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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Review

Mesua ferrea L.: A review of the medical evidence for its phytochemistry and pharmacological actions

Manoj Kumar Chahar*, Sanjaya Kumar D. S., Geetha L., Lokesh T. and Manohara K. P.

Sree Siddaganga College of Pharmacy, B. H. Road, Tumkur- 572 102, Karnataka, India.

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The plant kingdom provides many plants with properties which are conducive to health and to secure the best results from the use of the plants as remedial agencies. *Mesua ferrea* Linn (Nagakesar) is a rare plant which is traditionally being used for its antiseptic, anti-inflammatory, blood purifier, anthelmintic, cardiotoxic, diuretic, expectorant, antipyretic, purgative, antiasthmatic, antiallergic and several other effects. The scientific screening of the plant confirms its antioxidant, hepatoprotective, anti-inflammatory, central nervous system (CNS) depressant, analgesic, antimicrobial, antispasmodic, antineoplastic, antivenom and immunostimulant activity. The phytochemical screening confirms the presence of phenyl coumarins, xanthenes, triterpenoids, fats and flavanoids as main constituents responsible for its biological activity. It is a substitute for petroleum gasoline. It is also used in cosmetics, as fire wood and the polymer obtained from seed oil is used in the preparation of resins. The present review summarizes the phyto-pharmacological role of this valuable medicinal plant.

Key words: *Mesua ferrea*, traditional medicinal uses, phytochemical screening, pharmacological activities.

INTRODUCTION

Ayurveda has related research efforts which have led to generation of enormous amount of scientific information concerning plants, crude plant extracts, and various substances from plants as medicinal agents during last 30 to 40 years. Although herbal medicine has existed since the dawn of time, our knowledge of how plants actually affect human physiology remains largely unexplored.

The research is going on with a view to provide the scientific evidence for the ethnomedical claim and for their clinical application. *Mesua* is a large genus consisting of about 48 species but the extensive research work has been carried out only on *M. ferrea* L. This review provides insight on the phytochemical and pharmacological profile with other useful information on the plant (Kirtikar, 1935). It is native to tropical Sri Lanka and a state tree of Tripura but it is disappearing from India. *M. ferrea* L. (Figure 1) is locally known as Cobra's saffron (English), Nagakeshara (Hindi), Nagasampige (Kannada), Nageshwar (Assam), Nagachampakam (Tamil). The tree

is found throughout Southeast Asia in tropical evergreen forests up to 1,500 m elevation (Dassanayake, 1980).

Distribution

It is widely distributed in tropical countries like India, Burma, Thailand, Indochina and New Guinea (Kirtikar, 1981). In India, it is distributed in the mountains of Eastern Himalaya and East Bengal, Assam, Burma, Andaman, evergreen rain forests of North Canara and South Konkan, the Forests of Western Ghats from South Canara to Travancore (Anonymous, 2004).

Botany

Nagakesara is a medium to large sized tree that can attain a height between 18 and 30 m, with reddish-brown to grey colored bark that peels off in thin flakes, the wood is extremely hard. The leaves are simple, lanceolate, acute, and leathery, covered in a waxy bloom below, red when young, oppositely arranged, 7 to 13 cm long by 2 to 4 cm wide. The flowers are white with a floral fragrance,

*Corresponding author. E-mail: manojchahar7@gmail.com. Tel: 9964498676.

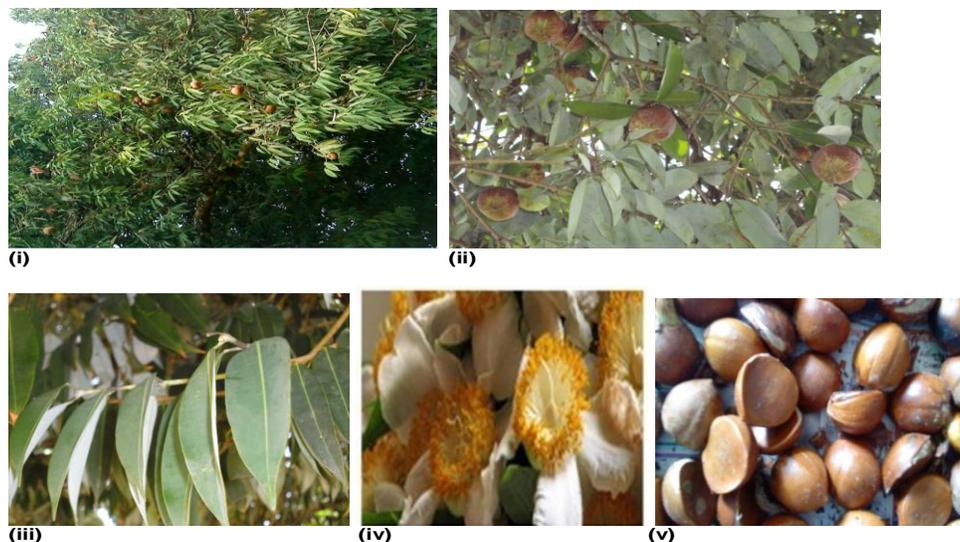


Figure 1. *Mesua ferrea* L. (i) Tree; (ii) Fruit; (iii) Leaves; (iv) Flower and; (v) Seeds

up to 7.5 cm in diameter, with numerous golden-colored stamens shorter than the length of the petals, the style is twice as long as the stamens, borne singly or in pairs, axillary or terminal. The fruits are ovoid with a conical point, 2.5 to 5 cm long; with a woody pericarp that contains one to four seeds (Dassanayake, 1980).

Uses

It is known for shade creation and radiation modification in improving human thermal comfort (Shahidana et al., 2010). The seed oil is substitute for petroleum gasoline, the fraction distilling between 200 and 300°C may be used as fuel for diesel engines (Konwer et al., 1984; Kallappa et al., 2003). The polymers obtained from seed oil are used in the preparation of resins (Dutta et al., 2004; Mahapatra et al., 2004, 2007; Dutta et al., 2005, 2006; Das et al., 2010).

Aqueous leaf extract was used to prepare silver nano particles (Konwarh et al., 2010). The seeds are brunt like candles, the wood is used for golf club heads, flowers and stamens are used to stuff pillows for the bridles bed (Sahni, 1998).

TRADITIONAL MEDICINAL USES

The plant is used in inflammation and septic conditions (Rai et al., 2000). The tribal's of Assam use this plant for its antiseptic, purgative, blood purifier, worm control, tonic properties (Parukutty et al., 1984). In Thai traditional medicine, it is used to treat fever, cold, asthma and as carminative, expectorant, cardiotoxic, diuretic and antipyretic agent (Foundation of Resuscitate and Encourage Thai Traditional Medicine, 2005). The ashes

of leaves are used for sore eyes. Kernels are used to poultice wounds and in skin eruptions (Burkill, 1966; Kumar et al., 2006). Leaf and flower are antidotes for snake bite and scorpion sting. The fixed oil is used for cutaneous infection, sores, scabies, wounds and rheumatism. The flower is stomachic, expectorant and astringent. The decoction or infusion or tincture of bark and roots is a bitter tonic and useful in gastritis, bronchitis (Sahni, 1998; Husain et al., 1992; Joy et al., 1992; Nadkarni, 1976) and to cure snake bite (Santamaría, 1978). The aerial parts are Chorionic villus sampling (CVS) active, spasmolytic, diuretic, (Husain et al., 1992; Joy et al., 1992), abortifacient (Nath et al., 1992) and used in fever, dyspepsia, renal disorders and in cosmetics (Kumar et al., 2006). *M. ferrea* is an ingredient of various ayurvedic formulations like dasamoolarishta (Nishteshwar et al., 2008), mahakaleshwara rasa (Das et al., 2001) and in various churnas (Sharangadhara, 2000) used to cure many diseases (Rosly et al., 2010). Some important ayurvedic formulations containing *M. ferrea* are listed in Table 1. An Ayurvedic formulation containing *M. ferrea* exhibited haemostatic and astringent properties and is particularly useful in uterine bleeding (Husain et al., 1992; Joy et al., 1992). In Unani system, the drug is an ingredient of large number of recipes like, "Jawarish Shehryaran" a stomach and liver tonic, "Hab Pachaluna", an appetiser, "Halwa-i-supari pack" a general tonic (Joy et al., 1992; Thakur et al., 1989).

PHARMACOLOGICAL ACTIVITIES

Disinfection studies

Nahar (*M. ferrea*) seed kernel oil was investigated for its potential as natural disinfectant and disinfection kinetics. Heterotrophic plate count using CFU/ml, pour plate method

Table 1. Ayurvedic formulations containing *M. ferrea*.

Formulation	Use	Reference
Nagakeshara-adi-churna	In bacillary dysentery	Joy et al. (1998) and Thakur et al. (1989)
Nagakeshara yoga	In piles	Joy et al. (1998) and Thakur et al. (1989)
Vyaghrihareetaki avaleha	Shwasa, kasa, pinasa	Roshy et al. (2010)
Eladi churna	Carminative, in vomiting, indigestion anorexia	Tambekar et al. (2010)
Lavangadi churna	In cough, diarrhoea, dysentery, mouth diseases, dental caries, anemia and fever	Tambekar et al. (2010)

method at 35°C/48 h, plate count agar were employed to evaluate the disinfection and its kinetics. Oil-water emulsion used as test and surface water samples were used for comparison of colonies produced using pour plate method. The crude oil emulsion showed total disinfection at a concentration of 2 mg/ml and above, while the data generated from the disinfection at 1 mg/ml fits first-order model. The study concluded that nahar seed kernel oil has a remarkable disinfection potential and the kinetics studies indicated that the oil fitted first-order model with a k value of -0.040 (Adewale et al., 2011).

Antioxidant and hepatoprotective activity

The methanolic extract of dried flowers of *M. ferrea* (100 and 200 mg/kg) was screened for *in vivo* antioxidant and hepatoprotective activity in experimental female Wistar mice. An artificial infection was induced by administration of *S. aureus* in drinking water for 24 h at the onset of experiment, sampling was done once a day and after one week. The biochemical parameters Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Creatinine phosphokinase (CPK), Alkaline phosphatase (ALKP), Creatinine, Urea, Super oxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPX), Glutathione reductase (GR) were measured. There was significant increase in liver SOD and AST in treated groups. There was significant reduction in catalase (CAT), GPX, GR, and ALT activity. No significant difference was observed in CPK and creatinine activity (Garg et al., 2009).

The ethanolic extract of flowers showed potent inhibitory activity (96.03%) at 100 µg/ml against nitric oxide (NO) assay (Makchuchit et al., 2010). The water-ethanol (1:1) leaf extract showed potent inhibition on lipid peroxidation (Yadav, 2010). The ayurvedic formulations Brahma rasayana (Ramnath et al., 2009) and Maharishi AK-4 (Cullen et al., 1997) containing *M. ferrea* have reported to exhibit significant antioxidant activity in cold stressed chicken and isolated rat heart, respectively. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (56.67 mmol/100 g DW), 2,2'-azino-bis (ABTS) (35.22 mmol/100 g DW), ferric reducing/antioxidant power (FRAP) (8.99 µmol/g DW) assay and determination of total phenolic content (4.18 g/100 g DW) in *M. ferrea* seeds and pericarp showed

antioxidant activity (Surveswaran et al., 2007). *Phomopsis* sp. GJJM07 (an endophyte) was isolated from *M. ferrea* and examined for the *in vitro* antioxidant activity by DPPH radical scavenging assay. It showed potent antioxidant activity with the half maximal inhibitory concentration (IC₅₀) value of 31.25 µg/ml compared to the IC₅₀ value of standard ascorbic acid, 11.11 µg/ml (Jayanthi et al., 2011).

Analgesic activity

n-Hexane, ethyl acetate and methanol extracts of *M. ferrea* leaves (125 and 250 mg/kg) exhibited significant analgesic activity in acetic acid induced writhing response in mouse. The reduction in writhing response for lower dose of above extracts was 36.08, 16.33 and 10.21%, respectively and for higher dose it was 42.21, 19.63 and 17.06%, respectively (Hassan et al., 2006).

Antispasmodic activity

The petroleum extract of *M. ferrea* seed oil was evaluated for antispasmodic activity on isolated rat ileum *in vitro*. The contraction of rat ileum was measured on kymograph. Acetylcholine and Carbachol caused contraction of 2.61 and 3.20 cm, respectively. The crude oil at concentration, which is 1:5 and 1:10, and the normal contraction of acetylcholine was reduced to 70 and 86%, respectively. Normal response of acetylcholine in presence of atropine was reduced to 55% (Prasad et al., 1999).

Anti-venom activity

The aqueous extract of *M. ferrea* leaves was screened for its activity against fibroblast cell lysis after *Heterometrus laoticus* scorpion venom treatment. The extract was evaluated against viability of fibroblast cells after 30 min treatment with mock control or with 0.706 mg/ml plant extracts preincubated with *H. laoticus* venom. Viability of fibroblast cells after 30 min treatment with mock control or with 0.706 and 0.406 mg/ml showed efficiency in protecting against venom induced lysis (Uawonggul et al., 2006).

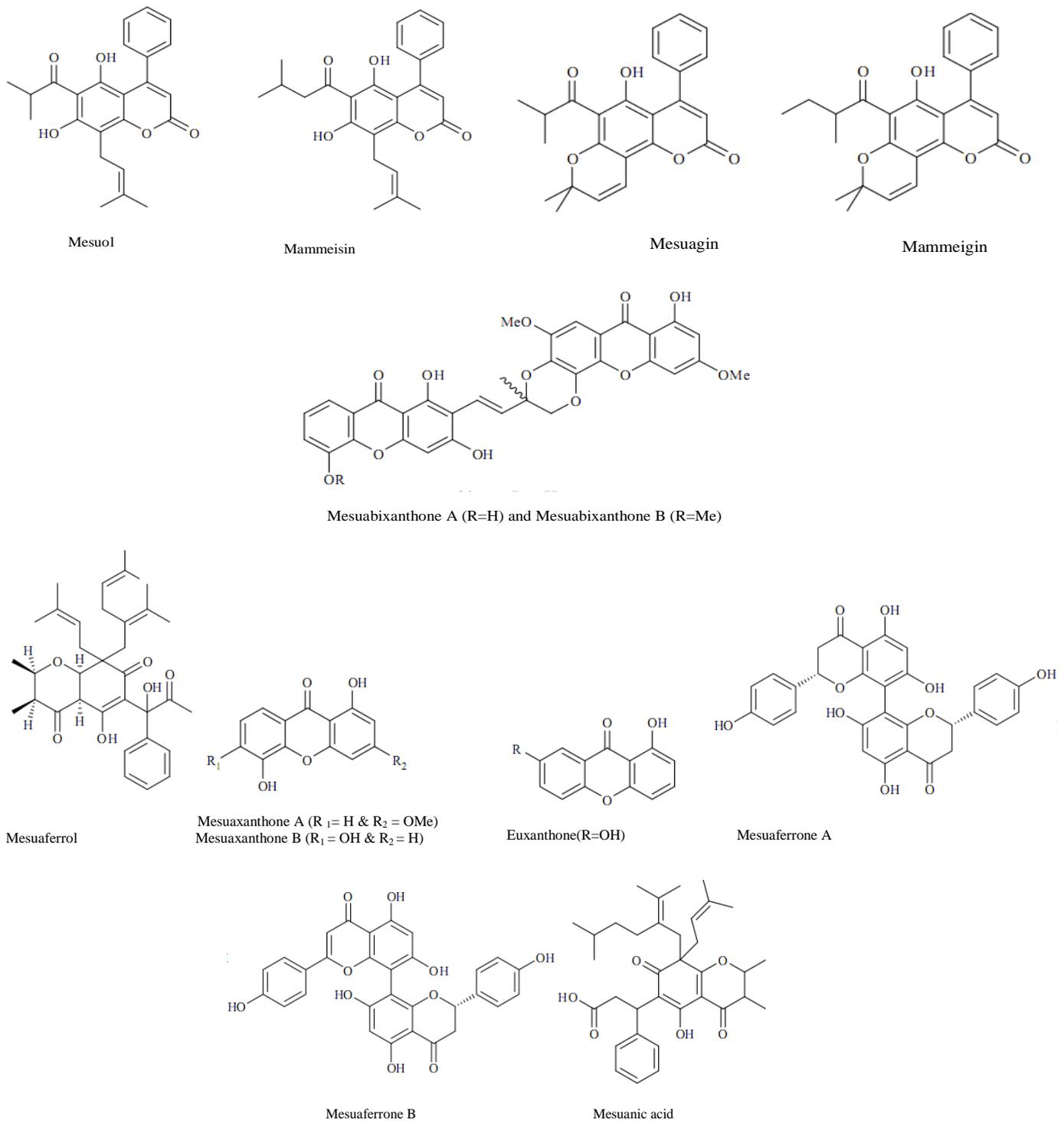


Figure 2. (i) Mesuol; (ii) Mammeisin; (iii) Mesuagin; (iv) Mammeigin; (v) Mesuabixanthone A (R=H) and Mesuabixanthone B (R=Me); (vi) Mesuaferrol; (vii) Mesuaxanthone A (R₁= H and R₂ = OMe); Mesuaxanthone B (R₁ = OH and R₂ = H); (viii) Euxanthone(R=OH); (ix) Mesuaferrone A; (x) Mesuaferrone B; (xi) Mesuanic acid.

