascular events due to the presence of antioxidants able to scavenge free radicals, thereby reducing oxidative damage of cellular macromolecules (lipids, proteins and nucleic acids). Recently, Zhang et al. (2009) identified seven phenolic compounds in *J. regia* by spectroscopic methods which are pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, protocatechuic acid, gallic acid and 3,4,8,9,10-pentahydroxydibenzo(b,d)pyran-6-one with significant antioxidant activities.

The results of the antioxidant properties of ethyl acetate extract are summarized in Table 3. The study reveals that the antioxidant activity of “Swak” extract was about 329 mg GAE.g^{-1} DW and the total phenolic content was 33,833 mg GAE.g^{-1} DW. Similarly, total condensed tannin content of ethyl acetate extract was estimated at 16.167 mg EC.g^{-1} DW. The determination of the concentration corresponding to 50% of inhibition of radical DPPH showed that showed that the ethyl acetate extract possess high antioxidant activities comparatively to the BHT (IC_{50} 3 µg/ml and IC_{50} 11 µg/ml respectively). In the reducing power assay, our results showed that the value of EC_{50} was about 99 µg.ml^{-1} for the ethyl acetate extract which not differ largely with the result of the standard BHT (EC_{50}, 75 µg.ml^{-1}). The antioxidant activity of ethyl acetate extract of *J. regia* bark’s measured by the bleaching of β-carotene and the superoxide anion radical-scavenging activity were estimated at IC_{50} = 280 µg.ml^{-1}

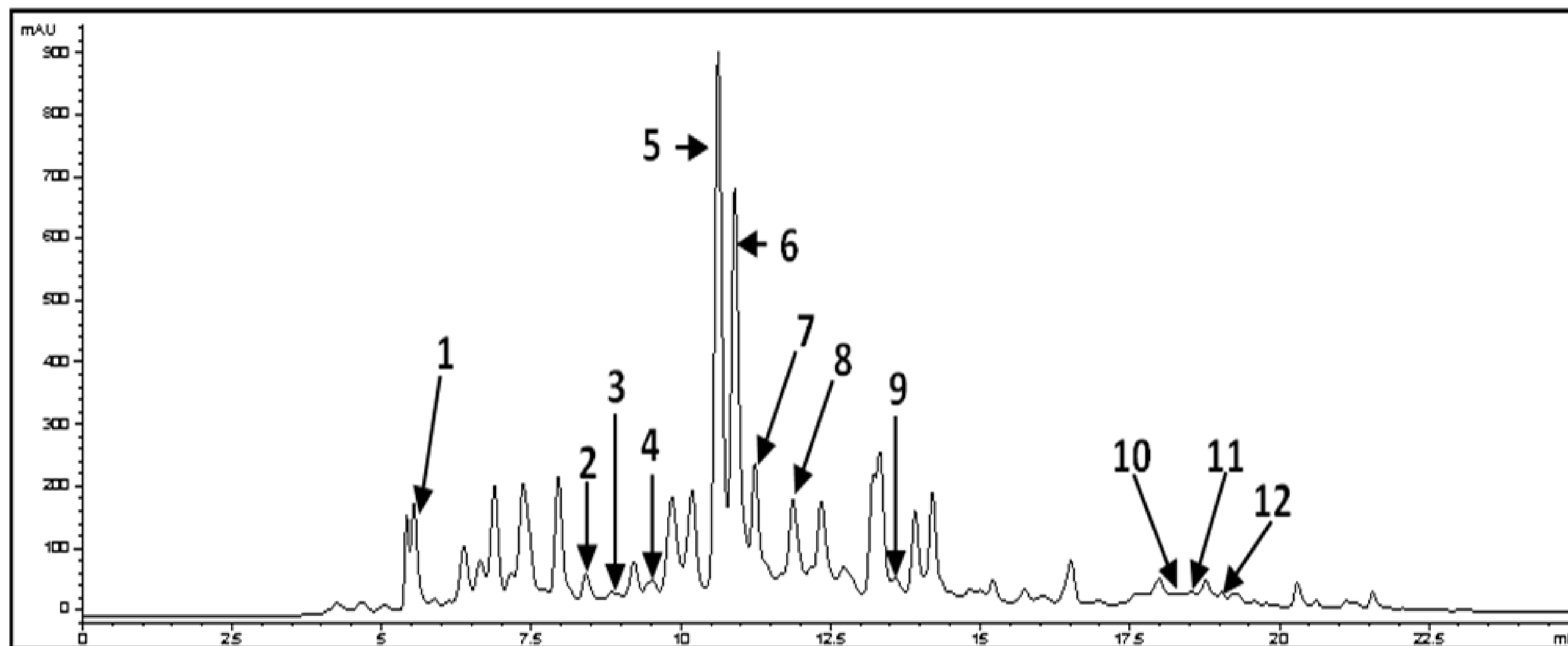

Figure 2. RP-HPLC Chromatographic profiles of phenolic acids and flavonoids in the ethyl acetate extract of *J. regia* barks monitored at 280 nm. The peak numbers correspond to: 1, gallic acid; 2, chlorogenic acid; 3, catechine hydrae; 4, resorcinol; 5, caffeic acid; 6, rutin trihydrate; 7, syringic acid; 8, vanillic acid; 9, *p*-coumaric acid; 10, quercitine dihydrate; 11, naphto-resorcinol; 12, trans-cinnamic acid.

and 70 $\mu g.ml^{-1}$ which differ largely with those of the standard BHT (75 and 1.5 $\mu g.ml^{-1}$, respectively). Several studies have been done to study the antioxidant activities of the *J. regia* organs using different solvent (Table 2). In fact, no study was found to report the antioxidant activities of the treated *J. regia* bark either in Tunisia or from the rest of the world. Comparing our results with the results obtained by Kornsteiner et al. (2006), that registered 1025 mg GAE/100 g, we concluded that Tunisian *J. regia* (bark) exhibited higher amounts of phenols in a ratio of 3.4 folds more. However, these authors also observed that walnut fruits showed the highest total phenolic contents when 10 nuts extracted the phenolic fraction with a solution of 75% acetone and 25% of 526 µmol/L sodium metabisulfite. Probably, the differences in the results could be explained by the different extraction methodologies.

In fact, Pereira et al. (2008) reported that the study of phenolic content present in different walnut cultivars aqueous extracts revealed values between 58.78 mg GAES/g in cv. Lara and 95.06 mg GAES/g in cv. Mayette. The determination of the concentration corresponding to 50% of inhibition of radical DPPH showed that the antiradical activity of ethyl acetate extract of J. regia (IC_{50}, 3 µg/ml) is very significant comparing to BHT as a synthetic antioxidant used in this study (IC_{50}, 11.5 µg/ml). Pereira et al. (2008)

Table 2. Review of the chemical composition and the biological activities of different organs of *J. regia* (Green husks, nuts, leaves and bark).

Organ	Chemical composition/references	Biological activities/references
Green Husks	*Juglone (Mahoney et al., 2000) *Phenolic compounds: Chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, gallic acid, ellagic acid, proto-catechuic acid, syringic acid, vanillic acid, catechin, epicatechin, myrecitin (Stampar et al., 2006) *Juglanin B (Huang et al., 2010) *Rhoiptelol, Juglanin (A, B, and C), α-tetralone derivative (sclerone): (Li et al., 2008)	*Antiproliferative, antioxidant and anti-hemolytic activities (Carvalho et al., 2010) *Antiradicalar and antimicrobial activities (Oliveira et al., 2008)
Nuts	*Fatty acids (linoleic and linolenic acids), tocopherols (γ-tocopherol), tocotrienols, proteins, fibers, melatonin, folate, sterols, tannins (Pereira et al., 2008) *Polyphenols: gallic, ellagic, syringic, 5-O-cafeoylquinic, caffeic, p-coumaric, ferulic and sinapic acids (Fukuda et al., 2006) *Tannins: glansrins (A, B and C), casuarinin, steophyllarin (Colaric et al., 2005) *Pyrogallol, p-hydroxybenzoic acid, vanillic acid, ethyl gallate, protocatechuic acid, gallic acid and 3,4,8,9,10-pentahydroxydibenzo[b,d]pyran-6-one (Zhang et al., 2009) *Nut oil: phospholipids, sphingolipids, sterols and tocopherols (Miraliakbari and Shahidi, 2008)	*Antiproliferative, antioxidant and anti-hemolytic activities (Carvalho et al., 2010) *Reduce the risk of coronary heart disease (Blomhoff et al., 2006) *Antibacterial effect (Pereira et al., 2008) *Antiatherogenic effect (Zambón et al., 200) *Anticancer activities (Kaur et al., 2003; Hardmn and Ion, 2008; Yang et al., 2009) *Antioxidant activities of the nut oil (Miraliakbari and Shahidi, 2008)
Leaves	*Juglone as the major compound I the fresh leaves (Bruneton, 1993) **p*-coumaric acid, 5-O-caffeoylquinic acid (Pereira et al., 2007) *3-O-coumaroylquinic acid, 4-O-*p*-coumaroylquinic acid, 3-O-ccaffeoylquinic acid, quercitin 3-O- galactoside (the major compound), quercitin 3-O-pentoside derivates, quercitin 3-O- arabinoside, quercitin 3-O- xyloside, quercitin 3-O- ramnoside and kaempferol 3-O- pentoside (Amaral et al., 2004)	*Infusion (Amaral et al., 2004) *Antiproliferative, antioxidant and anti-hemolytic activities Carvalho et al., 2010) *Keratolytic, antifungal, hypoglycemic, hypotensive, anti-scrafulous and sedatve activities (Valnet, 1992; Girzu et al., 1998) *Treatment of : skin inflammation, hyperdrosis and ulcers, antidiarrheic, antihelmitic, antiseptic and astringent activities (Bruneton, 1999) *Antiradicalar (Pereira et al., 2007) and antibacterial activities (Almeida et al., 2008)
Bark	*Tannins, polyphenols and flavonoids (Alkhawajah, 1997) *Polyphenols in the treated bark: caffeic acid, rutin trihydrate, syringic acid, gallic acid, chlorogenic acid, resorcinol, vanillic acid, naphtho-resorcinol, quercetin dihydrate, *p*-coumaric acid, trans-cinnamic acid and catechine hydrate (This study)	*Antibacterial activities (Alkhawajah, 1997) *Antifungal activities (Noumi et al., 2010) *Antioxidant activities (This study)

Table 3. Antioxidant activities of ethyl acetate extract of *J. regia* treated barks.

	***J. regia* (Ethyl acetate extract)**	**BHT**
TAA: (mg $GAE.g^{-1}$ DW)	329	-
Total Polyphenols: (mg $GAE.g^{-1}$ DW)	34.833	-
Tannins : (mg $EC.g^{-1}$ DW)	16.167	-
DPPH: IC_{50} ($\mu g.ml^{-1}$)	3	11.5
β- carotenes: IC_{50} ($\mu g.ml^{-1}$)	280	75
RP: EC_{50} ($\mu g.ml^{-1}$)	99	75
$O_2^{\cdot -}$: IC_{50} ($\mu g.ml^{-1}$)	70	1.5

TAA: Total antioxidant activity is expressed as mg gallic acid equivalents per gram of dry weight; Total polyphenols is expressed as mg gallic acid equivalents per gram of dry weight; Tannins is expressed as mg (+)-catechin/g DW; DPPH radical–scavenging activity is expressed as IC_{50} values (µg/ml); β-Carotens bleaching test is expressed as IC_{50} values (µg.ml-1); RP: reducing power was expressed as EC_{50} values (µg/ml); O_2.-: Superoxide anion radical-scavenging activity is expressed as IC_{50} values (µg/ml).

founded that the cultivar Parisienne presented the lowest EC_{25} value (EC_{25}, 1.56 mg/ml). As seen in Table 3, the walnut ethyl acetate extract exhibit a considerable O_2^- scavenging activity (IC_{50}, 70 µg.ml-1) but 46.6 folds less than BHT (IC_{50}, 1.5 $\mu g.ml^{-1}$). The same team reported that walnut's kernel showed high reducing power, even at concentrations below 1 mg/mL, being even more potent than BHA (3.6 mg/mL) and α-tocopherol (8.6 mg/mL) standards.

Conclusions

In conclusion, the results of this study represent the first evidence that walnut treated barks in Tunisia (ethyl acetate extract) possess high antioxidant activities correlated with a high concentration of tannins and polyphenols. Additionally to the known molecules with beneficial interest for Human health described in the different organs of *J.regia* tree, the identified polyphenols in the present work contribute to highlight and reinforce the large scale of biological properties of this plant and therefore suggested that *J. regia* can be used as an inexpensive and easily accessible source of effective natural antioxidants and chemopreventive agents. Future clinical investigations on this medicinal plant should be encouraged especially the comparison between the chemical composition and the biological activities of both treated and untreated walnut bark and their role to prevent the formation of oral *Candida* spp. biofilm.

REFERENCES

Alkhawajah AM (1997). Studies on the antimicrobial activity of *Juglans regia*. Am. J. Chin. Med., 25: 175-180.

Almeida IF, Fernandes E, Lima JLFC, Costa PC, Bahia MF (2008). Walnut (*Juglans regia*) leaf extracts are strong scavenger of pro-oxidant reactive species. Food Chem., 106: 1014-1020.

Amaral JS, Alves M, Seabra R, Oliveira B (2005). Vitamin E composition of walnuts (*Juglans regia* L.): a 3-year comparative study of different cultivars. J. Agric. Food. Chem., 53: 5467-5472.

Amaral JS, Casal S, Pereira J, Seabra R, Oliveira B (2003). Determination of sterol and fatty acid compositions, oxidative stability, and nutritional value of six walnut (*Juglans regia* L.) cultivars grown in Portugal. J. Agric. Food. Chem., 51: 7698-7702.

Amaral JS, Cunha S, Alves MR, Pereira JA, Seabra RM, Oliveira BPP (2004). Triacyl glycerol composition of walnut (*Juglans regia* L.) cultivars: Characterization by HPLC-ELSD and chemometrics J. Agric. Food Chem., 52: 7964-7969.

Amaral JS, Valentão P, Andrade PB, Martins RC, Seabra RM (2008). Do Cultivar, Geographical Location and Crop Season Influence Phenolic Profile of Walnut Leaves? Molecules, 13: 1321-1332.

Blomhoff R, Carlsen M, Andersen L, Jacobs Jr D (2006). Health benefits of nuts: potential role of antioxidants. Br. J. Nutr., 96: 52-60.

Bruneton J (1993). Pharmacogosie, phytochimie, plantes médicinales. Paris: Tec. and Doc.-Lavoisier, p. 348.

Bruneton J (1999). Pharmacognosie, phytochimie, plantes médicinales. In: Technique et Documentation Lavoisier, Paris, pp. 418-419.

Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jerónimo C, Silva BM (2010). Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. Food Chem. Toxicol., 48(1): 441-447.

Colaric M, Veberic R, Solar A, Hudina M, Stampar F (2005). Phenolic acids, syringaldehyde, and juglone in fruits of different cultivars of *Juglans regia* L.. J. Agric. Food Chem., 53: 6390-6396.

Cosmolescu S, Trandafir I, Achim GH, Botu M, Baciu A, Gruia M (2010). Phenolics of Green Husk in Mature Walnut Fruits. Not. Bot. Hort. Agrobot. Cluj., 38(1): 53-56.

Dewanto V, Wu X, Adom KK, Liu RH (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. J. Agric. Food. Chem., 50: 3010-3014.

Fukuda T, Ito H, Yoshida T (2003). Antioxidative polyphenols from walnuts (*Juglans regia* L.). Phytochemistry, 63: 795-801.

Gîrzu M, Carnat A, Privat AM, Fiaplip J, Carnat AP, Lamaison JL (1998). Sedative effect of walnut leaf extract and juglone, an isolated constituent. Pharm. Biol., 36: 280-286.

Hanato T, Kagawa H, Yasuhara T, Okuda T (1988). Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. Chem. Pharm. Bull., 36: 2090-2097.

Hardman WE, Ion G (2008). Suppression of implanted MDA–MB 231 human breast cancer growth in nude mice by dietary walnut. Nutr. Cancer, 60: 666-674.

Huang XY, Duan QY, Liu JX, Di DL (2010). Determination of a novel diarylheptanoid (Juglanin B) from green walnut husks (*Juglans regia* L.) in rat plasma by high-performance liquid chromatography. Biomed. Chromatogr., 24(3): 307–311.

Jaimand K, Baghai P, Rezaee MB, Sajadipoor SA, Nasrabadi M (2004). Determination of Juglone from Leaves and fresh peels of

J*uglans regia L*. by High Performance Liquid Chromatography. Iran. J. Med. Aromat. Plan. Res., 20: 323-331.

Kaur K, Michael H, Arora S, Härkönen PL, Kumar S (2003). Studies on correlation of antimutagenic and antiproliferative activities of *Juglans regia* L.. J. Environ. Pathol. Toxicol. Oncol., 22: 59-67.

Koleva II, Teris AB, Jozef PH, Linssen AG, Lyuba NE (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal., 13: 8-17.

Kornsteiner M, Wagner KH, Elmadfa I (2006). Tocopherol and total phenolics in 10 different nut types. Food Chem., 98: 381-387.

Li C, Liu JX, Zhao L, Di DL, Meng M, Jiang SX (2008). Capillary zone electrophoresis for separation and analysis of four diarylheptanoids and an alpha-tetralone derivative in the green walnut husks (*Juglans regia* L.). J. Pharm. Biomed. Anal*.,* 48(3): 749-753.

Li L, Tsao R, Yang R, Kramer JKG, Hernandez M (2007). Fatty acid profiles, tocopheol contents, and antioxidant activities of heartnut (*Juglans ailanthiofolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.). J. Agric. Food Chem., 55: 1164-1169.

Li L, Tsao R, Yang R, Liu CM, Zhu HH, Young JC (2006). Polyphenolic profiles and antioxidant activities of heartnut (*Juglans ailanthifolia* var. *cordiformis*) and Persian walnut (*Juglans regia* L.). J. Agric. Food Chem., 54: 8033-8040.

Mahoney N, Molyneux RJ, Campbell BC (2000). Regulation of aflotoxin production by naphtoquinones of walnut (*Juglans regia*). J. Agric. Food Chem., 48: 4418-4421.

Miraliakbari H, Shahidi F (2008). Oxidative stability of tree nut oils. Food Chem., 111(2): 421-427.

Noumi E, Hajlaoui H, Trabelsi N, Ksouri R, Bakhrouf A, Snoussi M (2011). Antioxidant activities and RP-HPLC identification of polyphenols in the acetone 80 extract of *Salvadora persica*. J.P.P., 5(7): 966-971.