M. ferrea in the cancer chemotherapy

Maharisi amrit kalash-4 (MAK-4) containing *M. ferrea* was

evaluated for its role in reduction of chemotherapy toxicity among women with breast cancer. 214 patients with breast carcinoma received cyclophosphamide, adriamycine,

and 5-fluorocil (CAF) adjuvant or neo-adjuvant chemotherapy. All patients received same antiemetic therapy with ondansetron and dexamethasone. The Anorexia, Karnofsky performance status, vomiting, weight stomatitis, leucopenia and other side effect such as diarrhea, alopecia, tumour response were checked. There was significant reduction in toxicities observed in MAK group throughout chemotherapy cycles: poor performance status was prevented by concomitant administration of MAK along with chemotherapy. {Prevented fraction (PF) = 6% (95% confidence interval, 22.1 to 80.1; p value = 0.005)}. Vomiting was prevented by MAK {PF = 40.3%, (95% confidence interval, 15.1 to 58.1; p value = 0.002)}. Similarly, anorexia was reduced with PF = 35.6% (95% confidence interval, 17.6 to 49.7; p value = 0.0001) in MAK group. No overgrowth of tumors occurred in the group treated with neoadjuvant chemotherapy receiving MAK (Saxena et al., 2008).

Immunomodulatory activity

A poly herbal formulation, ACII containing *M. ferrea* flower buds was evaluated for immunomodulation effect on radiation induced immunosuppression. There was a significant increase in the amount of circulating antibody in animals treated with ACCII (250 mg and 1 g/kg). There was no significant change in body weight in treated as well as irradiated animals.

The lowered total white blood cells (WBC) count was significantly increased. There was no significant change in the hemoglobin content of irradiated animals when compared with drug treated or normal animals. ACII also did not produce any change on differential count especially in lymphocyte-neutrophil ratio. The bone marrow cellularity was improved significantly and α -esterase positive cells were found to be improved. The weight of thymus was found to increase in ACII treated animals compared to irradiated animals (Tharakan et al., 2006). Moreover, ACII was found to have an immunomodulatory effect in normal (Tharakan et al., 2004) as well as in cyclophosphamide treated animals (Tharakan et al., 2003).

Mesulol isolated from *M. ferrea* seed oil was evaluated for immunomodulatory activity in experimental animals by using specific and non-specific immune response models. In humoral immune response model, mesulol evoked a significant dose dependent increase in antibody titer values in cyclophosphamide (50 mg/kg, i.p, 9th and 16th day) induced immunosuppression which was sensitized with sheep red blood cells (SRBC) on the 7th and 14th day of experiment. In cellular immune response model, an increase in paw volume was recorded on the 23rd day in cyclophosphamide-induced immune-suppressed rats treated with SRBC (0.03 ml 2% v/v, s.c.) on the 21st day. Mesulol restored the hematological profile in cyclophosphamide induced myelosuppression model.

Mesulol potentiated percentage of neutrophil adhesion in neutrophil adhesion test in rats and phagocytosis in carbon clearance assay. The study indicated immunomodulatory activity of mesulol (Chahar et al., 2012).

Anti-neoplastic activity

The crude ethanolic extract of *M. ferrea* was evaluated against human cholangio carcinoma (CL-6), human laryngeal (Hep-2), and human hepatocarcinoma (HepG2) cell lines *in vitro*. The extract showed promising activity against cholangio carcinoma (CL-6), with survival of less than 50% at the concentration of 50 μ g/ml. There was potent cytotoxic activity against Hep-2 and HepG2 also (Mahavorasirikul et al., 2010). The methanolic extract was evaluated against Ehrlich Ascites carcinoma in mice. There was significant inhibition in tumour growth inhibition (Rana et al., 2004). Muthu Marunthu, a poly herbal formulation containing *M. ferrea* flowers (100 mg) was evaluated for antitumour effect on experimental fibrosarcoma in rats. Significant reduction in the levels of DNA and RNA were noticed after Muthu Marunthu treatment. A significant reduction in the levels of vitamins A, C and E were observed in fibrosarcoma rats. Muthu Marunthu treatment was able to enhance the levels of vitamins A, C and E. An elevated level of copper and decreased levels of zinc and selenium were noticed in fibrosarcoma rats.

Treatment with Muthu Marunthu for 20 days brought back the altered levels of these trace elements to near normal levels. When compared between normal and Muthu Marunthu treated control rats, there was no significant changes in the blood levels of glucose, urea, plasma protein and cholesterol, and activities of serum enzymes such as Lactate dehydrogenase (LDH), Glutamate oxaloacetate transaminase (GOT) and Glutamic-pyruvic transaminase (GPT), alkaline and acid phosphatase (Palani et al., 1999).

Anti-convulsant activity

The ethanolic extract of *M. ferrea* flowers was evaluated for anticonvulsant activity at 3 different dose levels (200, 400 and 600 mg/kg p.o.) by Maximum electroshock seizure (MES) test using albino mice. The extract reduced the duration of Hind limb tonic extension (HLTE) in a dose dependent manner against MES model. The ethanolic extract of *M. ferrea* inhibited MES-induced convulsions. The percentage inhibition achieved at the doses 200, 400, and 600 mg/kg were 100% (p < 0.01), 60% (p < 0.01) and 100% (p < 0.001), respectively. Data from this study showed that *M. ferrea* flowers significantly increased the onset time and decreased the duration of seizures by electroconvulsive shock (Tiwari et al., 2012).

Effect of xanthenes isolated from *M. ferrea* on central nervous system

Gross behaviour

Xanthenes from *M. ferrea* were screened for their effect on gross behaviour in mice in a dose of 10, 25, 50, 100, 200 and 500 mg/kg. Gross behavioural changes were recorded at 15, 30, 60 and 120 min. All the xanthenes of the *M. ferrea* produced signs of CNS depression characterised by ptosis, sedation, decreased spontaneous motor activity and loss of muscle tone. The CNS depressant effect was predominant at the dose level of 200 mg/kg.

Anti-inflammatory activity

Mesuxanthone A and mesuxanthone B (MXA and MXB) from *M. ferrea* were evaluated using albino rats by carrageenan induced hind paw oedema, cotton pellet implantation and granuloma pouch tests. In all the methods, xanthenes were administered at the dose level of 50 mg/kg. *M. ferrea* xanthenes upon oral administration in carrageenan induced hind paw oedema test showed MXA (37%) and MXB (49%) reduction when compared to normal control group. The xanthenes produced significant anti-inflammatory activity in normal, as well as in adrenalectomised rats, as the inflammation reduced significantly by MXA (38%) and MXB (22%) when compared to normal control group. In granuloma pouch tests, these xanthenes showed MXA (46%) and MXB (49%) reduction in inflammation, and 47% reduction was observed in cotton pellets granuloma tests. The xanthenes used in the present study have been found to produce significant anti-inflammatory activity (Gopalakrishnan et al., 1980).

Anti-ulcer activity

Xanthenes from *M. ferrea* were screened for antiulcer activity by pyloric ligation method in albino rats. The ulcer scoring for the gum acacia treated rats was found to be 3.50 ± 0.27 which was significantly lesser than that of standards. The control animals showed extensive ulceration, haemorrhage and perforation, while the xanthenes pre-treated animals exhibited only scattered areas of hyperemia and occasional haemorrhagic spots (Gopalakrishnan et al., 1980).

Anti-arthritic activity

M. ferrea seed extracts (petroleum ether, ethyl acetate and alcohol) were evaluated in the formaldehyde and Complete Freund's Adjuvant (CFA)-induced arthritis in rats. The results indicate that *M. ferrea* protects rats

against formaldehyde and CFA induced arthritis. The body weight changes and the CFA-induced haematological perturbations, such as an increase in the WBC count, a decreased RBC count, a decreased haemoglobin (Hb) count and an increased erythrocyte sedimentation rate (ESR) which were significantly altered by *M. ferrea* treatment. The overall results indicated that *M. ferrea* extract has a potent protective effect against formaldehyde and adjuvant-induced arthritis in rats (Jalalpure et al., 2011).

Anti-microbial activity

In an *in vivo* experiment, the methanol extract of *M. ferrea* flowers protected mice challenged with *S. typhimurium* ATCC 6539.2 and 4 mg/mouse of the extract reduced the mice mortality. There was a significant reduction in the viable bacteria of blood, liver and spleen in the animals treated with the extract. In *in vitro* experiment, the extract inhibited 30 strains of *Staphylococcus aureus*, and all the tested strains of *Bacillus* spp., *Salmonella* spp., *Pseudomonas* spp., *Streptococcus pneumoniae*, *Sarcina lutea*, *Proteus mirabilis*, *Lactobacillus arabinosus* at 50 µg/ml concentration. One and two strains of *Staphylococcus* were inhibited by 100 and 200 µg/ml whereas 8 strains were resistant, strains of *Klebsiella*, *Vibrio cholera*, *Escherichia coli*, *Shigella* spp. were less sensitive (Mazumder et al., 2004).

Dichloromethane and methanol (1:1 v/v) extract of *M. ferrea* flowers showed complete inhibition against all tested bacteria at 500 and 1000 µg/ml. The screening was carried by agar dilution-streak method against *B. cereus varmycoides*, *B. pumilus*, *B. subtilis*, *Bordetella bronchiseptica*, *Micrococcus luteus*, *Sta. aureus*, *Sta. epidermidis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Str. faecalis*, *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae* (Prashanth et al., 2006).

The light petroleum ether, chloroform and ethanol extracts of *M. ferrea* seeds, leaves and stem bark were evaluated against antibacterial and antifungal study by disk diffusion method at 400 µg disk⁻¹ against 14 pathogenic bacteria including 5 gram positive like *B. subtilis*, *B. megaterium*, *Str. β-haemolyticus*, *Str. aureus*, *Sarcina lutea* and 9 gram negative as *Shigella sonnei*, *E. coli*, *Klebsiella* species, *Shigella shiga*, *S. boydii*, *S. flexneriae*, *S. dysenteriae*, *Salmonella typhi* and *Pseudomonas aeruginosa* and 6 pathogenic fungi *Penicillium notatum*, *A. niger*, *Trichoderma viride*, *A. flavus*, *C. albican* and *Hensinela californica*. Chloroform extract of *M. ferrea* stem bark displayed strong activity against gram-positive *Str. aureus* (16 mm) and gram-negative *E. coli* (19 mm). The extracts of *M. ferrea* leaves were found to be mild to moderate active against most of the bacteria strains. Antifungal activities of *M. ferrea* extracts were not significant enough against most of the tested fungal strains (Ali et al., 2004).

Antibacterial activity of aqueous and alcoholic extracts of *M. ferrea* seeds were tested at 100 µl by the agar disc diffusion and agar well diffusion methods against *Enterobacter aerogenes* ATCC13048, *E. coli* ATCC25922, *K. pneumoniae* NCIM2719, *Proteus mirabilis* NCIM 2241, *P. vulgaris* NCTC8313, and *Salmonella typhimurium* ATCC23564. Maximum antibacterial activity was against *P. mirabilis* (23 mm) and *K. pneumoniae* (20 mm). The ethanol/methanol extracts were more active than aqueous extracts (Parekh et al., 2007).

The methanolic extract of *M. ferrea* seeds was evaluated for antifungal activity *in vitro*. The test fungal strains investigated include 7 yeasts; *C. albicans* (1) ATCC2091, *C. albicans* (2) ATCC18804, *C. glabrata* NCIM3448, *C. tropicalis* ATCC4563, *C. luteolus* ATCC32044, *C. neoformans* ATCC34664, *Trichosporon beigelli* NCIM3404, and 4 moulds; *A. candidus* NCIM883, *A. flavus* NCIM538, *A. niger* ATCC6275 and *Mucorheimalis* NCIM873. The methanolic extract was effective at the concentration of 125 µg/disc for *C. albicans* (1) ATCC2091, *C. albicans* (2) ATCC18804 and *Trichosporon beigelli* NCIM3404 (125, 250 and 500 µg/disc). The extract was effective against moulds as *A. candidus* NCIM883 (500 µg/disc), *A. flavus* NCIM538 (125 & 250 µg/disc), *A. niger* ATCC6275 (125 and 250) and *Mucorheimalis* NCIM873 (250 and 500 µg/disc) (Parekh et al., 2008).

A series of 4-Alkyl and 4-phenyl coumarins like 6-acyl-8-prenyl derivatives (MF1 and MF2), 8-acyl-6-prenyl derivatives (MF3 and MF4), 6-acyl-7, 8-dihydro furano derivatives (MF5 and MF7) and 6-acyl-7, 8-pyran derivatives (MF8 and MF9) from *M. ferrea* blossoms were evaluated against gram negative bacteria, gram positive *Staphylococcus*, *Enterococcus* and a strain of *Str. durans*, fungi *P. falciparum*. All the samples tested, except MF5, MF8 and MF9, showed an activity of potential interest either against the *Enterococci* or against organisms of the genus *Staphylococcus*. The Mesua derivatives exhibited a higher minimum inhibitory concentration (MIC) 50% (2 to 4 µg/ml). When the concentrations inhibiting 90% of the strains (MIC 90%) were compared, some of the Mesua derivatives exhibited the best activity. There was a weak inhibitory activity against *P. falciparum* (Verotta et al., 2004).

M. ferrea leaves extracts were evaluated for the antibacterial activity (ethanol and methanol extract) by agar disc diffusion method and cytotoxicity activity (methanol extract). The micro broth dilution method was employed for the determination MIC and minimum bactericidal concentration (MBC), while Brine shrimp (*Artemia salina*) lethality bioassay was made use of for the cytotoxicity assay. The extract showed a remarkable antibacterial property against all the selected microbes (*E. coli*, *P. aeruginosa*, *B. subtilis* and *Sta. aureus*) with the inhibition zones ranging from 16.0 ± 0.5 to 18.0 ± 0.5 mm for all the tested bacteria. The MIC range of 2.5 to 0.625 mg/ml with MBC value of 5 mg/ml was obtained for

the gram-negative bacteria while MIC range of 1.3 to 0.313 mg/ml with MBC value of 2.5 mg/ml was obtained for the gram-positive bacteria. The leaves extracts were found to be toxic to the Brine shrimps with LC₅₀ of 500 ppm (µg/ml), suggesting that the extracts may contain bioactive compounds of potential therapeutic and prophylactic significance (Adewale et al., 2012).