Noumi E, Snoussi M, Hajlaoui H, Valentin E, Bakhrouf A (2010). Antifungal properties of *Salvadora persica* and *Juglans regia* L. extracts against oral *Candida* strains. Eur. J. Microbiol. Infect. Dis., 29: 81-88.

Oliveira I, Sousa A, Ferreira I, Bento A, Estevinho L, Pereira JA (2008). Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. Food Chem. Toxicol., 46: 2326-2331.

Oyaizu M (1986). Studies on products of browning reaction: Antioxidative activity of products of browning reaction. Jpn. J. Nutr., 44: 307-315.

Pereira JA, Oliveira I, Sousa A, Ferreira I, Bento A, Estevinho L (2008). Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. Food Chem. Toxicol., 46: 2103-2111.

Pereira JA*,* Oliveira I, Sousa A*,* Valentao P, Andrade P, Ferreira I, Ferreres F, Bento A, Seabra R, Estevinho L (2007)*.* Walnut (*Juglans regia L.*) leaves*:* phenolic compound*,* antibacterial activity *and* antioxidant potential of different cultivars. Food Chem. Toxicol*.,* 45: 2287-2295.

Prieto P, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal. Biochem., 269: 337-341.

Reiter RJ, Manchester LC, Dun-Xian TMD (2005). Melatonin in walnuts: influence on levels of melatonin and total antioxidant capacity of blood. Nutrition, 21: 920-924.

Stampar F, Solar A, Hudina M, Veberic R, Colaric M (2006). Traditional walnut liqueur – cocktail of phenolics. Food Chem., 95: 627-631.

Sun B, Richardo-Da-Silvia JM, Spranger I (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food. Chem., 46: 4267-4274.

Trabelsi N, Megdiche W, Ksouri R, Falleh H, Oueslati S, Soumaya B, Hajlaoui H, Abdelly C (2010). Solvent effects on phenolic contents and biological activities of the halophyte *Limoniastrum monopetalum* leaves. LWT-Food Sci. Technol., 43: 632-639.

Valnet J (1992). Phytotherapie: Traitement des maladies par les plantes. Paris: Maloine, pp. 476-478.

Yang J, Liu R, Halim L (2009). Antioxidant and antiproliferative activities of common edible nut seeds. Food Sci. Technol., 42: 1-8.

Zambón D, Sabaté J, Muñoz S, Campero B, Casals E, Merlos M, Laguna JC, Ros E (2000). Substituting walnuts for monounsaturated fat improves the serum lipid profile of hypercholesterolemic men and women. A randomized crossover trial. Ann. Int. Med., 132: 538-546.

Zhang Z, Liao L, Moore J, Wu T, Wang Z (2009). Antioxidant phenolic compounds from walnut kernels (*Juglans regia* L.). Food Chem., 113: 160-165.

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Full Length Research Paper

Bioactivity of the acetone extract of the stem bark of *Combretum molle* on selected bacterial pathogens: Preliminary phytochemical screening

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The antimicrobial activity of fractionated acetone extracts of the stem bark of *Combretum molle* was evaluated against *Helicobacter pylori* PE 252C, *Streptococcus pyogenes* ATCC 49399, *Pseudomonas aeruginosa* ATCC 15442 and *Plesiomonas shigelloides* ATCC 51903 in a bid to identify the active constituents of this plant. Fractionation of the acetone extract was done using thin layer chromatography (TLC) and bioautography to ascertain the presence of compounds and determine their antimicrobial activity, respectively. Four of the seventeen eluted fractions [E.A3, E.A4 and ethyl acetate/ methanol/ water (EMW) 1 and 3] were active against all the test organisms except *H. pylori* PE 252C with a minimum inhibitory concentration $(MIC)_{50}$ ranging from 0.0097 to 2.5 mg/ml; however E. A 4 was the only fraction which demonstrated broad spectrum activity. We conclude that the acetone extract and fractions of the stem bark of *C. molle* possess *in-vitro* antibacterial activity and therefore confirm the rationale behind the use of this plant in traditional medicine.

Key words: Phytochemical analysis, *Combretum molle*, antimicrobial activity, bioautography, minimum inhibition concentration.

INTRODUCTION

Medicinal plants have been used for centuries as remedies for human diseases and they are the richest bio-resources of drugs of traditional medicinal systems, modern medicines, food supplements, and folk medicines (Das et al., 2009). Herbal medicine has been shown to have profound utility with about 80% of rural population dependence on it for their primary health care; hence World Health Organization (WHO) has advocated the need for interaction between modern and traditional medicine with a view to exploiting and identifying compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins (Ndip et al., 2008). Since the discovery of antibiotics in the 1950s, the use of plant derivatives as antimicrobial agents was to a lesser extent, nonexistent; the interest has increased in the late 1990s as conventional antibiotics became ineffective and also many of the antimicrobial drugs in use have undesirable toxic effects (Cowan, 1999). This interest primarily stems from the belief that green medicine is safe and dependable, compared with costly synthetic drugs that have adverse effects (Prach and Shilpa, 2010). *Combretum molle* (R. Br. Ex G. Don) Velvet bush willow in English (*Basterrooibos* in Afrikaans and *umBondwe* in Zulu), is one of the herbal plants belonging to the genus *Combretum*. The plant is widely used in African traditional medicine for treatment of various ailments and diseases (Tan et al., 2002; Bessong et al., 2004).

Decoctions of the roots of *C. molle* seem to have a variety of uses against hookworm, stomach pains, snake bite, leprosy, fever, dysentery, general body swellings, arthritic and other inflammatory conditions, and abortion as well as for swelling of the abdomen, sterility and constipation (Fyhrquist et al., 2002; Ojewole, 2008). *C. molle* like other *Combretum* species has been studied by

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a number of researchers and compounds such as ellagitannins, triterpenes/ triterpenoids, arjunolic acid and glycosides have been isolated; which have demonstrated antifungal, antimicrobial, anti-parasitic, antioxidant, and anti-inflammatory activity (Asres et al., 2004; Eloff et al., 2005; Chaabi et al., 2006). Notwithstanding, there are few reports on the medicinal use of the stem bark of *C. molle*, an aqueous suspension of the stem bark is drunk to treat angina and gargling; decoctions of the inner bark is used for the treatment of stomach problems (Fyhrquist et al., 2007). Therefore there is a dearth of information regarding the bioactivity of the stem bark of this plant which merits further investigation. The present study evaluated the antimicrobial activity of the fractionated acetone extracts of the stem bark of *C. molle* against some bacterial pathogens reported to be a growing problem associated with drug resistance (Eloff et al., 2005). This constitutes part of an effort to identify potential sources of active molecules for the synthesis of new drugs to circumvent the problem of increasing drug resistance.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains used in the study consists of reference strains; *S. pyogenes* ATCC 49399, *P. shigelloides* ATCC 51903, *P. aeruginosa* ATCC 15442 and *H. pylori* PE 252C, clinical isolate which was isolated from gastric biopsies, lyophilized and kept at –80℃ in our laboratory (Tanih et al., 2010). These organisms were selected based on their disease burden and increasing trend of antibiotic resistance in the developing world (Eloff et al., 2005).

Preparation of the plant extract

The plant was selected based on ethno-botanical information. The stem bark was harvested in the vicinity of Venda, Limpopo Province. Identification was done by botanists at the University of Venda where voucher specimen (CNU FHO 5) has been deposited. The bark was washed and dried at room temperature for 2 weeks, then ground to fine powder using a mechanical blender (ATO MSE mix, 702732, England). The method previously described by Ndip et al. (2009) was employed with modifications. Briefly, six solvents (hexane, dichloromethane (DCM), ethyl acetate, acetone, ethanol and methanol) were used for extraction. Ground plant material (300 g) was macerated in three folds excess of the solvent in extraction bottles such that the solvent was above the plant material. The slurry was placed in a shaker (Edison, N.J., USA) for 48 h then centrifuged at 3000 rpm for 5 min and filtered using filter paper of pore size 60A. The process was repeated twice for a total of three extractions (exhaustive extraction) for each solvent. The collected extracts were concentrated under reduced pressure in a rotavapor (Strike 202, Steroglass, Italy) to recover the solvents. The yielded extracts were weighed and stored in a labelled tight lid container. The extracts were evaluated for their antimicrobial activity as previously described (Nyenje and Ndip, 2011) and retained for further bioassay.