Phomopsis sp. GJJM07 (an endophyte) was isolated from *M. ferrea*. The crude ethyl acetate extract of the fungus was evaluated for antimicrobial activity. The endophytic fungus were grown in different media and were tested against the test pathogens, gram positive bacteria; *B. subtilis*, *Micrococcus luteus*; gram negative bacteria; *E. coli*, *K. pneumoniae* and yeast, *C. albicans*. Among the different media, M1D medium showed good growth 1.57 g MDW/100 ml and broad spectrum of antimicrobial activity by exhibiting prominent zone of inhibition against the test pathogens such as *E. coli* (16 ± 0.14), *K. pneumoniae* (16 ± 0.19), *B. subtilis* (18 ± 0.13), *M. luteus* (12 ± 0.18) and *C. albicans* (12 ± 0.20) (Jayanthi et al., 2011).

Other therapeutic considerations

Ethanol and petroleum ether extract is used for sore throat, cough and asthma (Singhe et al., 1975; Bala et al., 1971; Sharma et al., 2002). The syrup of flower buds is used to cure dysentery. The leaves are used in the form of poultice which is applied to head in severe colds (Husain et al., 1992; Joy et al., 1998; Nadkarni, 1976). The LD₅₀ of ether extract of whole plant in mice is 500 mg/kg IP, LD₅₀ of acetone extract of stamens in mice was 400 mg/kg i.v. and non toxic up to 1600 mg/kg p.o. (Sharma et al., 2002).

PHYTOCONSTITUENTS

M. ferrea is the only species that has been chemically studied from the genus *Mesua* (Kirtikar, 1935; Rao et al., 1981). Phytochemical studies have revealed plants from this genus to be rich in many classes of secondary metabolites including phenylcoumarins, xanthenes and triterpenoids (Chow et al., 1968; Bandaranayake et al., 1975; Raju et al., 1976). The kernels contain about 75% of yellowish oil, constituted by the glycerides of common fatty acids: linoleic, oleic, stearic, and arachidic acids. An oil called nahor is extracted from the seeds (Husain et al., 1992; Joy et al., 1998). 4-Phenylcoumarins like mesuol, mesuagin, mammeisin, mammeigin and mesuone were isolated from the seed oil of *M. ferrea*. The trunk bark and the heartwood yielded 4-alkylcoumarins ferruols A and B, a lupeol-type triterpenoid guttiferol, mesuaxanthenes A and B, ferraxanthone 1,7-dihydroxyxanthone, 1,5-dihydroxy-3-methoxyxanthone, 1,6-trihydroxyxanthone, 1,5-dihydroxyxanthone, 1-hydroxy-7-methoxyxanthone and β-sitosterol. Stamens give α and β-amyirin, β-sitosterol, biflavonoids- mesuaferones A and B, mesuanic

acid, 1,5-dihydroxyxanthone, euxanthone 7-methyl ether and β -sitosterol. Other isolated constituents were mesuaferrol, leuco anthocyanidin, mesuone, euxanthone, etc. Presence of xanthone derivative and essential oil had also been reported from various parts of the plant (Sharma et al., 2002; Chow et al., 1968; Govindchari et al., 1967). Two new yellow pigments, meauxanthone A and memaxanthone B have been isolated from the heartwood extracts of *M. ferrea* (Govindchari et al., 1967). The stamens which yield the drug Nagakeshara contain mesuferrone-A and B, mesuaferrol, mesuanic acid, α and β -amyrin (Handa et al., 1992).

CONCLUSION

M. ferrea is being used in India and several parts of world for its potential medicinal and several other properties. The plant is known for its antioxidant, analgesic, anti-inflammatory, antitumor, immunostimulant, antimicrobial, and several other activities. It is an ingredient of several ayurvedic and unani formulations. The phytochemical screening confirms the presence of phenyl coumarins, xanthenes, triterpenoids, fats and flavanoids as main constituents of the plant. Apart from medicinal uses it is also being used commercially in polymer industry, painting, as a firewood and substitute for gasoline, preparation of nanoparticles. Therefore further studies may be carried out to prove the potential of this plant as well as the isolated products. Besides this, the systemic studies of pharmacological aspects of the plant are under way by our research team.

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Full Length Research Paper

The protective effect of silymarin on the antioxidant system at rat renal ischemia/reperfusion injury model

Ayhanci Adnan¹, Ozkal Bilge¹, Kabay Sahin^{2*}, Burukoglu Dilek³, Guven Gül⁴, Bayramoglu Gökhan¹, Senturk Hakan¹, Ustuner M Cengiz⁵, Akyuz Fahrettin⁶ and Ozden Hilmi, MD⁴

¹Department of Biology, Faculty of Art and Science, Osmangazi University, Turkey.

²Department of Urology, Faculty of Medicine, Dumlupinar University, Turkey.

³Department of Histology and Embryology, Faculty of Medicine, Osmangazi University, Turkey.

⁴Department of Anatomy, Faculty of Medicine, Osmangazi University, Turkey.

⁵Department of Medical Biology, Faculty of Medicine, Osmangazi University, Turkey.

⁶Department of Biochemistry, Faculty of Medicine, Osmangazi University, Turkey.

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The aim of this study is to reveal the protective effects of silymarin (SM) treatment on the generation of oxidative stress with rat renal ischemia/reperfusion (I/R) injury model. Thirty-two (32) Sprague-Dawley rats were evaluated in four groups. Group I (Sham), Group II (renal I/R), Group III (renal I/R injury + SM 100 mg per kg) and Group IV (renal I/R injury + SM 200 mg per kg) were designed to evaluate dose-dependent effects of SM in renal I/R injury on the morphological and biochemical parameters changes. Renal I/R significantly decreased the enzymatic activity of catalase (CAT) and superoxide dismutase (SOD), whereas the malondialdehyde (MDA) levels increased. After renal I/R injury, significant tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury was observed. In the Groups III and IV, in which the rats were treated with SM before renal I/R, tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury were observed to be significantly protected with the treatment. The results of this study have demonstrated that SM significantly prevents the generation of oxidative stress and renal I/R injury induced renal changes in the rat.

Key words: Kidney, oxidative stress, pathology, rat, silymarin, morphology.

INTRODUCTION

Renal ischemia/reperfusion (I/R) injury, which occurs in many clinical during the course such as partial nephrectomy, renal artery angioplasty, trauma, shock, major vascular surgery, sepsis and renal transplantation, is associated with increased mortality and morbidity rates due to acute renal failure (ARF) (Thadhani et al., 1996; Takada et al., 1997; Matin and Novick, 2001; Avlan et al., 2006). Reperfusion of the ischemic tissue may produce

reactive oxygen species (ROS), which are known to have deleterious effects such as increased microvascular permeability, interstitial edema, impaired vasoregulation, inflammatory cell infiltration and necrosis (Granger and Korhuis, 1995). In ischemic, ARF leads to a complex cascade of events which are also known to include the activation of nuclear factor kappa B (NF- κ B), which controls cytokine, chemokines and adhesion molecules (Rodrigo and Bosco, 2006). Oxidative stress is a relative excess of oxidants caused by increased free radical production and/or decreased antioxidant defense systems that impairs cellular function and contributes to the pathophysiology of many diseases (Karimi et al.,

*Corresponding author. E-mail: skabay@yahoo.com. Tel: +90 274 265 2031/1716. Fax: +90 274 265 22 77.

2005; Zhao, 2005). The antioxidant defense systems, none enzymatic free radical scavengers (vitamin E, vitamin C, uric acid and bilirubin) and the antioxidant scavenging enzymes, [catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx)] protect cells and tissues against oxidative injury (Granger and Korhuis, 1995; Marubayashi and Dohi, 1996; Zhao, 2005). Naturally occurring flavonoids have antioxidant effects due to their phenolic structures and have been reported to inhibit some free radical-mediated processes (Havsteen, 1983; Mora et al., 1990; Zhao, 2005). Silymarin (SM) is a mixture of three isomeric flavonolignans extracted from the milk thistle *Silibum marianum*. SM has been used in most of the remedies for liver disease. Hepatoprotective effects of SM have been attributed to its scavenging ROS, reduction of lipoperoxidation of cell membranes (Farghali et al., 2000). However; very few studies have been performed on oxidative stress with SM in relation to the kidney (Turgut et al., 2008). SM has also been reported to have beneficial effects to protect acute cisplatin nephrotoxicity (Karimi et al., 2005). In our previous study, we demonstrated that SM significantly prevents renal I/R injury induced histopathological changes in the rat kidney (Senturk et al., 2008). This study aimed to re-investigate the possible protective effect of SM against oxidative stress-induced during kidney I/R injury, by determining biochemical parameters and evaluating histological examinations.

MATERIALS AND METHODS

The experimental protocols were approved by the Institutional Animal Ethics Committee. Animals were obtained from Medical and Surgical Experimental Research Center (Eskisehir-TURKEY) and all experiments were carried in same center.

Animals

Thirty-two (32) adult male Sprague-Dawley rats weighting 220 to 250 g were used in the experiment. Rats were housed in polycarbonate cages in a room with controlled temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 5\%$), and a 12 h cycle of light and dark; they were fed with laboratory pellet chows and water was given *ad libitum*. The experiment was performed after a stabilization period in the laboratory for 5 days.

Experimental design

Four groups were designed. Group I (Sham) was designed as the control group. Group II (renal I/R) was designed to renal I/R injury. Groups III (renal I/R injury + SM 100 mg per kg) and Group IV (renal I/R injury + SM 200 mg per kg) were designed to evaluate SM on the morphological and biochemical changes in the rats kidney in renal I/R injury.

Right nephrectomies

Right nephrectomies were performed under xylazine (10 mg per kg)

and ketamine (70 mg per kg) anesthesia in all rats in all Groups (I to IV). Thereafter, rats were let to recover for 15 days in the standard laboratory.

Drug administration

Seven (7) days prior to I/R induction, 0.5 ml of 100 and 200 mg/kg SM solution (Sigma-Aldrich, S0292-50G, Italy) were administered orally (p.o.) to the rats in Groups III and IV, respectively. Rats in Groups I and II received 0.5 ml normal (0.9%) saline p.o for 7 days prior to sham operation and I/R induction, respectively.

Induction of renal I/R injury

All surgical procedures were performed under xylazine (10 mg per kg) and ketamine (70 mg per kg) anesthesia. Renal I/R injury were induced with left renal pedicle occlusion with a vascular clamp for 45 min followed with reperfusion for 6 h through a median laparotomy under anesthesia. Sham procedures were same beyond vascular occlusion in the Group I. After induction of I/R injury in Groups II, III and IV, left kidneys were dissected for both biochemical and histopathological examinations.

Histopathological evaluation

Left kidneys specimens were processed routinely in 10% formalin solution, and embedded in paraffin. Tissue sections of 5 μm were obtained and stained with hematoxylin and eosin (H&E). Histopathological examinations were performed under a light microscope (NIKON, Japan). All histopathological examinations were performed by the same pathologist of the institute, who was blinded to all the tissue specimens. A minimum of 10 fields for each kidney slide with minimum $\times 50$ magnification were examined to assign the severity of these morphological changes. The morphological changes were scored on a scale of none (-), mild (+), moderate (++) and severe (+++) damage in order to perform a comparison between the groups.

Biochemical analysis

Postmitochondrial supernatant preparation (PMS)

After sacrificing the animals, isolated areas of the nephron of their kidneys were quickly removed and wash immediately with ice-cold normal saline, and homogenized in chilled potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at $800 \times g$ for 5 min at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at $10,500 \times g$ for 20 min at 4°C to get the PMS which was used to assay malondialdehyde (MDA), CAT and SOD activity.

The protocols of lipid peroxidation and enzyme activities measurement

Determination of lipid peroxides (measured as MDA)

MDA, a reactive aldehyde, that is, a measure of lipid peroxidation, was determined according to the method of Uchiyama and Mihara (1978). The adducts formed following the reaction of tissue homogenate with thiobarbituric acid in boiling water bath, were extracted with n-butanol. The difference in optical density developed at two distinct wavelengths, 535 nm and 525 nm was a measure of the tissue MDA content. Tissue MDA content was

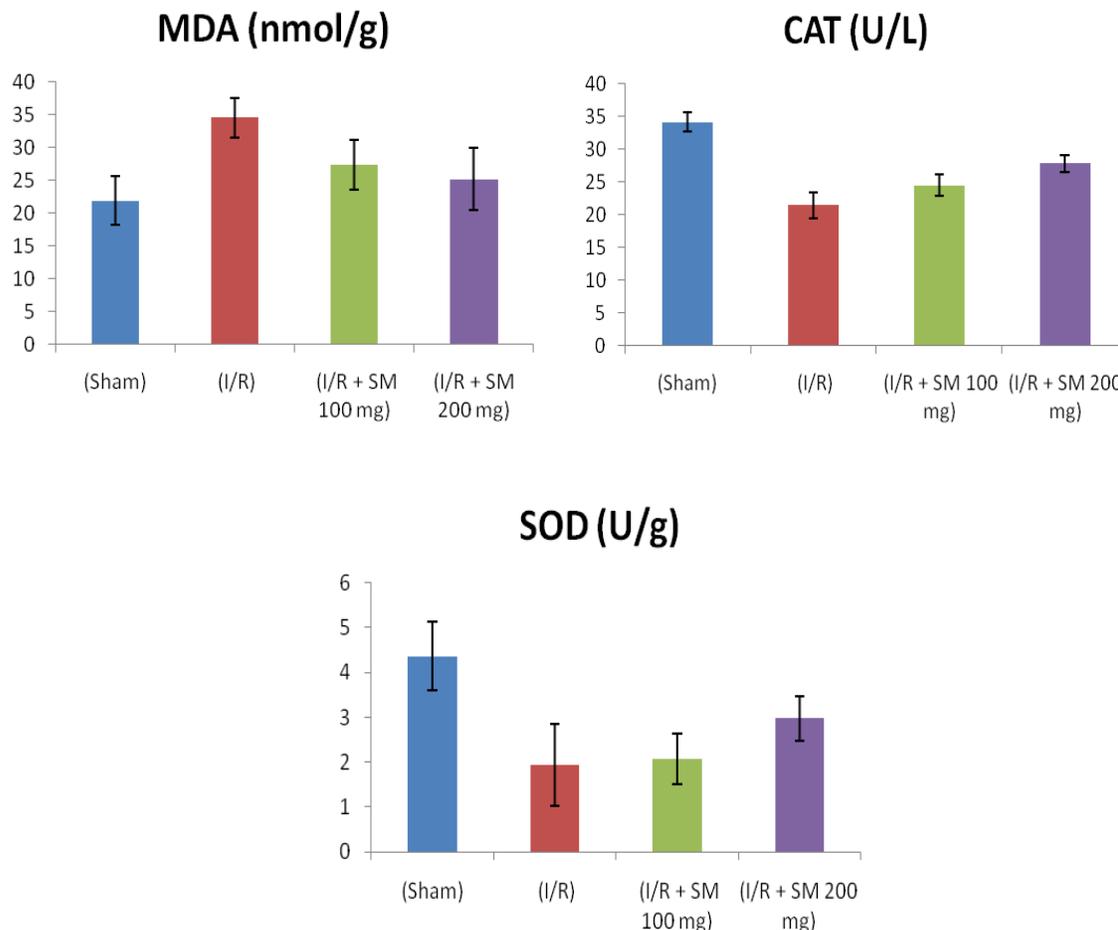


Figure 1. Effect of SM treatment on kidney tissue content of MDA, SOD and CAT. Rats in sham and I/R groups were administered normal saline 7 days prior to I/R induction. Rats in Groups III and IV were administered 100 and 200 mg/kg SM 7 days prior to I/R induction. Data were expressed as means \pm SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

expressed as nmol/g tissue (Mihara and Uchiyama, 1978).

Determination of SOD activity

SOD activity was spectrophotometrically assayed with commercial kits. The Fluka SOD kit USA contains all reagents and solutions required for determining SOD activity in an indirect assay method based on xanthine oxidase and a novel color reagent. The homogenate SOD activity was determined by inhibition of Formosan dye (450 nm) employing the xanthin-xanthin oxidase enzymatic method to generate superoxide radicals and expressed as U/g.

Determination of CAT activity

One unit (1 U) of CAT equals the enzyme activity that recognized 1 μ mol of hydrogen peroxide in 60 s at 37°C. The three blank samples were prepared according to Goth, 1991. CAT activity was measured with determination of absorbance of three blank samples at 405 nm in spectrophotometer. CAT activity (kU/L) was calculated as = $[(Abs_{blank1} - Abs_{blanksample}) / Abs_{blank2} - Abs_{blank3}] \times 271$ (Goth, 1991).

Statistical analysis

All statistical analysis was performed with the computer program "SPSS for Windows" (SPSS Inc; Release 11.5; Sep 6, 2002). All of the data were expressed as means \pm SD. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The significance was tested at $p > 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

Renal I/R significantly decreased the enzymatic activity of CAT and SOD, whereas the MDA levels increased. This enzymatic activity levels was significantly improved by treatment with both SM 100 (Group III) and 200 (Group IV) mg (Figure 1).

Light microscopic evaluation revealed that normal renal morphology in the Group I (Sham), and some of the histopathological findings, which were observed in renal I/R injury in Groups II, III and IV (renal I/R; renal I/R injury + SM 100 mg per kg; and renal I/R injury+SM 200 mg per

Table 1. Effect of SM (100 and 200 mg per kg, per oral) treatment on morphological changes as assessed by histopathological examination of kidneys of the rats exposed to renal I/R.

Group	Tubular necrosis	Glomerular necrosis	Tubular dilatation	Necrosis of epithelium	Hyaline casts	Interstitial inflammation
Group I (Sham)	-	-	-	-	-	-
Group II (I/R)	+++	+++	+++	+++	+++	+++
Group III (I/R + SM 100 mg)	-	+/-	+/-	-	+/-	+/-
Group IV (I/R + SM 200 mg)	-	+/-	+/-	-	+/-	+/-

Silymarin, SM; (-), none; (+), mild; (+/-), mild/none; (++) moderate; (+++), severe.

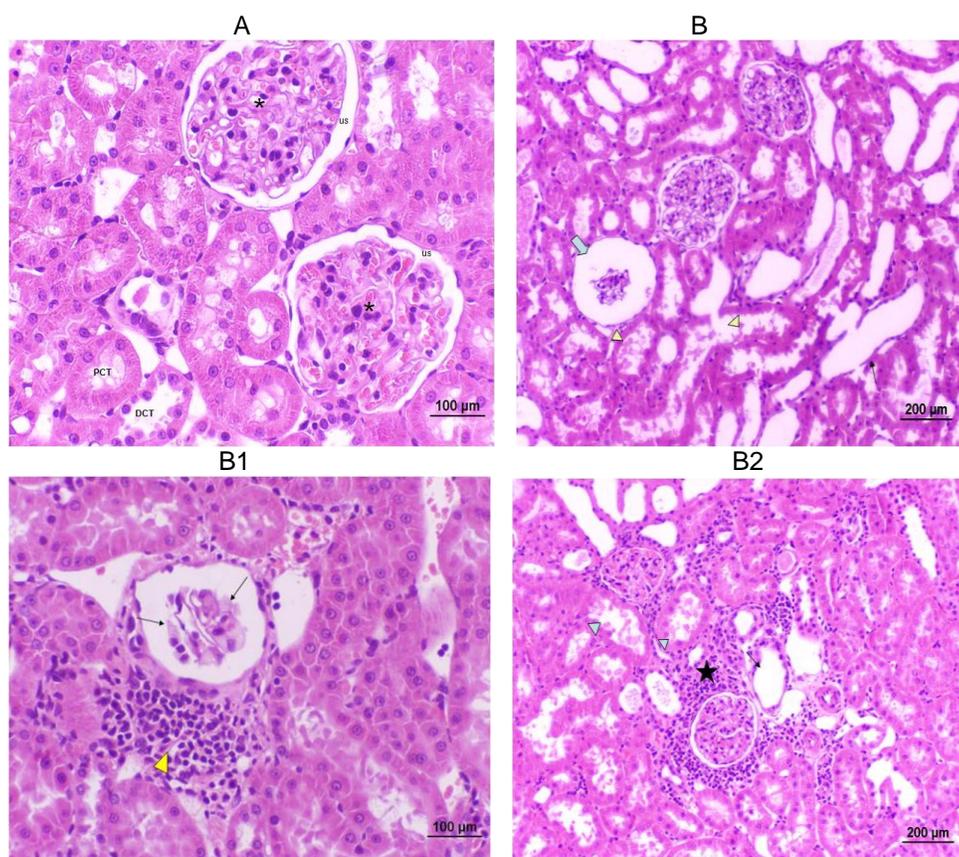


Figure 2. (A), Control group: Renal corpuscle and tubules were observed normal histological structure. (Glomerulus (*), urinary space (US), proximal convoluted tubules (PCT) and distal convoluted tubules (DCT), original magnification $\times 100$). (B), Group II (renal I/R); some tubules were desquamation of its epithelial cells (arrow head) and tubular dilatation (thin arrow). Displacement and shrinkage of glomerular tuft is also seen in this figure (thick arrow). (B1), Glomerular tuft was observed shrinkage and degeneration (arrow). Inflammatory cells were observed in the intertubular spaces of this figure (arrow head). (B2), Some renal tubules were observed desquamation of its epithelial cells (arrow head) and tubular dilatation (arrow). Inflammatory cells were observed in the intertubular spaces of this figure (*).

kg, respectively) (Table 1).

In Group II (renal I/R injury), significant tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium were observed due to renal I/R injury. In the Groups III and IV, in which the rats were

treated with SM 100 and 200 mg per kg before renal I/R, tubular dilatation, tubular necrosis, glomerular necrosis, tubular vacuolization, hyaline casts, interstitial inflammation and necrosis of epithelium due to I/R injury were observed to be protected with the treatment (Figures 2 and 3).

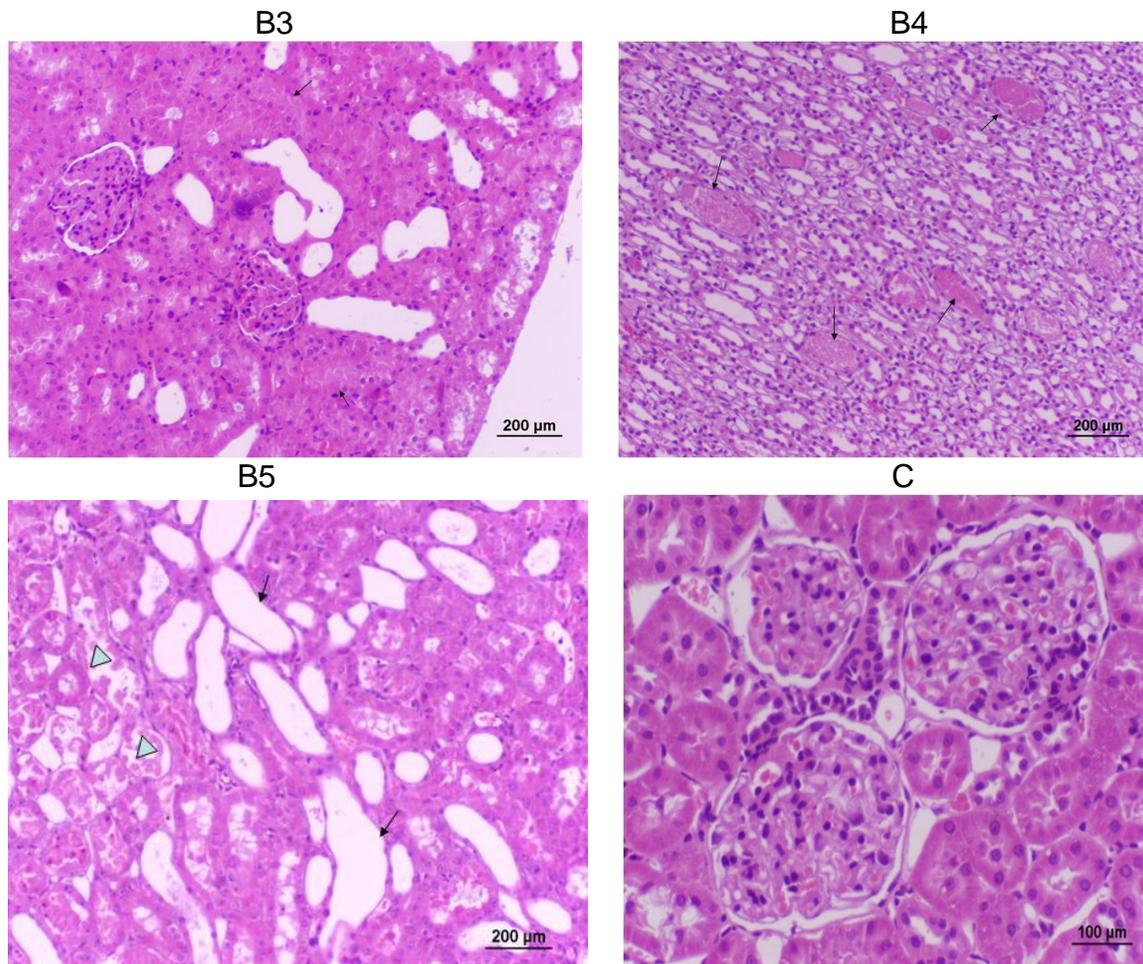


Figure 3. (B3), Widespread tubular necrosis and necrotic cells of the proximal tubules were observed in this figure (arrow). (B4), Hyaline casts were observed tubular structure (arrow). (B5), Epithelial cells of renal tubules were observed desquamation. Necrotic cells were seen in tubule lumen (arrow head). Also, some tubules were observed tubular dilatation (arrow). (C), Kidney section of SM (100 mg per kg, per oral) + renal I/R treated rat showing normal renal corpuscle and tubules.

DISCUSSION

Oxidative stress plays an important role in kidney I/R injury (Granger and Korthuis, 1995). Thus, increasing the kidney antioxidant capacity could be a promising therapeutic approach. Despite improvements in organ preservation and surgical techniques, I/R injury remains a significant clinical problem, and there is considerable interest in its prevention.

Several studies have been reported on the protective effects of antioxidants in different organ and renal I/R injury (Huang et al., 1995; Sehirli et al., 2003; Sener et al., 2004; Sener et al., 2006). Recent approaches advocated to control the production of ROS, which may directly lead to per oxidation of cell membrane lipids and permanent cellular damage, have been generally designed as the therapies including antioxidants such as *n*-acetylcystein (NAC), resveratrol, vitamin E, and others

(Sener et al., 2006; Thurman, 2007).

Tissue ischemia not only leads to the over production of ROS which directly induces tissue damage, but also triggers an aggravated local and systemic inflammatory response that causes multiple organ failure. Several studies demonstrated a recruitment of the neutrophils into post ischemic tissue, but activated neutrophils are also reported to be a potential source of ROS (Zimmerman et al., 1990; Granger and Korthuis, 1995). Chemokines, such as Interleukin-1 (IL-1), IL-6 and tumor necrosis factor- α (TNF- α) released from cellular elements, nitric oxide synthase (NOS) which modulates nitric oxide (NO) levels, adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and NF- κ B have been recently studied in several organ I/R injury models (Thurman, 2007). Results of these studies suggest that treatment and even protection of tissue and organ damage due to I/R may be possible with modulation of

the elements mentioned previously (Weight et al., 1996; Donnahoo et al., 1999).

The active constituents of milk thistle are flavonolignans including silybin, silydianin and silychristine, collectively known as silymarin. Medical use of milk thistle as a liver protecting herb dates back to the earliest Greek references to the plant. Hepatoprotective effects of SM are mainly attributed to its antioxidant, anti-inflammatory and antifibrotic activity (Ferenci et al., 1989; Luper, 1998). Also, recently, it has been reported that, induction of NF- κ B with TNF- α and IL-1 β was mediated through intracellular calcium but not ROS. The same report has showed that SM inhibited TNF- α -induced calcium-dependent NF- κ B activation irrespective of its antioxidant effect in human mesangial cells (Chang et al., 2006).

These protective effects may be related to different mechanisms such as the scavenger activity of the free radicals that induce lipid peroxidation (LP), and also stimulating antioxidant regeneration through increased protein synthesis (Sonnenbichler and Zetl, 1986). In experimental hepatic injury models, SM was reported to be effective on LP which mainly leads to destruction of plasma membrane (Moscarella et al., 1993; Farghali et al., 2000).

Further study is needed to overcome the limitations of this current study and to verify the significance of the results. The authors believe that the limitation of this study is quantitative measurement of apoptosis and may add some objective supporting data to our results to clarify the effect of SM in renal I/R injury. I/R injury caused an impairment in renal function (increased serum creatinine and blood urea nitrogen (BUN) levels along with significant decrease in creatinine clearance), in our study was not evaluated in this issue.

The protective effects of SM on the primary inflammatory cell, renal tubular epithelium, in renal I/R is thought to be both due to the inhibition of NF- κ B and anti-oxidative activity of SM. Further specific study is needed to clarify this issue such as measurement of tissue myeloperoxidase activity (MPO). SM has been reported to be safe to use in various conditions with minimal adverse effects (Jacobs et al., 2002). However, the adverse effects and the safety of SM were not in the scope of our study. The protective effects of SM was observed in even with 100 mg/kg in Group III (SM 100 mg/kg + renal I/R), with increased dose of SM in Group IV (SM 200 mg/kg + renal I/R) prevent the morphological changes in all rats. However, SOD and CAT levels suggested that the higher levels of SM may be more effective in preventing oxidative injury.

Conclusion

The results of our study have demonstrated that SM significantly prevents renal I/R injury-induced renal changes in the rat. The clinical implications of these results merits further experimental and clinical studies to

be performed.

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Full Length Research Paper

The survey of look alike/sound alike (LASA) drugs available in hospitals in Thailand

Teeraporn Chanakit*, Jintana Napaporn, Todsapon Chiempattanakajohn,
Somphon Sangkhawan and Sujitra Wichakot

¹Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand.

²5th year Pharmacy Student, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand

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A cross-sectional survey was designed to study look-alike, sound-alike (LASA) drugs in hospitals in Thailand. The questionnaires were developed and mailed to 1,380 hospitals throughout Thailand. The return rate was 11.16% or 154 hospitals, consisting of 5 tertiary hospitals (3.25%), 3 university hospitals (1.95%), 16 secondary hospitals (10.39%), 96 primary hospitals (62.34%), 26 private hospitals (16.88%) and 8 others (5.20%). A total of 5,327 pairs of drugs were identified as LASA drugs, including 3,695 tablets/capsules (Ranitidine-Roxithromycin pair in the highest frequency), 944 injections (Diazepam-Furosemide pair in the highest frequency), 307 liquid dosage forms (Alum milk-Milk of magnesia pair in the highest frequency), 367 external drugs (0.02% Triamcinolone cream and 0.1% Triamcinolone cream pair in the highest frequency) and 14 pairs of chemotherapeutic agents. This LASA report could be integrated into a suitable program used in hospitals in order to identify and prevent medication errors in the future.