Thin layer chromatography analysis of crude extract

Fractionation of the acetone extract, which were earlier determined to be very active (data not shown) was done using Silica gel TLC plates (Kieselgel 60 F_{254}, Merck, Germany) according to the method of Masoko and Eloff (2006). Acetone extract (5 µl) of different concentrations (25, 50 and 100 mg) were spotted on TLC plates, and eluted using three different mobile solvent systems, EMW in the ratio of 40: 5.4 :4 v/v to separate the polar compounds; chloroform/ethyl acetate/fomic acid (CEF) in the ratio 5:4:1 v/v to separate intermediate compounds and benzene/ethanol/ammonium hydroxide (BEA) in the ratio 90:10:1 v/v to separate non polar compounds. These solvent systems have been optimized to separate components of the family Combretaceae (Eloff, 1998). Samples were applied rapidly and developed without delay to minimize oxidation or photo-oxidation of constituents. The plates were done in duplictes (plate A for TLC and plate B for bioautography screening). The developed plates (plate A) were visuallized under ultraviolet light (254 and 360 nm), which was subsequently sprayed with vanillin sulphuric acid reagent (2 mg of vanillin in 28 ml of methanol plus 1 ml of concentrated sulphuric acid) and carefully heated for 5 min at 100℃ to allow colour development. The retention factor value (R_f) of the visible bands were marked under daylight.

$$Rf = \frac{\text{Distance moved by analyte (compound)}}{\text{Distance moved by solvent}}$$

Bioautography analysis for bioactivity

Developed TLC plates (plate B) were dried under a stream of fast moving air for three days to remove traces of solvent on the plates. *P. shigelloides*, *P. aeruginosa* and *S. pyogenes* were sub-cultured in 20 ml of freshly prepared brain heart infusion (BHI) broth and incubated overnight, to ensure that actively growing bacteria is sprayed. The plates were sprayed with the bacterial suspension until wet, incubated overnight at 37℃ and 100% relative humidity. The plates were sprayed with 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) [0.2 mg of INT + 99 ml of water + 1ml of methanol] and further incubated for 1h. White bands indicated that reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of organisms. The retention factor (Rf) of the inhibition zones on the plate (plate B) was compared with the Rf of the reference chromatogram (plate A) to determine the Rf of the active compound (Masoko and Eloff, 2006). *H. pylori* is a fastidious organism which requires nutritional supplements as well as special growth conditions, as such, the organism did not grow on the TLC plate; indirect bioautography (agar overlay) method was then used to develop the dried TLC plates and were placed in sterile Petri dish. A thin layer of BHI agar containing 10^5CFU/ml of *H. pylori* isolates, Skirrow's supplement and 7% horse serum was poured over the plates. After solidification of the medium, the plates were incubated for 3 to 5 days under micro-aerophilic conditions (5 to 6% O_2 and 10% CO_2) [Anaerocult, Basingstoke, England]. Inhibition of growth was indicated by zone of inhibition around the active compounds on the chromatogram. The Rf of the zones on the plate was compared with that on the reference plate to find the Rf of the active compound (Valgas et al., 2007).

Fractionation of crude extract by column chromatography

A 40 cm long × 2.5 cm diameter glass column was packed to a height of 31cm with a slurry of silica gel 60 (Merck, Germany; particle size 0.063 to 0.2 mm/ 70 to 230 mesh). The column was equilibrated with 100% ethyl acetate for 30 min. Six grams of acetone extract and 12 g of silica gel powder were mixed and ground to very fine particles. The mixture was then loaded onto a

silica gel column equilibrated with ethyl acetate. The combination which gave good activity, ethyl acetate/methanol/water (40:5.4:4) was used to elute the column; fractions (200 ml) were collected and concentrated on a rotary evaporator (Strike 202 Steroglass, Italy). Fractions were weighed and stored in air tight containers for further bioassay (Ndip et al., 2009).

Determination of MIC_{50} of fractions

The MIC of the fractions was determined by micro broth dilution method performed in 96-well plates (Ndip et al., 2009) and active fractions were further analyzed on TLC to determine the purity. Briefly, test fractions were prepared at a concentration of 5.0 mg/ml. Two-fold dilutions of each fraction was made in the test wells in BHI broth supplemented with 5% horse serum and Skirrow's supplement (for *H. pylori*) (Oxoid, England). The final concentration ranged from 2 to 2500 µg/ml. Twenty microlitres of an 18 h old broth culture of the test organisms (McFarland turbidity standard 9 and 2 for *H. pylori*) was added to 100 µL of fraction-containing culture medium. Control wells were prepared with culture medium only and bacterial suspension and broth only, respectively. Ciprofloxacin was used as a positive control antibiotic and run alongside each batch of tests at a concentration range of 0.0002 to 2 µg/ml. An automatic ELISA microplate reader (SynergyMx, Biotek^R USA) adjusted to 620 nm was used to measure the absorbance of the plates before and after incubation at 37℃ and under micro- aerophilic conditions for *H. pylori*. The absorbencies were compared to detect an increase or decrease in bacterial growth and the values plotted against concentration. The lowest concentration of the fraction resulting in inhibition of 50% bacterial growth was recorded as the MIC_{50}.

Statistical analysis

The statistical package used for analysis was PASW version 18.0 (Chicago, Illinois USA, 2009). One way analysis of variance (ANOVA) was used to compare the difference in inhibitory activities of fractions and antibiotic, followed by Turkey's post-hoc test. The differences were considered significant at $P < 0.05$.

RESULTS

Fractionation of bioactive extracts by thin layer chromatography

The intermediate polarity solvent combination, chloroform/ethyl acetate/formic acid, separated 10 compounds, while the more polar combination, EMW, separated 9 compounds. The least polar combination benzene/ethanol/ammonium hydroxide had the least number of compounds (3). Twenty two compounds were observed with Vanillin spray while 9 were visualized under ultra-violet. Five compounds fluoresced on chloroform/ethyl acetate/formic acid, 3 on benzene/ethanol/ammonium hydroxide and only 1 on EMW.

Bioautography assay

Although, more compounds were observed on chloroform/ ethyl acetate/formic acid plate, EMW solvent system separated more active compounds (9) compared to 5 on chloroform/ethyl acetate/formic acid (Table 1). Since the Rf value is constant for the same compound under defined conditions, the presence of clear bands with the same Rf value may imply that the same compounds are probably responsible for the antimicrobial activity in the extract.

Column chromatography analysis and MIC_{50} determination of the fractions

Seventeen fractions (200 ml each) were collected from the column chromatography assay. Fractions E.A 1- 4 were eluted with 100% ethyl acetate and fractions EMW 1 to 13 eluted with EMW in the ratio 40:5.4: 4 v/v. To determine the purity, fractions were further analyzed on TLC plate. Fractions, EMW 8 and 13 presented one band on TLC plate and fractions EMW 6 and 9 had compounds with similar Rf value and different MIC values. The same was true for EMW 11 and 12.

Although, fractions EMW 8 and 13 presented one band on TLC, they demonstrated activity against *S. pyogenes* only; whereas fractions E.A 3, E.A 4, EMW 1 and 3 were active against all the test organisms except *H. pylori* with a MIC_{50} ranging from 0.0097 to 2.5 mg/ml which was not significantly different ($P>0.05$) from the control antibiotic, ciprofloxacin (Table 2). EA4 was the only fraction which had broad spectrum activity and the TLC profile showed more than one compound.

DISCUSSION

Plants have been used throughout history in traditional medicines for the treatment of diseases worldwide. Today, there has been an increasing interest in studying the biological properties of plants and their derivatives for discovering biologically active compounds (Das et al., 2009). The biological activity of a given plant extract reflects contributions from a number of constituents. Consequently, the initial observation of biological activity in a plant extract is typically followed by bioassay-guided fractionation which is designed to isolate and purify the bioactive constituents. The quantity and types of bio-molecules eluted from extracts will depend on the polarity of the solvents (Eloff et al., 1998; Masoko et al., 2006; Ndip et al., 2009). In a previous study (Nyenje and Ndip, 2011), we reported the acetone extract of *C. molle* to be active against the test organisms with an inhibition zone diameter ranging from 11 to 32 mm and MIC_{50} of 0.078 to 2.5 mg/ml. Consequently, the extract was subjected to phytochemical screening using TLC. The intermediate polarity solvent combination chloroform/ ethyl acetate/ formic acid gave good separation. The results are contrary to the findings of Eloff et al. (2005) who investigated the efficacy of the leaf of *Combretum woodii*

Table 1. Inhibition of bacterial growth by acetone extract by bioautography.

Rf-value					Test organisms			
	S. pyogenes		*P. shigelloides*		*P. aeruginosa*		*H. pylori 252C*	
	EMW	CEF	EMW	CEF	EMW	CEF	EMW	CEF
0.031	-	-	x	-	-	-	x	-
0.181	-	-	-	x	-	-	-	x
0.361	-	-	-	x	-	-	-	x
0.437	x	-	-	-	xx	-	-	-
0.515	-	-	-	x	-	-	-	x
0.562	-	-	-	-	xx	-	-	-
0.818	-	x	-	-	-	xx	-	-
0.878	-	-	-	-	-	x	-	-

x, active; xx, more active; -, no activity, CEF, chloroform/ ethyl acetate/formic acid; EMW, ethyl acetate/ methanol/water; Rf retention factor.

Table 2. Eluted fractions and their MIC_{50} against the test organisms.

Fraction/ antibiotic	Rf value of the compounds in fractions	MIC_{50} against test organisms			
		S. pyogenes	*P. shigelloides*	*P. aeruginosa*	*H. pylori* 252C
E.A 1	0.810, 0.878	NA	NA	NA	NA
E.A 2	0.135, 0.168, 0.439, 0.608	NA	NA	NA	NA
E.A 3	0.186, 0.202, 0.472, 0.506	0.195	0.078	1.25	NA
E.A 4	0.641, 0.810	0.195	0.078	0.156	2.5
EMW 1	0.472, 0.608, 0.709	0.0097	0.0097	0.625	NA
EMW 2	0.067, 0.202, 0.506	0.0195	0.156	NA	NA
EMW 3	0.168, 0.202, 0.405, 0.472	0.078	0.156	2.5	NA
EMW 4	0.033, 0.067, 0.202, 0.4391	0.078	0.312	NA	NA
EMW 5	0.033, 0.439, 0. 810, 0.844	0.312	0.625	NA	NA
EMW 6	0.013, 0.439, 0.810	0.312	0.625	NA	NA
EMW 7	0.031, 0.824	0.625	1.25	NA	NA
EMW 8	0.202	1.25	NA	NA	NA
EMW 9	0.013, 0.439, 0.810	1.25	1.25	NA	NA
EMW 10	0.439, 0.810	0.625	NA	NA	NA
EMW 11	0.013, 0.810	NA	0.156	NA	NA
EMW 12	0.013, 0.810	NA	NA	NA	NA
EMW 13	0.878	1.25	NA	NA	NA
Ciprofloxacin		0.024	0.048	0.024	0.075

E.A, ethyl acetate; EMW, ethyl acetate/ methanol/ water; NA, no activity at 5 mg/ml, *P. shigelloides, Plesiomonas shigelloides* ATCC 51903; *P. aerginosa, P. aeruginosa* ATCC 15442; *H. pylori* 252C, *H. pylori* PE 252C; *S. pyogenes, S. pyogenes* ATCC 49399.

and reported good separation with benzene/ethanol/ ammonium hydroxide. Species diversity and difference in climatic conditions of plant growth might explain this discrepancy. Prior studies have demonstrated that more polar solvents generally elute more active molecules (Eloff, 1998; Masoko and Eloff, 2006; Ndip et al., 2009) which is in agreement with our findings where more active compounds were separated with the polar solvent combination EMW. There was a variation in the Rf values of the active compounds on direct and overlay methods used in the study. There was one major antibacterial compound (Rf 0.818 from CEF) with activity against *S. pyogenes* and *P. aeruginosa.* It is also important to note that compounds eluted with EMW (Rf 0.562 and 0.437) were more active against *P. aeruginosa* (Table 1). The findings suggest that some of the antimicrobial compounds of this plant are of intermediate and high polarity. Worthy to note is that bioautography is not a quantitative measure of antimicrobial activity. It only indicates separated compounds with antimicrobial activity. It is also important to note that the absence of activity could be due to evaporation of the active compounds, photo-oxidation or due to very little amount of the active compound (Masoko and Eloff, 2005).

On the other hand it is also possible that synergism plays a major role in extracts that were active when the MIC of the mixture was determined, but no activity when the compounds are separated on bioautography (Masoko and Eloff, 2006). Although, fractions EMW 8 and 13 presented one band on TLC, they demonstrated activity against *S. pyogenes* only; whereas fractions E.A 3, E.A 4, EMW 1 and 3 were active against all the test organisms except *H. pylori* (Table 2). EA4 was the only fraction which had broad spectrum activity and the TLC profile showed more than one compound; hence it was postulated that these compounds work in synergism. Similar findings were reported by Luna- Herrera et al. (2007) who reported that further purification of extracts and fractions end up in loss of biological activity confirming the synergistic activity of the compounds. Previous studies on phytochemical analtsis of this plant led to the isolation of triterpenoid glycosides, tannins, alkaloids, saponins, stilbenes, triterpene saponin-oleanone trypetitepene, arjunolic acid and mollic acid glucosides which demonstrated cytotoxic, antifungal, antimicrobial and anti-inflammatory activity (Asres et al., 2001; Ponou et al., 2008; Ojewole, 2008). Asres et al. (2001) isolated triterpenoid glycosides and tannins from acetone extract of the stem bark of *C. molle*. Punicalagin, arjunglucoside and sericoside were isolated after further purification. These compounds were evaluated for antimycobacterial activity and only punicalagin inhibited the growth of *Mycobacterium tuberculosis* typus humanus ATCC 27294.

Burapadaja and Bunchoo (1995) reported antimicrobial activity of the same compound against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*. Studies into the effects of the terpenoids on isolated bacterial membranes revealed their site of action to be at the phospholipid bilayer. They affect bacterial processes that include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Seenivasan et al., 2006). Hydrolysable tannins have been reported to have anti-*H. pylori* and antiplasimodial activity (Asres and Bucar, 2004; Funtagawa et al., 2004). Tannins are found in large quantities in the bark of trees where they act as a barrier for micro-organisms like bacteria and fungi. They have been found to form irreversible complexes with proline rich protein (Shimada, 2006) resulting in the inhibition of cell protein synthesis. Herbs that have tannins as their main components are astringent in nature and are used for treating inflamed or ulcerated tissues, intestinal disorders such as diarrhoea and dysentery (Parekh and Chanda, 2007). Perhaps similar mechanisms of action were responsible for the antimicrobial actions of the plant extract under our present study.

Conclusion

We conclude that the acetone extract and fractions possess *in vitro* antibacterial activity and therefore confirm the rationale behind the use of this plant in traditional medicine. These may provide starting materials for the synthesis of new drugs.

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REFERENCES

Asres K, Bucar F (2004). Anti-HIV activity against immunodeficiency virus type 1 (HIV-1) and type 327 II (HIV-II) of compounds isolated from the stem bark of *Combretum molle*. Ethiop. Med. J., 43: 15-20.

Asres K, Bucar F, Knauder E, Yardley V, Kendrick H, Croft SL (2001). *In vitro* antiprotozoal activity of extract and compounds from the stem bark of *Combretum molle*. Phytother. Res., 15: 613-617.

Bessong PO, Obi CL, Igunibor E, Andreola M, Litvak S (2004). *In vitro* activity of three selected South African plants against human immunodeficiency virus type 1 reverse transcriptase. Afr. J. Biotechnol., 3: 555-559.

Bessong PO, Rojas LB, Obi CL, Tshisikawe MP, Igunbor EO (2006). Further screening of Venda medicinal plants for activity against HIV type 1 reverse transcriptase and integrase. Afr. J. Biotechnol., 5: 526-528.

Burapadaja S, Bunchoo A (1995). Antimicrobial activity of tannins from *Terminalia citrina*. Planta Med., 61: 365-366.

Chaabi M, Benayache S, Vonthron-Senecheau, E, Weinger B, Anton R, Lobstein A (2006). Antiprotozoa activity of saponins from *Anogeissus leiocorpus* (Combretaceae). Biochem. Syst. Ecol., 36: 59-62.

Cowan MM (1999). Plant Products as Antimicrobial Agents. Clin. Microbiol. Rev. 12: 564-582.

Das K, Tiwari RKS, Shrivastava DK (2009). Techniques for the evaluation of medicinal plants products as antimicrobial agents: Current methods and future trend. J. Med. Plant. Res., 492: 104-111.

Eloff JN (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. Ethnopharmacol., 1: 1-8.

Eloff JN, Famakin JO, Katerere DRP (2005). Isolation of antibacterial Stilbene from *Combretum woodii* (Combretaceae) leaves. Afr. J. Biotechnol., 4: 1167-1171.

Funtagawa K, Hayashi S, Shimomura H (2004). Antibacterial activity against *H. pylori*. J. Microbiol. Immunol., 48: 251-261.

Fyhrquist P, Mwasumbi L, Haeggstro CA, Vuorela H, Hiltunen R, Vuorela P (2002). Ethnobotanical and antimicrobial investigation on some species of *Terminalia* and *Combretum* (Combretaceae) growing in Tanzania. J. Ethnopharmacol., 79: 169-177.

Luna-Herrera JC, Costa MC, Gonzalez HG, Rodrigues AI, Castilho PC (2007). Synergistic antimyobacterial activities of sesquiterpene lactones from *Laurus* spp. J. Antimicrob. Chemother., 59: 548-552.

Masoko PJ Eloff JN (2005). Antifungal activities of six South African *Terminalia* species (Combretaceae). J. Ethnopharmacol., 99: 301-308.

Masoko PJ, Eloff JN (2006). Bioautography indicates the multiplicity of antifungal Compounds from twenty-four southern African *Combretum* species. Afr. J. Biotechnol., 5: 1625-1647.

Ndip RN, Ajonglefac AN, Mbullah SM, Tanih NF, Akoachere JFK, Ndip LM, Luma HN, Wirmum C, Ngwa F, Efange SMN (2008). *In vitro* anti-*Helicobacter pylori* activity of *Lycopodium cernuum*. Afr. J. Biotechnol., 7: 3989-3994.

Ndip RN, Ajonglefac AN, Wirna T, Luma HN, Wirmum C, Efange SMN (2009). *In-vitro* antimicrobial activity of *Ageratum conyzoides* on clinical isolates of *Helicobacter pylori.* Afr. J. Pharm. Pharmacol., 3: 585-592.

Nyenje ME, Ndip RN (2011). *In vitro* antimicrobial activity of crude acetone extract of the stem bark of *Combretum molle* against selected bacterial pathogens. J. Med. Plants Res., 5(21): 5315-5320.

Ojewole JAO (2008). Analgesic and anti-inflammatory effects of mollic acid glucoside, a 1a- hydroxycycloartenoid saponin extractive from *Combretum molle* R. Br. ex G. Don (Combretaceae) leaf. Phytother. Res., 22: 30-35.

Parekh J, Chanda S (2007). *In vitro* antibacterial activity of crude methanol extract of *Woodfordia fruticosa Kurz* flower (*Lythacease*). Braz. J. Microbiol., 38: 2-6.

Ponou BK, Barboni L, Teponno RB, Mbiantcha M, Nguelefack TB, Hee-Juhn P, Kyung-Tae L, Tapondjou LA (2008). Polyhydroxyoleanane-type triterpenoids from *Combretum molle* and their anti-inflammatory activity. Phytochem. Lett., 1: 183-187.