Key words: Look-alike, sound-alike, look-alike, sound-alike (LASA), hospital, Thailand.

INTRODUCTION

The confusion of similar drug names is one of the most common causes of medication errors worldwide that threatens patients' safety (WHO, 2007a; Lambert et al., 2001, 1999; Basco et al., 2010; Phatak et al., 2005). With tens of thousands of drugs currently available, both of brandname and generic in the market, the potential for medication error due to confusing drug names is significant (WHO, 2007a; The Joint Commission, 2001). Causes of look-alike, sound-alike (LASA) medication errors were identified; and include illegible handwriting, unfamiliarity with drug names, similarity in the spelling and/or pronunciation of drug names, newly available products, similar packaging or labeling, similar clinical use, similar strength, dosage forms, frequency of administration, incorrect selection of a similar name from a computerized product list, and the failure of

manufacturers and regulatory authorities to recognize the potential for error and to conduct rigorous risk assessments, both for nonproprietary and brand names, prior to approving new product names (WHO, 2007a; Lambert et al., 2001; McCoy, 2005; Hoffman and Proulx, 2003). More than 33,000 trademarked and 8,000 nonproprietary medication names were reported in the United States alone in 2004, and an estimated 24,000 therapeutic health products were reported in the Canadian market (WHO, 2007a). On June 2011, the Institute for Safe Medication Practices (ISMP) (2011a, b) reported a listing of confusing drug names involved in medication errors that were reported through the ISMP National Medication Errors Reporting Program (ISMP MERP) (ISMP, 2011a). The United States Pharmacopeia (USP) also publishes a list of look-alike and sound-alike drug names periodically. Name pairs that have been included in LASA medication error reports are listed alphabetically (Cohen et al., 2007; Carothers, 1995). The aim of our list is to help healthcare providers and the public to determine which medications require special

*Corresponding author. E-mail: chanatee_1@yahoo.com. Tel: 6645353630, 66846068745.

Table 1. Affiliation of the hospital.

Organisation	N	(n =154) %
Primary hospitals	96	62.34
Private hospitals	26	16.88
Secondary hospitals	16	10.39
Other hospitals	8	5.20
Tertiary hospitals	5	3.25
University hospitals	3	1.95

safeguards to reduce the risk of errors (ISMP, 2011). Sauberan et al. (2010) reported cases of medication dispensing errors with look-alike, sound alike medication in neonatal care units, where multidisciplinary collaboration within the system helped the pharmacy identify, resolve and prevent errors related to medication storage, labeling, delivery, knowledge, and administration documentation. Santell et al. (2009) found that look-alike, sound-alike drug names is one of the problems associated with computer entry and actions at the university of Pittsburgh Medical, causing of order-entry errors and adverse drug events. Number of errors from brand or generic name look-alike are 887 (1.6%) from Medmarx®, 22 (3.1%) from University of Pittsburgh Medical Center (UPMC). Actions included adding warning notes and mixed-case or uppercase lettering in the pharmacy computer system and informing pharmacy staff through e-mail notices of the potential for drug name confusion errors when drug products are added to the formulary or when drug mix-up errors occur. Additionally, the USP operates MEDMARX, an internet-accessible, anonymous error-reporting program specifically designed for hospital and health care system (Hicks et al., 2004; Nosek et al., 2010). A 2008 MEDMARX report by the USP identified 1,470 drugs involved in LASA errors, including categorizing medication errors (Hicks et al., 2008). A retrospective study revealed that one of the causes of medication errors in the inpatient department of Mahasarakham Hospital, Thailand, was LASA drugs, such as the confusing similar-sound of dobutamine injection and dopamine injection (Pattanajak, 2006).

In 2005, the World Health Organization (WHO) lunched the World Alliance for Patient Safety and identified six action areas which look-alike, sound-alike medication names is one of the inaugural patient safety solutions (WHO, 2007b). Similarly, in Thailand, the Thai Patient Safety Goals 2008 program was developed to improve medication safety with focus on LASA drugs. The program is based on WHO Collaborating Centre for Patient Safety Solutions (2007a, b) and is still used as a guideline in Thailand (The Healthcare Accreditation Institute, 2011). However, there have been few studies specifically addressing look-alike, sound-alike medication in Thailand, therefore this study surveys the pair list of LASA drugs in hospitals in Thailand.

METHODS

This was a cross-sectional survey research. The questionnaires were sent along with an accompanying letter by mail to 1,380 hospitals through out Thailand during December 2009 to January 2010. The questionnaire was designed to gather data in the area of personnel affiliation, the LASA drug list generic/brand name and the form of drug, including tablets/capsules, injectable drugs, liquid preparations, external use preparations and chemotherapy agents, as well as, a medication error categorization index (category A-I) for each LASA pair, according to the National Coordinating Council for Medication Error, Reporting and Prevention (NCC MERP's, 1998-2012) index for categorizing medication errors. Category A represents circumstances or events that have the capacity to cause error, category B indicates that a medication error occurred but it did not reach the patient, categories C and D designate medication errors that reached the patient but did not result in patient harm. The varying level of patient harm is reflected in categories E, F, G and H. Category E refers to errors that may have contributed to or resulted in temporary harm to the patient and required intervention. Category F contains errors that may have contributed to or resulted in temporary harm to the patient and required initial or prolonged hospitalization. Category G labels all errors that may have contributed to or resulted in permanent patient harm. Category H errors require intervention necessary to sustain life and any medication error that resulted in, or may have contributed to a patient's death is classified as category I (NCC MERP, 1998 to 2012; Hartwich et al., 1991; Dubey et al., 2006). All of the error reports were submitted using voluntary error reporting programs in each hospital, by health care professionals (pharmacists, nurses and physicians).

RESULTS

After a month of distribution, 154 completed questionnaires were returned (11.16% response rate). Data from questionnaires were evaluated. All respondents were affiliated with hospitals of various types, as shown in Table 1. Response was received, with most frequently response from primary hospitals (62.34%). Ninety percent (90.91%) of hospitals found LASA medication errors a relevant problem.

This study identified 5,327 pairs of LASA drugs. Tablet/capsule forms appeared most frequently (3,695, 69.36%), followed by injection preparation (944, 17.72%), external use preparations (367, 6.89%), liquid preparations (307, 5.76%) and chemotherapeutic agents (14, 0.26%), respectively.

The study revealed two distinct types of drug similarity, based on the source of confusion. Similar spelling or pronunciation (LASA) caused visual and auditory confusion, while similar packaging/labeling (LA1) or similar tablets/capsules (LA2) caused visual-only confusion.

The most frequently occurring similarity was LASA stemming from similar spelling or pronunciation, as shown in Table 2. From the surveyed medication error categories, category B error occurred but did not reach the patient and was most prevalently reported (Table 3). No category I (fatal) errors were reported. Harmful medication errors (category F, G) are shown in Table 4. From all the reported drugs, 3,695 pairs were tablets/

Table 2. Types of LASA (n = 6,550, one pair of LASA may have more than one type of LASA).

Preparations	Types of LASA pairs		
	LASA (Similar spelling/pronunciation)	LA	
		(Similar packaging/labeling)	(Similar tablets/capsules)
Tablets/Capsules	2745	868	1143
Injections	519	540	0
Liquid dosage forms	110	207	0
External use preparations	233	170	0
Chemotherapeutic agents	8	6	1
	3615 (55.19%)	1791 (27.34%)	1144 (17.47%)

Table 3. Cross-tabulation of type of medication by error category index (n = 3,161; some pairs of LASA did not report error category index).

Preparations	Error category (pairs)								
	A	B	C	D	E	F	G	H	I
Tablets/Capsules	435	1469	185	30	14	7	3	0	0
Injections	132	389	57	19	2	0	0	0	0
Liquid dosage forms	40	156	23	1	0	0	0	0	0
External use preparations	33	146	13	0	0	0	0	0	0
Chemotherapeutic agents	2	5	0	0	0	0	0	0	0
	642 (20.31%)	2165 (68.49%)	278 (8.79%)	50 (1.58%)	16 (0.51%)	7 (0.22%)	3 (0.09%)	0 (0.00%)	0 (0.00%)

Table 4. Harmful medication error (category F, G) of LASA pairs (N = 3,161).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs*	Error Category	Affiliation (hospital)
Atarax	Ativan	1	0.03	Tablets/capsules	LASA	G	Private
Clotrimazole	Cotrimoxazole	1	0.03	Tablets/capsules	LASA	G	Private
Voltaren	Ventolin	1	0.03	Tablets/capsules	LASA	G	Private
Clozapine	Carbamazepine	1	0.03	Tablets/capsules	LASA	F	Primary
Haloperidol	Propranolol	1	0.03	Tablets/capsules	LASA	F	Others
Ranitidine	Roxithromycin	1	0.03	Tablets/capsules	LASA	F	Others
Terbutaline (2.5 mg)	Tianeptine (12.5 mg)	1	0.03	Tablets/capsules	LASA	F	Others
Gemfibrozil	Glibenclamide	1	0.03	Tablets/capsules	LASA	F	Primary
Atenolol (50 mg)	Allopurinol (100 mg)	1	0.03	Tablets/capsules	LASA, LA1	F	Primary
Spirolactone (Aldactone)	Methyldopa (Aldomet)	1	0.03	Tablets/capsules	LASA	F	Primary

*Types of LASA pairs: LASA = similar spelling/pronunciation; LA1 = similar packaging/labeling; LA2 = similar tablets/capsules.

capsules (Ranitidine and Roxithromycin pair in the highest frequency) (Table 5), 944 were injections (Diazepam injection and Furosemide injection pair in the highest frequency) (Table 6), 307 were liquid dosage forms (Alum milk and Milk of magnesia pair in the highest frequency) (Table 7), 367 were external drugs (0.02% Triamcinolone cream and 0.1% Triamcinolone cream pair in the highest frequency) (Table 8) and 14 pairs were

chemotherapeutic agents (Table 9). There were 323 pairs of high-alert medications, all in injection preparation form. However, the list of potential high-alert medications can vary by individuals and organizations. This study distinguished 6 distinct variations (Warinchumrab hospital, 2009; ISMP, 2011b) shown in Tables 10 to 14; including narrow therapeutic index medications (110, 34.06%), highly concentrated electrolytes (36, 11.15%), emergency

Table 5. Top ten LASA pairs in tablet/capsule form (N = 3,695).

Drug name	Drug name	No. (Drug pairs)	%	Types of LASA pairs	Error Category	Affiliation (hospital)
Ranitidine	Roxithromycin	29	0.78	LASA, LA1, LA2	A,B,C,F	Primary Secondary Private Others
Hydralazine	Hydroxyzine	23	0.62	LASA, LA1, LA2	A,B,D	Primary Secondary Tertiary Private Others
Glibenclamide	Glipizide	19	0.51	LASA, LA1, LA2	A,B,C,D	Primary Secondary Tertiary Private
Propranolol (10 mg)	Propranolol (40 mg)	18	0.49	LASA, LA1, LA2	A,B,C	Primary Tertiary Others
Lasix	Losec	17	0.46	LASA	A,B,C	Primary Secondary Tertiary Private
Loratadine	Lorazepam	17	0.46	LASA, LA1, LA2	A,B	Primary Secondary Private Others
Voltaren	Ventolin	17	0.46	LASA, LA1, LA2	B	Primary Secondary Private
Gliclazide	Glipizide	16	0.43	LASA, LA1, LA2	A,B	Primary Secondary Tertiary University Private Others
Merislon	Mestinon	14	0.38	LASA	A,B	Secondary University Private Others
Ibuprofen (200 mg)	Ibuprofen (400 mg)	11	0.30	LASA, LA1, LA2	A,B	Primary Secondary

*Types of LASA pairs: LASA = similar spelling/pronunciation; LA1 = similar packaging/labeling; LA2 = similar tablets/capsules.

Table 6. Top ten LASA pairs in injection form (N = 944).

Drug name	Drug name	No. (drug pairs)	%	Types of LASA pairs	Error category	Affiliation (hospital)
Diazepam	Furosemide	38	4.0	LASA, LA1	A, B	Primary Secondary Tertiary Private
Dobutamine	Dopamine	29	3.1	LASA, LA1	A, B	Primary Secondary Tertiary Private Others
Vitamin K1 (1 mg)	Vitamin K1 (10 mg)	27	2.9	LASA, LA1	A, B, C, D	Primary Secondary Tertiary Private Others
Vitamin K1	Vitamin B complex	16	1.7	LASA, LA1	A,B,C,D	Primary Secondary Private Others
Adenosine	Adrenaline	15	1.6	LASA, LA1	A, B	Primary Secondary Tertiary Private Others
Ceftazidime	Ceftriaxone	15	1.6	LASA, LA1	A, B, C	Primary Private
Lasix	Losec	14	1.5	LASA	A, B, C, D	Primary Secondary Private Others
Vitamin K1 (2 mg)	Vitamin K1 (10 mg)	11	1.2	LASA, LA1	A, B	Primary Secondary Tertiary Private
Ampicillin (500 mg)	Ampicillin (1000 mg)	10	1.1	LASA, LA1	A, B	Primary Private
Ceftazidime (1000 mg)	Ceftriaxone (1000 mg)	10	1.1	LASA	A, B, D	Primary Private

Table 7. Top ten LASA pairs in liquid dosage form (N=307).

Drug name	Drug name	No. (drug pairs)	%	Types of LASA pairs	Error Category	Affiliation (hospital)
Alum milk	Milk of magnesia	8	2.61	LA1	A,B,C	Primary Secondary Tertiary Private Others
Amoxicillin (125 mg)	Amoxicillin (250 mg)	7	2.28	LASA,LA1	A,B,C	Primary Secondary Private
Ammonium carbonate	M.tussis	5	1.63	LASA,LA1	B	Primary Secondary
Amoxicillin	Erythromycin	5	1.63	LA1	A,B	Primary
Amoxicillin	Cloxacillin	5	1.63	LA1	A,B	Primary Private
Domperidone	Hyoscine	3	0.98	LA1	N/A	Primary
Amoxicillin	Amoxicillin + Clavulanic acid	3	0.98	LASA	A,B	Primary
Bromhexine	Brown mixture	3	0.98	LASA	A,B,C	Primary
Domperidone	Hyoscine	3	0.98	LA1	B	Primary Secondary
Miotin	Meptin	3	0.98	LASA	A	Private

drugs (93,28.79%), antidotes (68, 21.05%), high-alert drugs in obstetric medications (15, 4.64%) and high-alert drugs in anesthetic agents (1, 0.31%; Methergin injection and Pancuronium injection which have similar packaging, category B error and were found in a tertiary hospital).

DISCUSSION

Confusing drugs with similar names constitute about 10% of all medication errors. The American Pharmacists Association reported that there are more than 33,000 trademarked medication names in the United States and more than 9,000 generic names (American Hospital Association, 2005). In this study, all participating hospitals reported LASA-related medication errors, both from the governmental and from the private sector. Most participants are affiliated with primary hospitals (62.34%) the most prevalent type of hospitals nationwide (730

hospitals, 91.7%) (Ministry of Public Health, 2011). These institutions provide primary health care for the most patients, resulting in highest workload for physicians in Thailand, followed by physicians in secondary, tertiary, university and private hospitals, respectively. Although most LASA reports came from respondents affiliated with primary or community hospitals, the overall response rate was low (11.16% or 154 completed questionnaires), perhaps due to Thailand's insufficient medication-error-reporting infrastructure, such as MEDMARX® and USP-ISMP and due the voluntary nature of reporting (Ministry of Public Health, 2011; Macagba, 2011). It is also worth noting that there are no comparable data available from specialty hospitals and military hospitals, because they did not return the questionnaire. The Joint Commission on Accreditation of Healthcare Organizations now requires that accredited health care organizations develop and maintain programs to minimize these LASA medication risks; a good example of this is the annual report list of

Table 8. Top ten LASA pairs in external form (N = 367).