Prach J, Shilpa S (2010). Antimicrobial properties and phytochemical analysis of *Emblica officinalisa.* Asian Biol., 10: 91-95.

Seenivasan P, Manickkam J, Savarimuthu I (2006). *In vitro* antibacterial activity of some plant essential oils. BMC Complement. Altern. Med., 6: 3-9.

Shimada T (2006). Salivary proteins as a defence against dietary tannins. J. Chem. Ecol., 32: 1149-1163.

Tan F, Shi S, Zhong Y, Gong X, Wang Y (2002). Phylogenetic relationships of Combretoideae (Combretaceae) inferred from plastid, nuclear gene and spacer sequences. J. Plant Res., 115: 475-481.

Tanih NF, Okeleye BI, Naidoo N, Clarke AM, Mkwetshana N, Green E, Ndip LM, Ndip RN (2011). Marked susceptibility of South African *Helicobacter pylori* strains to ciprofloxacin and amoxicillin: clinical implications. S. Afr. Med. J., 100: 49-52.

Valgas C, Machado de Souza S, Smania FAE Smania A (2007). Screening methods to determine antibacterial activity of natural products. Braz. J. Microbiol., 38: 1517-1526.

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Full Length Research Paper

Influence of medium composition on multiplication of walnut (*Juglans regia* L.) growth

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***In vitro* proliferation of walnut (*Juglans regia* var. zeiabadi) was studied in response to the four levels (1/3X, 1/2X, 1.0X and 1.5X) of macrominerals of DKW (Driver and kunjyukj, 1984) medium. Macrominerals (N, P, K and Mg) can be a limiting factor for growth quantity (fresh weight and dry weight) and growth quality (explants appearance) of walnut explants. Addition of 0.5X macrominerals to the basal DKW medium in which containing 4.0 µM 6-benzylaminopurine (BA) and 12 µM indole-3-butyric acid (IBA), significantly (P = 0.05) improved the proliferation. Better response of was found with 1.5X macrominerals concentration in respect of growth and proliferation. In contrary, the decrease in mineral availability observed in the depressed growth in the low macrominerals supply treatments (1/3X and 1/2X), (the restricted mineral availability was the main cause of the inhibition of the growth). However, root formation was worsted by supplemented additional minerals. For example, low mineral concentration treatment exhibit high rooting ability (75%) and high mineral concentration showed low (13%).**

Key words: Micropropagation, medium composition, walnut, mineral concentration.

INTRODUCTION

The most popular way of walnut (*Juglane regia* L.) asexual propagation is budding, which is labor intensive, time-consuming and costly. On the other hand, propagation by cuttings is very difficult due to their low rooting ability (McGranahan and Leslie, 1990). In the last decade, *in vitro* cultured techniques have been investigated for the successful large scale propagation of walnut. For example, Rodriguez et al. (1989) reported the establishment of walnut cultures *in vitro* and to describe the development of shoots or roots from cultured walnut embryos. Later, a large amount of works was conducted on different walnut species using different types of explants, media, and culture conditions and rooting techniques, with encouraging results (Jay-Allemand and Cornu, 1986; Gruselle et al., 1987; Cornu and Jay-Allemand, 1989). Most of the aforementioned work was based on a medium developed by DKW medium (Driver and Kuniyuki, 1984) for the *in vitro* culture of *Juglans* species. Several reports indicate that *Juglans* species suitable to a certain degree, to micropropagation (Somers et al., 1982; Driver and Kuniyuki, 1984; Heile-Sudholt et al., 1986; Gruselle et al., 1987; Lee et al., 1986; McGranahan et al., 1988; Revilla et al., 1989; Felaliev, 1990; Leslie and McGranahan, 1992). Somatic embryogenesis has been induced from immature cotyledons in a number of species of *Juglans* (Cornu, 1988; Cornu and Jay-Allemand, 1989; Long et al., 1992; Neuman et al., 1993; Pijut, 1993a, 1993b) and shoot tips rooted plantlets which originated from cotyledon segments with embryonic roots and histology of root structures (Jay-Allemand et al., 1991; Tulecke and McGranahan, 1985; Polito et al., 1989) and also root formation of nodal segments and embryonic axes (Revilla et al., 1989); shoot formation of apical and lateral buds (Felaliev, 1990); bud formation and shoot multiplication (Gruselle et al., 1987; Stephens et al., 1990); somatic embryo originated from ovules improved acclimatization of plantlets (Voyiatzis and McGranahan, 1994).

Weekly transfer of butternut nodal explants to fresh

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Table 1. Effects of mineral concentration on growth (fresh and dry weight), multiplication rate and root formation of walnut (*Juglans regia* var. Zeiabadi) explants.

Rooting	Multi. Rate2	DW	FW	Rel.min. conc.1
(%)	(no mon-1)	(g)	(g)	DKW medium
75	2.1	0.08	7.3	1/3X
52	2.9	0.14	12.9	1/2X
28	3.2	0.20	17.9	1.0X

[1]mineral supplied of DKW medium (1/3X, 1/2X, 1.0X and 1.5X macroelements). [2] Multiplication rat.no. mon^{-1}).

culture medium was necessary to maintain optimum growth and to limit the build up of phytotoxic exudates in the culture medium. The production of exudates from freshly cultured explants of walnuts has also been a problem, solved by employing explants presoaks and transferring explants frequently to fresh medium (Preece et al., 1989; Leslie and McGranahan, 1992). In contrast, DKW medium has proven to be suitable (and in many cases superior) for the culture of *J. regia* as well as other *Juglans* species (Driver and Kuniyuki, 1984; Heile-Sudholt et al., 1986; Lee et al., 1986; McGranahan et al., 1988; Leslie and McGranahan, 1992). As evidence show that many researches with *Juglans* spp. have focused on somatic embryogenesis and initial explant material for the purpose of clonal propagation and subsequent genetic improvement (Preece et al., 1989; Rodriguez et al., 1989). However, few reports have been published on the effects of mineral composition of *in vitro* cultured. Changes in the levels of mineral supply in the medium resulted to changes in the tissues of *in vitro* cultured plantlets are often associated with physiological disorder such as cessation (Barbas et al., 1993), or hyperhydric malformations (Kevers and gasper, 1986). In this work, it is shown that mineral is critical for the growth and root formation of walnut explants.

MATERALS AND METHODS

One month after fruit set, the immature walnut (*Juglans regia* var. Zeiabadi) nut, was cracked and the kernel was immersed in NaOCl (1.0% w/v) for 5 min then rinsed three times in sterile distilled water. Embryo was carefully isolated from the kernel and it was established on gelled basal medium and was incubated in a growth chamber in darkness. After three weeks germinated embryos *in vitro*, four uniform size explants were established on four (1/3X, 1/2X, 1.0X and 1.5X) levels of macroelements of DKW (Driver and kunjyukj, 1984) medium, supplemented with indolbutric acid (IBA) 12 µM, benzyladenine (BA) 2 µM, sucrose 3%, agar (Difco BiTek™ agar) 8%. The medium pH was adjusted to 5.6 by HCl 0.5 N and NaOH 0.5 N before autoclaving.

All explants were kept in a growth room at a temperature of 25 ± 2°C, with 55% relative humidity and 16 h 50 µ m^{-1} S^{-1} cool white fluorescent light. Dry and fresh weights (g), multiplication rate (no. month^{-1}), root formation (%) and adventitious roots per shoot were recorded one month after transfer to new development medium. Containers did not occupy fixed positions on culture shelves but were moved around randomly during visual examination every week.

RESULTS AND DISCUSION

Result of this experiment revealed that different aspects of 'Zeiabadi' walnut growth were mineral dependence in culture medium. As macromineral concentration increased, both growth (dry and fresh weights) and multiplication rate significantly (P=0.05) increased. Whereas, there was a negative relationship between macromineral increase and root formation. The greatest amount of biomass (dry weight 0.26 g, fresh weight 23.4 g) and highest multiplication rate (4.6 no. month^{-1}) were obtained in the high (1.5X) macrominerals supply treatment. In contrast, the highest percentage (78%) of root formation was obtained in low mineral concentration treatment (Table 1). In Table 1, the effects of different level of macrominerals on all growth aspects (fresh and dry weights, multiplication rate, root formation) have been shown. As the mineral supply increased root formation decreased. Biomass production (shoots elongation) was dependent upon mineral supply in the medium. Regarding the number of the newly formed axillary shoots, the highest amount of growth was obtained in 1.5X DKW medium.

Although, higher concentrations of IBA (that is, containing 12 mM IBA) gave the best respond to root formation. As for the elongation, it seems that the best combinations of mineral and growth regulators were those of 12 µM IBA. Great variation (P=0.5) on rooting percentage, and total root length was observed among treatments (Figure 1).

It should be mentioned that treatment with low rooting ability developed fewer but longer roots compared with the high rooting ability clones which formed many main and secondary roots.

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Figure 1. Influence of mineral concentration on multiplication rate (No. explants/month) of walnut (*Juglans regia* var. Zeiabadi) cultured on gelled medium.