Drug name	Drug name	No. (drug pairs)	%	Types of LASA pairs	Error Category	Affiliation (hospital)
Triamcinolone cream (0.02%)	Triamcinolone cream (0.1%)	21	5.72	LASA, LA1	A, B	Primary Secondary Private
Chloramphenicol ear drop	Chloramphenicol ED	14	3.81	LASA, LA1	A, B, C	Primary Private University
Berodual MDI	Budesonide MDI	11	3.00	LASA, LA1	A	Pyrimar Secondary Tertiary
Chloramphenicol ED	Chloramphenicol EO	8	2.18	LASA, LA1	B	Primary Secondary Private
Clobetasol cream	Clotrimazole cream	8	2.18	LASA, LA1	B	Primary Secondary
Nasocort nasal spray	Nasonex nasal spray	8	2.18	LASA, LA1	A, B	University Private Others
Hista oph	Dex oph	6	1.63	LASA, LA1	B	Primary Secondary Private
Spersallerg ED	Spersadexoline ED	6	1.63	LASA	A, B	Private, University Others
Clotrimazole cream	Clobetasol cream	5	1.36	LASA, LA1	B	Primary Tertiary
Fucidine	Fucidine H	4	1.09	LASA, LA1	B, C	Private

Table 9. Top ten LASA pairs in chemotherapeutic agents (N = 14).

Drug name	Drug name	No. (drug pairs)	%	Types of LASA pairs	Error category	Affiliation (hospital)
Carboplatin	Cisplatin	3	21.4	LASA, LA1	A	Secondary
Vinblastine	Vincristine	2	14.3	LASA	B	Private
5-FU (500 mg)	5-FU (250 mg)	1	7.14	LA1	N/A	Secondary
Cyclophosphamide	Methotrexate	1	7.14	LA1	B	Private
Cytosar	Vincristine	1	7.14	LA1	B	University
Doxorubicin	Farmorubicin	1	7.14	LASA	N/A	Tertiary
Hydralazine	Hydroxyurea	1	7.14	LASA	N/A	Private
Methotrexate	Multivitamin	1	7.14	LA1	B	Tertiary
Methotrexate	Tamoxifen	1	7.14	LASA	B	Secondary
Mybacin	Myleran	1	7.14	LA1, LA2	A	Primary

Table 10. Top ten of LASA pairs in high-alert narrow therapeutic index medications (N =110).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs	Error Category	Affiliation (hospital)
Diazepam	Furosemide	38	34.55	Injections	LA1	A, B	Primary Secondary Tertiary Private
Gentamicin	Metoclopramide	10	9.09	Injections	LA1	A, B	Primary Secondary Tertiary Private
Warfarin (3 mg)	Warfarin (5 mg)	5	4.55	Tablets	LA1, LA2	A, B	Primary Private others
Amikacin	Ampicillin	4	3.64	Injections	LASA	B	Private others
Warfarin (2 mg)	Warfarin (3 mg)	3	2.73	Tablets	LA2	N/A	Secondary
Dimenhydrinate	Diazepam	3	2.73	Injections	LASA	B	Primary
Furosemide	Gentamicin	3	2.73	Injections	LA1	B, D	Primary
Lidocaine (2%)	Lidocaine (2%) + Adrenaline	3	2.73	Injections	LASA, LA1	A	Primary
Gentamicin	Amikacin	2	1.82	Injections	LA1	B	Secondary Tertiary
Cimetidine	Gentamicin	2	1.82	Injections	LA1	B	Primary

LASA drug names (Joint Commission of Accreditation of Healthcare Organizations, 2005).

This study found the source of confusion to include not only drug names but also look-alike product labeling and packaging (LA1), and look-alike tablets/capsules (LA2), confirming earlier reports (McCoy, 2005; Levine and Cohen, 2007; Cohen, 2007b). Category E, F and G (harmful) errors were reported in tablet/capsule preparations; however no category H and I (fatal) errors have been reported in our study. This could be the result of the obstacle of system, especially in light of 2006 MEDMARX® report whose 176,407 examined records 14 cases, where medication errors may have caused or contributed to patient deaths. The report also noted that the percentage of harmful errors has remained above 1% for more than seven years (Hicks et al., 2008). This research identified 5,327 LASA pairs, most of which came from tablet/capsule preparations (3,695 pairs, 69.36%). This could likely be explained by the prevalence of these forms of drugs on the market in general; with 15,404 and 4,287 preparations, tablets and capsules make up 43 and 12% of drugs available on the market, respectively (Thai Drug Control Division, 1999). Of all these, Ranitidine-Roxithromycin pair was reported the

most frequently. Reported LASA-related errors fell into categories A, B, C, D, E, F and G. The highest harmful medication error was G, found only in tablet/capsule preparations. The study made a distinction in drug similarity, based on the source of confusion: similar spelling/pronunciation (LASA), similar packaging/labeling (LA1) and similar tablets/capsules (LA2). Two pairs of high-alert tablet drugs with a narrow therapeutic index are Warfarin (3 mg)-Warfarin (5 mg) pair and Warfarin (2 mg)-Warfarin (3 mg) pair; fortunately their LASA-associated error categories were only A and B.

From the 944 reported injection LASA pairs, Diazepam-Furosemide pair occurred in the highest frequency (34.55%). Diazepam is a high-alert drug with a narrow therapeutic range; however, the associated error categories were only A and B. From the 307 liquid dosage forms drug pairs, Alum milk-Milk of Magnesia pair was reported frequently. Among the 367 external drug pairs, the one occurred most frequently was 0.02% Triamcinolone cream-0.1% Triamcinolone cream. This study also identified confusing medication names and packaging in oncology medications. The names of several chemotherapy agents can look or sound alike (Carboplatin and Cisplatin, Vinblastine and Vincristine) or

Table 11. Top ten LASA pairs in high-alert highly concentrated electrolytes (N = 36).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs	Error category	Affiliation (hospital)
KCl	Dopamine	6	16.67	Injections	LA1	A, B, C	Primary Secondary Private
MgSO ₄	Dimenhydrinate	5	13.89	Injections	LA1	B	Primary Secondary Tertiary
KCl	Calcium gluconate	4	11.11	Injections	LASA, LA1	A,B	Primary Secondary Tertiary Private
Glucose	Sodium bicarbonate	4	11.11	Injections	LA1	A,B	Primary Private
KCl	Aminophylline	3	8.33	Injections	LA1	N/A	Primary Secondary
MgSO ₄	Aminophylline	2	5.56	Injections	LA1	B	Primary
MgSO ₄ (50%)	Dexamethasone (5 mg)	2	5.56	Injections	LA1	B	Primary Private
MgSO ₄ (10%)	MgSO ₄ (50%)	2	5.56	Injections	LASA	A,B	Primary Private
CaCO ₃	Calcium gluconate	1	2.78	Injections	LASA	B	Primary
KCl	Dipotassium chloride (20 mEq)	1	2.78	Injections	LASA	A	Private

can be confused with unrelated medications (for example, Methotrexate and Multivitamin, found in this study), as noted also by Schulmeister (2006). Most of the high-alert drugs were found in injection preparations, a trend also shown in ASHP and other literature reports (ASHP, 2008; Hicks and Becker, 2006; Taxis and Barber, 2003a; Taxis and Barber, 2003b; Parshuram et al., 2008). James et al. (2009) investigated dispensing errors in the UK, US, Australia, Spain and Brazil. The most common dispensing errors identified by community and hospital pharmacies were dispensing the wrong drug, strength, form or quantity, or labeling medication with incorrect directions. They revealed that factors subjectively reported as contributing to dispensing errors were look-alike, sound-alike drugs, low staffing and computer software. High workload, interruptions, distractions and inadequate lighting were objectively shown to increase the occurrence of dispensing errors (James et al., 2009). Other factors associated with dispensing errors may be communication failures, problems related to package labels, work overload, the physical structure

of the working environment, distraction and interruption, the use of incorrect and outdated information sources and the lack of patient knowledge and education about the drugs they use (Anacleto et al., 2005). The ISMP's landmark article by Leape et al., 1995, on systems analysis of adverse drug events defined broad categories, where the underlying problems that result in medication errors identified the following proximal causes of medication errors: lack of knowledge of the drug, lack of information about the patient, violations of rules, slips and memory lapses, transcription errors, faulty identity checking, faulty interaction with other services, faulty dose checking, infusion pump and parenteral delivery problems, inadequate patient monitoring, preparation errors and lack of standardization (Cohen, 2007a).

Risk reduction strategies include being aware of medications which look or sound like other drugs, installing pop-up alerts in computer systems, prescribing medications both by their generic and trade names, placing eye-catching labels and warning stickers on

Table 12. Top ten LASA pairs in high-alert emergency drugs (N = 93).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs	Error Category	Affiliation (hospital)
Dobutamine	Dopamine	29	31.18	Injections	LASA, LA1	A, B	Primary Secondary Tertiary Private Others
Adenosine	Adrenaline	15	16.13	Injections	LASA, LA1	A,B	Primary Secondary Tertiary Private Others
Dopamine	KCl	6	6.45	Injections	LASA, LA1	A, B, C	Primary Secondary Tertiary Private Others
Morphine	Pethidine	5	5.38	Injections	LASA, LA1	A, B, C	Primary Tertiary Private
Adrenaline	Atropine	4	4.30	Injections	LASA, LA1	A, B	Primary Secondary Tertiary Private Others
Enoxaparin	Fraxiparine	4	4.30	Injections	LASA, LA1	B, C	Secondary Tertiary Private
Pethidine	Ranitidine	4	4.30	Injections	LASA	B, C	Primary Secondary Private
Regular insulin	Mixtard	3	3.23	Injections	LA1	B, C	Primary
Adrenaline	Vitamin B complex	2	2.15	Injections	LA1	N/A	Primary
Adrenaline	Methergin	2	2.15	Injections	LASA, LA1	A, B	Primary Secondary Tertiary Private Others

storage bins, storing medication in nonadjacent areas, and advising patients to be alert for potential mix-ups with look-alike, sound-alike medications (Schulmeister, 2006). Further strategies could involve the use of uppercase letters which successfully alert health professionals to

potential for error with several generic name pairs, for example; acetoHEXAMIDE, acetaZOLAMIDE; lists of similar name pairs (Cohen et al., 2007), annual review and revision of a list of look-alike, sound-alike drugs and a proactive implementation of safety strategies to prevent

Table 13. Top ten LASA pairs in high-alert antidotes (N = 68).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs	Error Category	Affiliation (hospital)
Vitamin K1 (1 mg)	Vitamin K1 (10 mg)	27	39.71	Injections	LASA, LA1	A,B,C,D	Primary Secondary Tertiary Private Others
Vitamin K1	Vitamin B complex	16	23.53	Injections	LASA, LA1	A,B,C,D	Primary Secondary Tertiary Private
Vitamin K1 (2 mg)	Vitamin K1 (10 mg)	11	16.18	Injections	LASA, LA1	A,B	Primary Secondary Tertiary Private
Adrenaline	Atropine	4	5.88	Injections	LASA, LA1	B	Primary University
Hyoscine	Neostigmine	2	2.94	Injections	LA1	N/A	Secondary Tertiary
Nalador	Naloxone	2	2.94	Injections	LASA	A	Primary Private
Atropine	Hyoscine	1	1.47	Injections	LASA, LA1	N/A	Primary
Atropine	Oxytocin	1	1.47	Injections	LA1	N/A	Primary
Epinephrine	Atropine	1	1.47	Injections	LA1	N/A	Primary
Vitamin K1	Gentamicin	1	1.47	Injections	LA1	B	Tertiary

medication errors involving these drug combinations. Proactive assessment of potential for LASA medication errors should evaluate for potential look-alike packaging problems in addition to the drug names (McCoy, 2005). Furthermore, organizations could develop and implement process enhancements, including moving and reorganizing shelf storage bins, improving labeling for intravenous medications or other dosage forms with similar packaging, revising processes for selecting and maintaining the list of LASA medications to include real-time review of new medications added to the formulary and changes in packaging resulting from contract changes or drug storage.

Another important factor to consider is multidisciplinary collaboration within the system; increasing organisations' ability to identify, resolve and prevent errors related to medication storage, labeling, delivery, knowledge, and administration documentation (McCoy, 2005; Cohen, 2007a; Sauberan et al., 2010). The World Health

Organization is currently developing global patient safety taxonomy that could facilitate consistent data collection and assist the development of error-reduction strategies (James et al., 2009). Little research is available on the topic, but regulators and the industry have begun discussing what level of similarity leads to confusion and errors (Cohen et al., 2007). The Food and Drug Administration (FDA) also has a role in preventing drug confusion. The FDA Office of Drug Safety, Division of Medication Errors and Technical Support (DMETS), reviews all trademarks prior to marketing and disapproves trademarks proposals that are deemed too similar to existing drug names (Cohen et al., 2007). Patient safety clearly requires improved methods for naming pharmaceutical products. FDA, the pharmaceutical industry, USP, ISMP, and others need to reach consensus and collaborate on a guidance document for product naming (Cohen et al., 2007). Such guidance should propose a common nomenclature with

Table 14. LASA pairs in high-alert obstetric medications (N = 15).

Drug name	Drug name	No. (drug pairs)	%	Preparations	Types of LASA pairs	Error category	Affiliation (hospital)
Hyoscine	Terbutaline	6	40.00	Injections	LA1	A	Primary Secondary
Dimenhydrinate	Terbutaline	3	20.00	Injections	LA1	N/A	Primary, Private
Methergin	Adrenaline	2	13.33	Injections	LA1	A	Primary
Atropine	Oxytocin	1	6.67	Injections	LA1	N/A	Primary
Dexamethasone	Terbutaline	1	6.67	Injections	LA1	A	Primary
Methergin	Pancuronium	1	6.67	Injections	LA1	B	Tertiary
Methergin	Vitamin K1	1	6.67	Injections	LA1	N/A	Primary

standardized abbreviations, acronyms, and terms. Although there is no single or simple answer to reducing medication errors, a coordinated effort to research problems associated with drug naming would produce timely and measurable results (Cohen et al., 2007).

Like other voluntary error reporting systems, this study is limited by the voluntary nature in capturing medication errors, therefore significant errors may go unreported and thus may not be reflected in this report, a limitation that is also applicable to UPMC or MEDMARX data (Santell et al., 2009). While a detailed analysis of the of the root causes of LASA medication errors is outside the scope of this study, discernible patterns indicate that the potential for LASA drug errors is present at every step of the patient care process; prescription, dispensing and administration of drugs. Recommendations for preventing LASA medication errors at the prescription stage include maintaining awareness of LASA information, clearly indicating drug name, strength and dosage form on prescriptions, and abstaining from verbal-only orders. At the dispensing stage, pharmacy and therapeutic committees should regularly provide LASA information to health-care staff and consider the possibility of name confusion when adding a new drug product to the hospital formulary, avoiding LASA in both presentation and packaging. Furthermore, when it comes to computerized systems, the appearance of LASA drug names could be emphasized with highlighting, boldface, unique colors, uppercase lettering or a mix of upper and lowercase lettering at different in parts of the name, such as vinCRiStine, vinBLASStine. Pharmacy departments could store LASA products in different locations, or use alert stickers on shelves or places where LASA drugs are present. An independent double-check would also be useful to ensure LASA-error prevention at the dispensing process.

At the drug administration step, LASA-errors could be prevented by nurses reading prescriptions more carefully

and considering them in the context of patient status and diagnoses. It is also important that appropriate systems for reporting errors and potentially hazardous LASA conditions are present and their use is encouraged. Moreover, professional staff could educate patients and their families about the confusing names and indication of their medication, while encouraging patients to ask about their medication if it is unfamiliar, or looks or sounds different.

Conclusion

A cross-sectional survey was developed to study look-alike, sound-alike (LASA) drugs in hospitals in Thailand. This LASA report could be developed and integrated to suitable programs used in hospitals in order to identify and prevent medication errors in the future.

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Full Length Research Paper

The effect of blood stasis syndrome on the pharmacokinetics of hydroxysafflower yellow A in human

Yan-Yan Jia, Jing Yang, Jin-Wen Wang, Yun Tian, Ai-Dong Wen* and Zhi-Fu Yang

Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, China.

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Blood stasis, that is, the decrease of blood flow velocity and the increase of blood viscosity indicates hemorheological abnormalities and pharmacokinetic difference of drugs. Therefore, it is very important to investigate the pharmacokinetics of drugs in patients with blood stasis syndrome, which may influence absorption, distribution, metabolism, and excretion of drugs in blood. The aim of this study was to compare the pharmacokinetics of 140 mg hydroxysafflower yellow A (HSYA) in healthy subjects and patients with blood stasis syndrome due to stable angina pectoris (SAP), and indentify the therapeutic regimen for patients and promote clinical rational drug use. This study was carried out in 12 healthy volunteers and 24 patients with blood-stasis syndrome due to SAP using a single-dose of HSYA (140 mg) under fasting conditions. Venous blood samples were drawn through indwelling cannula from each volunteer prior to drug administration and at 0.5, 1, 1.25, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 15 h after the drug administration. Plasma obtained after centrifuge was analyzed to determine HSYA by high-performance liquid chromatography (HPLC). HSYA pharmacokinetics have a significant difference between healthy subjects and patients with blood-stasis syndrome due to SAP: ratios of C_{max} and $AUC_{(0-\infty)}$ were 116.5% (105.0 to 134.2) and 132.2% (116.5 to 153.7), respectively; mean terminal half-life ($t_{1/2}$) was 4.2 h as compared to 2.5 h in healthy subjects. The blood stasis syndrome has an impact on the pharmacokinetic parameters including C_{max} , $AUC_{0-\infty}$, and $t_{1/2}$, and dose adjustment should be required for patients with blood-stasis syndrome.

Key words: Hydroxysafflower yellow A, safflower, pharmacokinetics differences, blood stasis.

INTRODUCTION

Safflower yellow injection is a new Chinese drug made of extraction from single herb, *Flos Carthamus tinctorius* and prepared by Shandong Lvyue Natural Medicine Research and Development Center, China, having the effect of activating blood circulation and removing stasis. It is widely used to treat cardio-cerebral-vascular and gynecologic disease. Hydroxysafflower yellow A (HSYA), the main active component of safflower yellow injection, has been demonstrated to have the activities of antioxidation, and myocardial and cerebral protective effect (Chu et al.,

2006; Yang et al., 2009). HSYA was chosen as an active marker component for controlling the quality of safflower in Chinese Pharmacopoeia (The State Pharmacopoeia Commission of China, 2005; Nie et al., 2012; European Agency for the Evaluation of Medicinal Products, 2005).

Blood stasis is a pathological state resulting from a sluggish or impeded flow of blood in the body or abnormal blood outside the vessels that remains in the body and fails to disperse (Li et al., 2009). As soon as blood stasis develops, the blood circulation will further be affected and thus lead to new pathological changes. The clinical manifestations of blood stasis vary with the locale and the degree of blood stasis or blood stagnancy, such as abdominal pain, retarded menstruation, dysmenorrhea, dysmenorrhea, thrombosis or pricking pain in the

*Corresponding author. E-mail: adwen-2004@hotmail.com. Tel: +86-29-84773636. Fax: +86-29-84773636.

chest, hematochezia, etc (López and Chen, 2009). Pharmacokinetics is the study of what the body does to a drug and human's physical state has a close relationship to drug transport in human body (Pfeifers, 1999a). As we all know, diseases including hepatic impairment and kidney impairment have significant impact on the pharmacokinetic characteristic of drugs in human (Pfeifers, 1999b, c). Blood stasis, that is, the decrease of blood flow velocity and the increase of blood viscosity indicates hemorheological abnormalities and pharmacokinetic difference of drugs, so it is necessary to investigate the pharmacokinetics of drugs in patients with blood stasis syndrome. Our previous study indicated that the pharmacokinetic characteristic of HSYA has altered in rats with blood stasis syndrome, as compared to the healthy rats. And the pharmacokinetic character of HSYA in healthy Chinese female volunteers has been reported by our team too (Yang et al., 2009). But, there was no report about whether the blood stasis, this especial pathological condition has impact on the absorption, distribution, metabolism, and excretion of drugs in human body.

The aim of this study was to assess the pharmacokinetic characteristic of HSYA in patients with blood stasis syndrome due to stable angina pectoris (SAP) and healthy volunteers, and to design the rational dose regimen for patients with blood stasis syndrome.

SUBJECTS AND METHODS

This study was conducted in Xijing Hospital, Fourth Military Medical University, China in accordance with the Helsinki Declaration (Kimmelman et al., 2009), and the protocol was approved by the institutional review boards from Xijing Hospital. Written informed consent was obtained from the subjects prior to enrolment.

Study population

This study was conducted in 12 normal subjects and 24 patients with blood stasis syndrome due to SAP. Normal subjects (12) were healthy volunteers. Their health condition was measured based on their medical records and physical examination, vital signs, 24 h dynamic electrocardiography (ECG) monitoring, routine clinical laboratory tests (hematology, serum biochemistry, urinalysis, hepatitis B surface antigen screen, hepatitis C antibody screen, and human immunodeficiency virus (HIV) antibody screen). None consumed alcohol or tobacco. No drug was taken, including contraceptives, within 2 weeks during the test.

Patients (24) with blood stasis syndrome due to SAP were enrolled into the study. The diagnosis of blood stasis syndrome due to SAP was based on: conforming to coronary heart disease angina pectoris with blood stagnation syndrome (CHD-AP-BSS) diagnostic criteria, with over 2 episodes of grades I or II; patients differentiated by hemorheological disorders and ultrasonic diagnosis; all the patients enrolled in this study received ECG examination, both general and exercise tolerance test (except those with contraindication), and the load used in the test should be up to elevating or deepening ST segment by 0.05 mV in changes that occurred in general ECG. The result showed: positive figures of general ECG, either during angina episode or not, with ischemic changes (ST segment lower than ≥ 0.05 mV and/or T wave inverted

and deeper than 0.2 mV in R dominative leads); normal figure of general ECG, but positive figures in submaximal exercise test ECG. Patients were excluded if they: had acute myocardial infarction, hypertension (systolic pressure ≥ 160 mmHg, diastolic pressure ≥ 100 mmHg), severe cardiovascular dysfunction, or severe arrhythmia (rapid atrial fibrillation, atrial flutter, paroxysmal ventricular tachycardia, etc.); underwent complete vascular reconstruction through coronary bridging or intervention therapy; had serious liver, renal or hematopoietic system diseases, or psychic disease.

All procedures were performed in accordance with the principles of the declaration of Helsinki for biomedical research involving human subjects (Kimmelman et al., 2009). All subjects gave written informed consent before enrollment in the study.

Study design

An open-label clinical study was performed at Xijing Hospital, Fourth Military Medical University. Volunteers were allocated into two groups: the normal control group enrolling healthy volunteers and the experimental group enrolling patients with blood stasis syndrome due to SAP. All subjects were required to fast overnight. A single 140 mg dose of HSYA was given to all subjects by intravenous drip of safflower yellow injection diluted with 150 ml 0.9% normal saline (NS) in 60 min. Blood samples (5 ml) were drawn from antecubital vein and collected into tubes at 0.5, 1, 1.25, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 15 h after administration. All samples were separated and kept at -80°C until analysis. All subjects were prohibited from drinking and participating in vigorous exercise for 24 h before dosing until discharge. No food or drinks was allowed for 4 h after HSYA administration. Smoking and consumption of grapefruit and caffeine-containing beverages were also prohibited during hospitalization. All subjects were discharged on the morning of the day after dosing.

Measurement of drug concentrations

HSYA levels in plasma were measured by high-performance liquid chromatography (HPLC). After the addition of 40 μl of 20 $\mu\text{g/ml}$ riboflavin and 40 μl of 50% methanol to a 200 μl plasma sample, the mixture was stirred immediately for 30 s, and 120 μl of 6% perchloric acid was added, then vortexed for 2 min, and was centrifuged at 12,000 rpm for 10 min. The supernatant (50 μl) was injected into the HPLC for analysis.

The HPLC system consisted of a binary pump (LC-10Avp, Tokyo, Japan), an ultraviolet (UV) detector (SPD-10A, Shimadzu Corporation, Kyoto, Japan), and a computer system for data acquisition (LC-Solution). The systems were operated with LC solution (version 1.2.1, Shimadzu Corporation), and signals from the UV detector were recorded using the SPD-10A. The detection wavelength was 403 nm. A Shim-pack CLC-ODS C₁₈ column (150 \times 4.6 mm, inner diameter (i.d.), 5 μm) was used for analysis with a guard column packed with the same packing material. The mobile phase consisted of acetonitrile for pump A, and 0.02 mol/L KH₂PO₄, adjusted to pH 3.0 with ortho-phosphoric acid for pump B. Gradient elution was employed and the gradient program was used as follows: initial 0 min at 10% solvent A; then 0 to 17 min, linear increase from 10 to 22% solvent A. The system was balanced for 5 min by the initial mobile phase (A:B = 10:90) after detecting each sample. The column temperature was 35 $^{\circ}\text{C}$, and the flow rate was 0.8 ml/min.

Concentrations of standard reference samples for the calibration curve were from 0.04 to 20 $\mu\text{g/ml}$ for HSYA. The within-run and between-run accuracy (relative error) ranged from -1.4 to 2.5% and from 1.2 to 6.8%, respectively, and the within-run and between-run precision (percentage coefficient of variation (CV)) ranged from 1.2

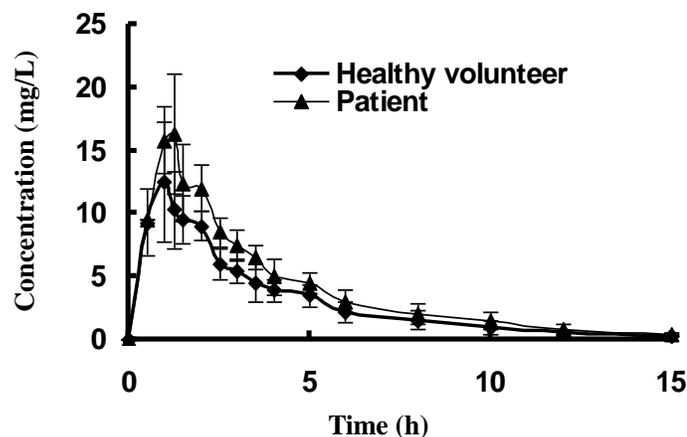


Figure 1. Plasma concentration–time curves of HSYA in 12 healthy volunteers and 24 patients with blood-stasis syndrome due to SAP after administration of 140 mg HSYA injection (mean \pm SD).

to 3.3% and from 4.5 to 6.8%, respectively. Freeze-thaw, short-term, and autosampler stability experiments were evaluated, and these results were within 9.5%.

Pharmacokinetic evaluation

Blood samples (5 ml) were obtained via an indwelling IV cannula inserted into a forearm predose and at 0.5, 1, 1.25, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, and 15 h after drug administration. The samples were centrifuged at 5000 rpm for 10 min, and then were immediately stored in polypropylene tubes at -20°C until required for analysis.

Non-compartmental pharmacokinetic analysis was performed using DAS 2.1 computer program. Actual blood sampling times were used in the pharmacokinetic analysis. C_{max} values in plasma were determined from the measured values, and values for T_{max} were obtained directly from the observed values. Individual plasma concentrations of the terminal phase were fitted to a log-linear line by the least squares method to obtain the elimination rate constant (λ_z).

Values for $t_{1/2}$ were calculated for each individual sample as follows:

$$t_{1/2} = \ln(2)/\lambda_z$$

The individual values for $\text{AUC}_{(0-t)}$ were calculated using the log-linear trapezoidal rule, and $\text{AUC}_{0-\infty}$ values were calculated as follows:

$$\text{AUC}_{(0-\infty)} = \text{AUC}_{(0-t)} + C_{\text{last}}/\lambda_z$$

where C_{last} = the last measurable concentration.

C_{max} and $\text{AUC}_{(0-\infty)}$ were normalized by dose and body weight ($C_{\text{max, norm}}$ and $\text{AUC}_{0-\infty, \text{ norm}}$). Apparent oral clearance (CL/F) was calculated as:

$$\text{dose}/\text{AUC}_{(0-\infty)}$$

Apparent volume of distribution (V) was calculated as

$$(\text{CL}/F)/\lambda_z.$$

RESULTS

Demographic characteristics

Twelve healthy subjects (6 males and 6 females) participated in the study (mean [standard deviation, SD] age, 49 [4.2] years [range, 45 to 60 years]; weight, 68.8 [4.5] kg [range, 52.0 to 82.0 kg] and height, 168.6 [6.5] cm [range, 160 to 178 cm]). Twenty-four patients (12 males and 12 females) with blood stasis syndrome due to stable angina pectoris (SAP) also participated (age, 50 [4.9] years [range, 43 to 60 years]; weight, 60.2 [4.4] kg [range, 52.4 to 67.0 kg] and height, 168.9 [6.4] cm [range, 162.8 to 184.9 cm]). There were no significant differences between healthy volunteers and patients in age, height, and sex.

Pharmacokinetic profiles

HSYA pharmacokinetic profiles are as shown in Figure 1. Pharmacokinetic parameter values are summarized in Table 1. The C_{max} and $\text{AUC}_{(0-\infty)}$ values for HSYA were higher in patients with blood stasis syndrome due to SAP than in healthy subjects. For patients with blood stasis syndrome due to SAP, the elimination $t_{1/2}$ of HSYA was nearly 4.5 h longer than in healthy subjects. Clearance of HSYA was approximately 25% lower for patients with blood stasis syndrome due to SAP than in healthy subjects. It is suggested that blood stasis syndrome has statistically significant effect on the pharmacokinetic variables of HSYA in patients with blood stasis syndrome due to SAP.

Tolerability

A total of 2 adverse reaction events occurred in 36 subjects; the events were mild in severity and were generally short in duration (resolved within 1 day). Dizziness (1 case) was reported in the healthy volunteers group and a feeling of being hot (1 case) was reported in the patients with blood stasis syndrome due to SAP.

No clinically significant changes in physical examination results or vital sign measurements were reported for any subject for the duration of the study. Physical examination results for healthy subjects and patients with blood stasis syndrome due to SAP were normal at all assessments.

DISCUSSION

Blood stasis syndrome, or blood stagnation (Chinese: Xue Yu) is an important underlying pathology for many disease processes according to traditional Chinese medicine (TCM) theory. Described in TCM theory as a slowing or pooling of the blood due to disruption of

Table 1. The pharmacokinetic variables of HSYA in 12 healthy volunteers and 24 patients with blood-stasis syndrome due to SAP after administration of 140 mg HSYA injection (mean \pm SD).