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REFERENCES

Barbas E, Chaillou S, Cornu D, Doumas P, Jay-Allemand C, Lamaze T (1993). Orthophosphate nutrition of *in vitro* propagated hybrid walnut *(Juglans nigra* x *Juglans regia)* trees: Pi (32Pi) uptake and transport in relation to callus and shoot development. Plant Physiol. Biochem., 31(1): p 4149

Cornu D (1988). Somatic embryogenesis in tissue cultures of walnut *(Juglans nigra, J. major* and hybrids *J. nigra* x *J. regia).* In: Ahuja MR (ed) Somatic cell genetics of woody plants. Kluwer, Dordrecht, Pp. 45-49

Cornu D, Jay-Allemand C (1989). Micropropagation of hybrid walnut trees *(Juglans nigra* x *luglans regia)* through culture and multiplication of embryos. Ann. Sci. For., 46 (Suppl): 113s : 116s

Driver J, Kuniyuki A (1984). *In vitro* propagation of Paradox walnut rootstock. HortScience, 19: 507-509.

Felaliev AS (1990). Morphogenesis of *Juglans regia* L. in vitro. Ukr. J. Bot., 47 (3): 85-87.

Gruselle R, Badia N, Boxus P (1987). Walnut micropropagation: first results. Acta Hortic., 212:511-515.

Heile-Sudholt C, Huetteman CA, Preece JE, van Sambeek JW,

GaffÕney GR (1986). *In vitro* embryonic axis and seedling shoot tip culture of *Juglans nigra* L. Plant Cell Tissue Organ Cult., 6: 189-197.

Jay-Allemand C, Cornu D (1986). Culture *in vitro* of embryos isolated of noyer commun (*Juglans regia* L.). Ann. Sci. For., 43: 189-192.

Jay-Allemand C, de Pons V, Doumas P, Capelli P, Sossountzov L, Cornu D (1991). Root formation in vitro. from cotyledons of nuts (lug lans sp.): so that at Â • rhizogenes to study the Spanish in the woody. C R Acad Sci Paris, 312, Ser III: 369-375.

Kevers C, Gaspar TH (1986). Vitrification of walnut in vitro: changes in water content, exteracellular space, air volume, and ion levels. Physiol. Veg., 24: 647-653.

Lee MH, Ahn CY, Park CS (1986) *In vitro* propagation of *Juglans sinensis* Dode from bud culture. Res. Rep. Inst. For. Gen. Korea, 22:159 : 163

Leslie C, McGranahan G (1992). Micropropagation of Persian walnut *(Juglans regia* L.). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, High-tech and micropropagation I 1. Springer, Berlin Heidelberg New York, 18: 136-150.

Long LM, Preece SE, Gaffney GR, van Sambeek JW (1992). Somatic embryogenesis and organogenesis of eastern black walnut *(Juglans nigra* L.) HortSci., 27 (6): 58489.

McGranahan G, Leslie C (1990). Walnuts *(Juglans).* In: Moore JN, Ballington JR (eds) Genetic resources of temperate fruit and nut crops, Int. Soc. Hortic. Sci, Wageningen, 2: 907-951

McGranahan G, Leslie CA, Driver JA (1988). *In vitro* propagation of

mature Persian walnut cultivars. HortSci., 23 (1): 220-228.
Neuman MC, Preece JE, van Sambeek JW, Gaffney GR (1993). Somatic embryogenesis and callus production from cotyledon explants of Eastern black walnut. Plant Cell Tissue Organ Cult, 32: 9-18.
Pijut PM (1993a). Somatic embryogenesis in butternut, *Juglans cinerea* L., Can J. For. Res., 23: 835-838.
Pijut PM (1993b). Regeneration of *Juglans cinerea*L. through somatic embryogenesis. *In vitro* Cell Dev. Biol., 29 (3): 69-75.
Polito VS, McGranahan G, Pinney K, Leslie C (1989). Origin of somatic embryos from repetitively embryogenic cultures of walnut *(Juglans regia* L.): implications for *Agrobacterium-mediated* transformation. Plant Cell Rep., 8: 219-221.
Preece JE, van Sambeek JW, Huetteman CA, Gaffney GR (1989). Biotechnology: *in vitro* studies with walnut *(Juglans)* species. In:
Phelps JE (ed) The continuing quest for quality. Proc. 4th Black Walnut Symp, Walnut Council, Indianapolis, Pp. 159-180.
Revilla MA, Majada J, Rodriguez R (1989). Walnut *(Juglans regia* L.) micropropagation. Ann. Sci. For., 46: 149-151.
Rodriguez R, Revilla A, Albuerne M, Perez C (1989). Walnut *(Juglans* spp). In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, Trees II. Springer, Berlin Heidelberg New York, 5: 99-126.
Somers PW, van Sambeek JW, Preece JE, Gaffney G, Myers O (1982). *In vitro* micropropagation of black walnut *(Juglans nigra* L.). University of Kentucky Press, Lexington. Proc 7th North Am. For. Biol., Pp. 224-230.
Stephens LC, Krel SL, Domoto PA (1990). In *vitro* propagation of *Juglans regia,* ISU71-3-18. Annu. Rep. North Nut. Grow. Assoc., 81: 122-126.
Tulecke W, McGranahan G (1985). Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L., Plant Sci., 40: 57- 63.
Voyiatzis DG, McGranahan GH (1994) An improved method for acclimatizing tissue-cultured walnut plantlets using an antitranspirant. HortSci., 29 (1): 42-45.

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Full Length Research Paper

Inhibition effects on Hepatitis B virus replication by hydrophobic extracts from *Ferula ferulaeoides* (Steud.) Korov

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Hepatitis B virus (HBV) infection is a worldwide public health problem, which can lead to life threatening chronic hepatitis, liver cirrhosis and cancer. Despite this, treatments for chronic HBV infection are still very limited. In our investigation of herbal medicines as antiviral agents, FF, the lipid soluble fraction of *Ferula ferulaeoides*, which has been used as a traditional Chinese medicine, showed a significant inhibitory effect against HBV in HBV-producing cell line HepG2.2.15. In the experiment, FF reduced the HBsAg level and HBV replication by 87 and 36%, respectively. The results suggest that the lipophilic fraction of root extracts from *F. ferulaeoides* could serve as an effective natural constituent of herbs for the search of novel anti-HBV agents.

Key words: Hepatitis B Virus, anti-HBV, HBsAg, *Ferula ferulaeoides*.

INTRODUCTION

Although efforts to prevent and control HBV with vaccines have met with increasing levels of success, there are still nearly two billion people worldwide with serologic evidence of past or present HBV infection and more than 350 million chronically infected by HBV (The World Health Report, 1997). Hepatitis B virus (HBV) persistence infection can cause chronic hepatitis, which often leads to liver cirrhosis and even hepatocellular carcinoma. Although prophylactic HBV vaccines have been effective, current treatment for chronic HBV infected patient remains limited. Contemporary clinical treatment for chronic HBV infection relies on two kinds of antivirals: interferon and nucleotide/nucleoside. Conventional and pegylated interferons act on the immune system to enhance host antiviral capacity, whereas nucleotide reverse transcriptase inhibitor (NRTI), nucleotide/ nucleoside analogue, targets viral polymerase and inhibit the replication of HBV DNA (Ganem and Prince, 2004). However, limitations exist for both interferon and NRTI for the treatment of HBV. Interferon shows a low response rate (<30%) and is poorly tolerated in a substantial number of patients. NRTI often encounters the occurance of antiviral resistance during long term treatment (Zoulim, 2004; Perrillo, 2009; Lok and Chotiyaputta, 2009). Thus, discovery of more effective antivirals agents is urgently needed.

HBV belongs to a group of hepatotropic, enveloped animal DNA viruses known as hepadnaviridae. HBV virion contains a circular, partially double-stranded genome which encodes four open reading frames (ORF), S/pre-S, core/pre-core, P and X. These ORFs encode three envelope proteins, core and E proteins, polymerase and HBV X transactivator, respectively (Seeger and Mason, 2000; Bouchard and Schneider, 2004). During HBV infection, envelope proteins and E protein are continuously secreted from infected cells, which are serologic markers of HBV infection and termed as HBsAg and HBeAg, respectively. The replication of HBV DNA is

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Abbreviations: FF, The lipid soluble fraction of *Ferula ferulaeoides;* **HBV,** hepatitis B virus; **ORF,** open reading frames; **DMSO,** dimethyl sulfoxide; **SD,** standard deviation; **RT-PCR,** real-time polymerase chain reaction.

unique in the utilization of a pregenomic RNA as a template for reverse transcription carried out by viral polymerase. Progeny HBV virions are secreted from infected cells and can be measured quantitatively by real-time PCR. The genus *Ferula* (Apiaceae) includes about 150 species grown in a vast geographical region ranging from Central Asia to the Mediterranean region (Mozaffarian, 1996), and 26 species distribute in China (She and Watson, 2005). Different parts of extract for plant of *Ferula* have a reputation in the treatment of various diseases such as neurological disorders, inflammations, dysentery, digestive disorders, rheumatism, headache, arthritis and dizziness (Tamemoto et al., 2001). Constituents from genus Ferula were summaried many biological effects including antiinflammatory, cytotoxicity and P-gp inhibitory, cancer chemopreventive, antibacterial, and antileishmanial activities (Nazari and Iranshahi, 2011). In addition, activities of influenza A (H_1N_1) antiviral from plants of the genus *Ferula* have also been reported (Lee et al., 2009). In our primary bioassay screening, dichloromethane extracts from *F. ferulaeoides* (Steud.) Korov, showed appreciable inhibition against HBV. With further study using an HBV-producing cell model HepG2.2.15, the extract possessed antiviral effect on reducing HBsAg expression and HBV DNA replication.

MATERIALS AND METHODS

Extraction and isolation of the herbal sample

Roots of *Ferula ferulaeoides* used in the experiment were collected in suburban of Shihezi City, Xinjiang province. The plant was identified by Dr. Hui-Qin Xie of the Department of Plant Protection, Agricultural School of Shihezi University, China, and the voucher specimen (XHQ-FF-1) was kept in the herbarium of the Department of Plant Protection. Air-dried and powdered roots (2 kg) were extracted with 95% ethanol and yielded 1 L extract after the ethanol was evaporated. The extract was suspended in 2 L of distilled water and then partitioned with dichloromethane (1 L × 4). The solvent was evaporated under vaccum at 50°C to yield FF (400 g). In the virological experiment the sample, FF, was solved in 1% dimethyl sulfoxide (DMSO) for tests.

Cell culture in virological experiments

The HepG2.2.15 cell line which harbors two copies of the HBV genome integrated in the chromosome in a head-to-tail form supports HBsAg and HBeAg production as well as viral replication. The cells were cultured at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-Glutamine, and 250 g/ml G418.

Detection of HBsAg and HBeAg

HepG2.2.15 cells were cultured in 96-well plate (10^4 cells/well). The sample FF was added into the medium to a final concentration. Wells containing only medium were conducted as Controls. Similarly, the cells with 1% DMSO added at the same volume of the sample in maintenance media are conducted as DMSO Controls. Supernatants of culture suspension were collected 4 days after the treatment. HBsAg and HBeAg in the supernatants were measured using ELISA kits (Kehua Inc., Shanghai, China) according to the manufacturer's instruction and were represented as absorbance values at A450, using an ELISA reader (Molecular Devices).

Real-time PCR for quantitating HBV DNA

HepG2.2.15 cells were cultured to a 10^4 cells/well. The sample was added into the test wells to a final concentration of 0.1 μg/μL. Control wells containing 1% DMSO in media was setup. After 4 days incubation, the supernatants were colledted. The concentration of HBV DNA were quantified by RT-PCR using a commercially available HBV RT Detection Kit (PG BIOTECH, Shenzhen, China). RT-PCR was performed on the Stratagene MX3000P Detection System (Agilent Inc., USA) using following PCR conditions: 50°C for 2 min for uracil N-glycosylase incubation, 95°C for 10 min for Hotstart Taq DNA polymerase activation, followed by 40 cycles of 95°C for 5s (denaturation), 60°C for 30 s (annealing and extension).

Statistical analysis

Statistical analysis was performed using the Sigmaplot software. Data from three separate experiments are presented as means ± standard deviation (SD). Student's t-test was performed to determine the difference between groups with $P<0.05$ considered significant.

RESULTS AND DISCUSSION

Effects of sample FF on HBsAg

HepG2.2.15 cells were cultured and the concentration of HBsAg and HBeAg in the supernatant was measured. Treated with the sample FF, the OD450 value of HBsAg vastly decreased as low as 0.237 ± 0.016, which indicated the HBsAg level reduced by 87% comparing with that of DMSO (1.408 ± 0.098); while the HBeAg level increase slightly. (Sample FF shows 1.769 ± 0.057, while the DMSO Control shows 1.399 ± 0.084) (Figure 1).

Effects of sample FF on reducing HBV DNA

To investigate the mechanism of the inhibition, the tissue culture supernatants were collected and purified, and the HBV DNA in the virions was quantified with the HBV-specific real-time polymerase chain reaction (RT-PCR). As shown in Figure 2, the treatment with FF resulted in 36% reduction of HBV DNA/virion in the supernatants ($P<0.05$).

F. ferulaeoides is a traditional ethnic medicine, and its gum obtained from the aerial parts has been used for detoxication, and for anti-inflammatory helminthicide, anti-convulsion, anti-epileptic, and antifertility treatments. In this study, the hydrophobic extract of roots from the plant was found to exhibit a significant inhibition of HBsAg production and HBV replication. It is noteworthy

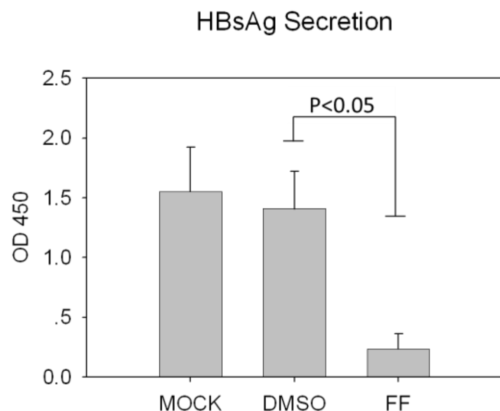

Figure 1. Effect of FF on the production of HBsAg.

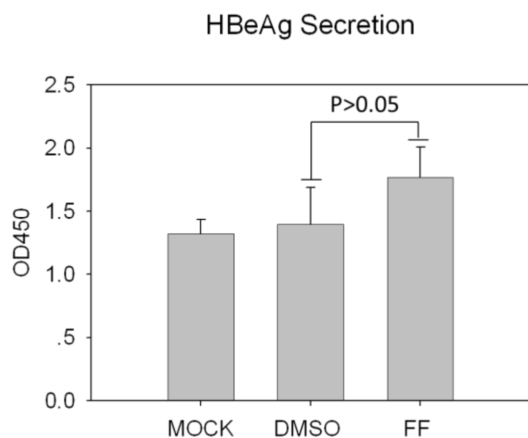

Figure 2. Effect of FF on the production of HBeAg.

that the drug had little inhibitory effect on the HBeAg production. The HepG2.2.15 cell used in this study contains HBV genome integrated in the cell chromosome. It is possible that in this cellular context, HBeAg expression is therefore not inhibited by the drug. Biological activities are almost attributed to sesquiterpene comarin constituents from studied *Ferula* species (Nazari, and Iranshahi, 2011). however, there exist a little differences in smell of *F. ferulaeoides* species– there was no such disgusted smell like common *Ferula* species. This plant have ever been isolated and identified a series of sesquiterpene comarin derivatives (Nagatsu et al., 2002), there was no biological activity investigation on those compounds. A recent research on this species reported two novel sesquiterpenoids (Hu et al., 2010). This suggested that more need on chemical constituents especially on pharmacology should be carried out on F. *ferulaeoides*.

The finding we reported suggests that this traditional drug might have the potential to act against HBV infection. It is encouraging to further isolate and identify the chemical in the fraction FF which is responsible for the antiviral effect shown in this study. Furthermore, investigation for the mechanism of inhibition by this drug on HBsAg and HBV replication will probably give new insight for the improvement of the antiviral activity.

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REFERENCES

Bouchard MJ, Schneider RJ (2004). The enigmatic X gene of hepatitis B virus. J. Virol., 78: 12725-12734.

Ganem D, Prince AM (2004). Hepatitis B virus infection - Natural history and clinical consequences. N. Engl. J. Med., 350: 1118-1129.

Hu Y, Li XD, Li GY, Li N, Zuo WJ, Zeng YM, Meng He, Li X, Wang JH (2010). Two Novel Sesquiterpenoids from the Roots of *Ferula ferulaeoides* (Steud.) Korov. Helv. Chim. Acta, 93: 1019-1023.

Lee CL, Chiang LC, Cheng LH, Liaw CC, El-Razek MHA, Chang FR, Wu YC (2009). Influenza A (H_1N_1) Antiviral and Cytotoxic Agents from *Ferula assa-foetida*. J. Nat. Prod., 72: 1568-1572.

Lok ASF, Chotiyaputta W (2009). Hepatitis B virus variants. Nat. Rev. Gastrol. Hepat., 6: 453-462.

Mozaffarian V (1996). A Dictionary of Iranian Plant Names. Tehran: Farhang Moaser Publications.

Nagatsu A, Isaka K, Kojima K, Ondognii P, Zevgeegiin O, Gombosurengyin P, Davgiin K, Irfan B, Iqubal CM, Ogihara Y (2002). New Sesquiterpenes from *Ferula ferulaeoides* (Steud.) Korovin. VI. Isolation and Identification of Three New Dihydrofuro[2,3-b]chromones. Chem. Pharm. Bull., 50(5): 675-677.

Nazari ZE, Iranshahi M (2011). Biologically Active Sesquiterpene Coumarins from *Ferula* Species. Phytother. Res., 25: 315-323.

Perrillo R (2009). Benefits and risks of interferon therapy for hepatitis B. Hepatology, 49: S103-111.

Seeger C, Mason WS (2000). Hepatitis B virus biology. Microbiol. Mol. Biol. Rev., 64: 51-68.

She ML, Watson MF (2005). Ferula. In Flora of China, 14: 174-180.

The World Health Report (1996). Fighting disease, fostering development (1997). World Health Forum, 18: 1-8.

Tamemoto K, Takaishi Y, Chen B, Kawazoe K, Shibata H, Higuti T, Honda G, Ito M, Takeda Y, Kodzhimatov OK, Ashurmetov O (2001). Sesquiterpenoids from the fruits of Ferula kuhistanica and antibacterial activity of the constituents of F. kuhistanica. Phytochemistry, 58: 763-767.

Zoulim F (2004). Mechanism of viral persistence and resistance to nucleoside and nucleotide analogs in chronic Hepatitis B virus infection. Antivir. Res., 64: 1-15.

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