Variable	Patients (n = 24)	Healthy volunteers (n = 12)	P-value	Geometric mean ratio	90% CI
C _{max} (mg/L)	16.3 \pm 4.4	12.5 \pm 4.7	0.04	116.5	105.0 - 134.2
T _{max} (h)	1.3 \pm 0.5	1.0 \pm 0.5	0.40	-	-
t _{1/2} (h)	4.2 \pm 0.8	2.5 \pm 0.2	0.01	-	-
AUC _(0-t) (mg/L/h)	75.2 \pm 10.2	48.3 \pm 12.8	0.001	131.7	116.6 - 153.2
AUC _(0-∞) (mg/L/h)	75.6 \pm 14.3	49.5 \pm 13.5	0.001	132.2	116.5 - 153.7
CL (L/h/kg)	0.25 \pm 0.06	0.35 \pm 0.08	0.04	-	-

Heart Qi, it is often understood in biomedical terms in terms of hematological disorders such as hemorrhage, congestion, thrombosis, and local ischemia (micro clots) and tissue changes (Gunter, 2007). Many researchers suggest that blood stasis syndrome exhibits a close relationship with the development of many diseases, like cardio-cerebral-vascular disease, diabetes II, and even tumor (Xue et al., 2011; Lu et al., 2007; Gouin-Thibault et al., 2001).

Blood stasis, that is, the decrease of blood flow velocity and the increase of blood viscosity indicates hemorheological abnormalities and pharmacokinetic difference of drugs. It is therefore very vital to investigate the pharmacokinetics of drugs in patients with blood stasis syndrome. In this study, the pharmacokinetics of HSYA was different in subjects with blood stasis as compared to healthy subjects. Investigations showed that blood stasis syndrome resulted in the increase of AUC and t_{1/2}. The increase in AUC_(0-∞) was moderate (>40%) and t_{1/2} of HSYA was about double in subjects with blood stasis syndrome as compared to healthy subjects. It also suggests that the rate and extent of drug metabolism was altered in patients with blood stasis syndrome and the bioavailability was significantly higher in patients with blood stasis syndrome than in healthy volunteers. Therefore, it is recommended that the dose of HSYA should be decreased for patients with blood stasis syndrome, and more attention should be paid on the clinical drug usage in patients with blood stasis syndrome, especially the drugs with narrow therapeutic index or severe toxicity to human body, such as digoxin (Wen et al., 2001). Moreover, therapeutic drug monitoring was needed for some patients with kidney damage, liver injury or blood circulation disorders (blood stagnation).

The number of subjects in this study was a relatively small sample and the digoxin pharmacokinetic difference between patients with blood stasis syndrome and normal subjects has been verified by our study team (Wen et al., 2001). Further research is therefore necessary on the pharmacokinetic character of drugs in patients with various types of blood circulation disease, and the relationship between pharmacokinetic characteristic and blood circulation disease needs to be examined further.

Conclusions

This study suggests that the pharmacokinetic profile of HSYA was significantly different between healthy Chinese volunteers and patients with blood stasis syndrome due to SAP. The dosage adjustment is expected to be necessary for patients with serious blood stasis syndrome.

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Full Length Research Paper

A comparison of pre-emptive with preventive epidural analgesia in the patients undergoing major gynecologic surgery

Simin Atashkhoyi*, Mehri Jafari Shobeiri, Alireza Azari Kalejahi and Pouya Hatami Marandi

Department of Anesthesiology, Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

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Pain management is a crucial component in the care of the postoperative patient. Although pre-emptive analgesia is commonly used for the management of postoperative pain, timing the analgesic administration is unclear. This study was designed to compare the efficacy of pre-emptive epidural analgesia (EA) with preventive EA in the patients undergoing major gynecologic surgery. A randomized, double-blinded trial was performed in 50 women of physical status American Society of Anesthesiologists (ASA) 1-3 undergoing major gynecologic surgery. Prior to induction of general anesthesia an epidural catheter was inserted in the patients of the two groups. Patients were allocated randomly into one of two groups; pre-emptive group (n = 25) received 12 ml of 0.125% bupivacaine and 50 µg fentanyl epidurally 20 min before the incision of surgery and the preventive group (n = 25) received the same of agents 20 min before the end of surgery via the epidural catheter. Preventive compared to pre-emptive EA had a significantly increased interval between the analgesic requests (P<0.001). The preventive group compared to the pre-emptive group had significantly decreased postoperative visual analog scale (VAS) in post anesthesia care unit (PACU) and up to 3 h after surgery (P<0.001). A preventive EA before the end of operation provides an improved postoperative analgesia in comparison to pre-emptive EA with no side effects in patients undergoing major gynecologic surgery.

Key words: Gynecologic surgery, postoperative pain, epidural administration, pre-emptive analgesia, bupivacaine, fentanyl.

INTRODUCTION

Peripheral and central nervous system sensitization results in postoperative pain hypersensitivity at the incision site and in surrounding tissues (Updike et al., 2003; Farouk, 2008). "Pre-emptive" analgesia describes the concept of being able to reduce pain perception and overall analgesic needs by using agents to inhibit central nervous system sensitization before the application of painful stimuli (Akural et al., 2002; Hony et al., 2008; Atashkhoyi et al., 2011). "Preventive" analgesia includes any peri-operative analgesic regimen that is able to control pain-induced sensitization of the central nervous system (CNS), hence

reducing both the development and the persistence of pathologic pain (Lavand'homme et al., 2005).

There are studies concerning different routes of pre-emptive analgesia, such as wound infiltration, intravenous, intrathecally or epidurally, that improve anesthetic and analgesic quality (Shapiro et al., 2003; Wilson et al., 2008; Bong et al., 2005). Superior analgesia after different operations was achieved with epidural injection of analgesics compared to other routes of analgesic administration (Zutshi et al., 2005; Klasen et al., 2005; Wu et al., 2000). The timing of epidural analgesic administration (pre-incision, during operation, at emergence from anesthesia or after operation) is also another impact factor of the efficacy of analgesia. Richards et al. (1998) was unable to detect any significant difference in either of

*Corresponding author. E-mail: satashkhoyi@gmail.com.

the outcome of pain measures for the two groups that received epidural local anesthetic with combination of opioid 15 min prior to surgical incision or 15 min prior to skin closure. Katz et al. (2004) showed the short term beneficial effects of preventive epidural analgesia in patients undergoing gynecologic laparotomy. Another study showed that fentanyl administered epidurally before awaking at the end of the elective abdominal surgery versus prior to surgical incision produce lower pain scores (Esmaglu et al., 2001).

In this study, we compared the epidural administration of local anesthetic with combination of fentanyl before the surgical incision (pre-emptive analgesia) or at the end of surgery (preventive analgesia) in reducing the postoperative pain following major gynecologic surgery.

MATERIALS AND METHODS

Fifty female patients were enrolled in this prospective, randomized, and double-blind clinical trial, with approval from the medical Ethics Committee and written consent obtained from the participants. The inclusion criteria were physical status American Society of Anesthesiologists (ASA) I-III, aged between 20 and 80 years undergoing elective major gynecologic surgery. Exclusion criteria were contraindication for epidural puncture, known allergy to local anesthetics, the patients consumed opioids and non-steroidal anti-inflammatory drugs (NSAIDs) preoperatively, and patients with physiological and psychological disorders.

Patients were pre-medicated with oral 5 mg diazepam 1 h before operation. Before the induction of anesthesia, an epidural catheter was placed at the L₃₋₄ intervertebral space in the patients. Patients were allocated randomly into one of two groups; patients in the pre-emptive group (n=25) received 12 ml of 0.125% bupivacaine (Bupivacaine mylan, Delpharm, France) and 50 µg fentanyl (Aburaihan Pharmaceutical, Iran) epidurally 20 min before the incision of surgery. Patients in preventive group (n = 25) received the same agents 20 min before the end of surgery via the epidural catheter. General anesthesia (GA) was induced in all patients with fentanyl 1 - 2 µg/kg, thiopentone 4 mg/kg, and succinylcholine 0.6 mg/kg was used to facilitate tracheal intubation. Anesthesia was maintained with nitrous oxide (N₂O) 50% in oxygen (O₂), isoflurane 0.5 - 1 minimum alveolar concentration (MAC), and pancronium 0.05 mg/kg. Fentanyl (1 µg/kg intravenous (i.v.)) was used as supplement of analgesia during surgery, if necessary. At the end of surgery, patients were transferred to post anesthesia care unit (PACU) and then referred to the intensive care unit (ICU). All patients received patient-controlled epidural analgesia (PCEA) with 0.125% of bupivacaine (1.25 mg/ml) and 5 µg/ml fentanyl delivered with a patient controlled analgesia (PCA) device (Abbott Pain Management Provider; Abbott Laboratories, North Chicago, IL) for 24 h postoperatively. No background infusion was used in either group. PCA pump was programmed to deliver 2 ml bolus with a lockout interval of 15 min in both groups. If analgesia was inadequate and visual analogue scale (VAS) >4 for 30 min, meperidine (0.5 - 1 mg/kg every 6 h) was given intramuscularly as rescue measure of pain management.

Intraoperative monitoring consisted of electrocardiogram (ECG), pulse oximetry, end tidal CO₂ concentration (ETCO₂), urine output and hemodynamic variables. Hydration was achieved with lactated Ringer's solution. Three surgeons performed the surgical procedures, and the anesthesia was provided by two anesthesiologists who were responsible for monitoring of anesthesia and postoperative analgesia management. The surgeons, second anesthesiologist (Azari A) and patients were blinded to the study.

All study-related measurements were taken by the same anesthesiologist who was not aware of the treatment allocation of the patients. Pain scores on a visual analogue scale (VAS; 0= no pain, 10= worst pain imaginable) were recorded in the PACU (at emergence and during 24 h post-operatively). First request to analgesia, total analgesic consumption (epidurally or systemically administered drugs) and the presences of side effects (nausea-vomiting, drowsiness, dizziness, and respiratory depression) were also recorded during the first 24 h after operation.

The sample size was based on a power calculation which showed that 50 patients were necessary to achieve 80% power to detect a prolongation of postoperative first analgesic demand of 3 h between patients treated with pre-emptive or preventive epidural analgesia with $\alpha = 0.05$. Data were presented as mean \pm SD. Demographic data, dose of intra-operative fentanyl, duration of surgery and anesthesia, IV fluid were compared using the student *t* test. Postoperative pain scores and incidence of side effects were compared by χ^2 - square test between two groups. A *p* value ≤ 0.05 was considered statistically significant.

RESULTS

There were no significant differences between the groups with regards to age, weight, height, duration of surgery, type of surgery, and ASA status of classification (Table 1). Moreover, there were no significant differences between the two groups regarding to intraoperative data such as intravenous fluid, urine output, and blood loss (Table 1). Intraoperative fentanyl consumption was lower in the pre-emptive group than those in the preventive group (128.00 \pm 38.40 vs. 158.00 \pm 37.30 µg; *p*= 0.007, Table 1). The hemodynamic changes during anesthesia were presented in Figure 1. Decreased of mean arterial pressure (MAP) and heart rate (HR) occurred more often in the pre-emptive group than in the preventive group, although this difference was not significant.

Additionally, the VAS was significantly lower for the patients in the preventive group at emergence (*p*<0.001), and up to 3 hours postoperatively (*p*<0.001) than those in the pre-emptive group, but not at 6, 12, and 24 h after surgery (Table 2 and Figure 1). Table 2 shows that the time to first request for analgesics were significantly longer in the preventive group (5.06 \pm 2.54 vs. 2.44 \pm 1.58 h; *p*<0.001). The frequency of rescue epidural analgesia by using the PCEA was significantly lower in preventive group than those in the patients of pre-emptive group (7 in preventive group vs. 12 in pre-emptive group; *p*<0.001). There were no differences in the consumption of additional analgesics (meperidine) between two groups. Also, there were no differences in hemodynamic or respiratory instability among patients of two groups during 24 h post operation. Other post-operative side effects also were absent in both groups. Furthermore, no patient experienced motor block during postoperative period.

DISCUSSION

The results of the present study indicate that epidural

Table 1. Patient's characteristics and intraoperative variables in the two groups [number (%) and mean \pm SD].

Parameter	Pre-emptive group (n = 25)	Preventive group (n = 25)	p-value
Age (years)	56.24 \pm 10.45	56.16 \pm 16.87	0.98
Weight (kg)	73.12 \pm 15.91	70.80 \pm 12.67	0.57
Height(cm)	159.36 \pm 07.33	159.84 \pm 07.00	0.81
ASA (I/II/III)	12/6/7	13/7/5	0.42
Duration of surgery(min)	171.00 \pm 46.02	191.40 \pm 40.22	0.10
Type of surgery (%)			
Lymphadenectomy	15(60)	16(64)	
Abdominal hysterectomy	8(32)	7(28)	0.76
Vaginal hysterectomy	2(8)	2(8)	
Total fluid during anesthesia (ml)	3240.00 \pm 737.67	3484.00 \pm 655.54	0.22
Intraoperative blood loss (ml)	304.00 \pm 113.57	345.00 \pm 121.55	0.87
Urine output (ml)	594.00 \pm 323.01	604.80 \pm 309.75	0.6
Intraoperative fentanyl consumption (μ g)	128.00 \pm 38.40	158.00 \pm 37.30	0.007
Intraoperative hypotension	0	0	1.0
Intraoperative bradycardia	0	0	1

Table 2. Postoperative pain variables in two groups [number (%) and mean \pm SD].

Parameter	Pre-emptive (n = 25)group	Preventive (n = 25)group	p-value
Mean VAS score			
in PACU	1.84 \pm 0.34	0.16 \pm 0.07	<0.001
3 h later	4.44 \pm 0.30	1.82 \pm 0.39	<0.001
6 h later	3.00 \pm 1.50	2.78 \pm 1.62	0.11
12 h later	2.16 \pm 1.14	2.56 \pm 1.63	0.32
24 h later	1.28 \pm 0.28	1.24 \pm 0.29	0.87
Time to first analgesic request (h)	2.44 \pm 1.58	5.06 \pm 2.54	<0.001
Number of PCEA rescue	7	12	<0.001

injection of bupivacaine and fentanyl 20 min before the end of surgery (preventive) provided better analgesia, which was referred as lower pain scores, longer duration of the first analgesic request, and lower postoperative analgesic consumption during the first 24 h after major gynecologic surgery compared with pre-incisional (pre-emptive) epidural analgesia. Although some studies have shown that administration of local or regional anesthesia just minutes before surgical procedures seems to be ineffective for prevention of postoperative pain (Updike et al., 2003), most studies succeeded to show the benefits pre-emptive analgesia by intravenous or epidural analgesia (Farouk, 2008; Akural et al., 2002; Hony, 2008). Studies have also showed that long-lasting (on the day before operation) pre-emptive epidural analgesia with local anesthetic and opioid reduced postoperative consump-

tion of analgesics with improvement pain rating (Wu et al., 2000; Richards et al., 1998) were unable to detect any significant difference in either of the postoperative pain outcomes for the pre-incisional epidural analgesia when it was compared with injection of epidural analgesics at the end of surgery. Others reported that analgesics administered epidurally prior awaking at the end of operation is more effective for postoperative pain control than those given before the induction of anesthesia (Lavand'homme et al., 2005; Katz et al., 2004; Esmaoglu et al., 2001; Gottschalk et al., 2008).

The present study findings demonstrates that injection of analgesics epidurally prior the end of surgery effectively suppresses all possible nociceptor transduction pathways, preventing central sensitization and improving postoperative pain management. Epidural anesthesia

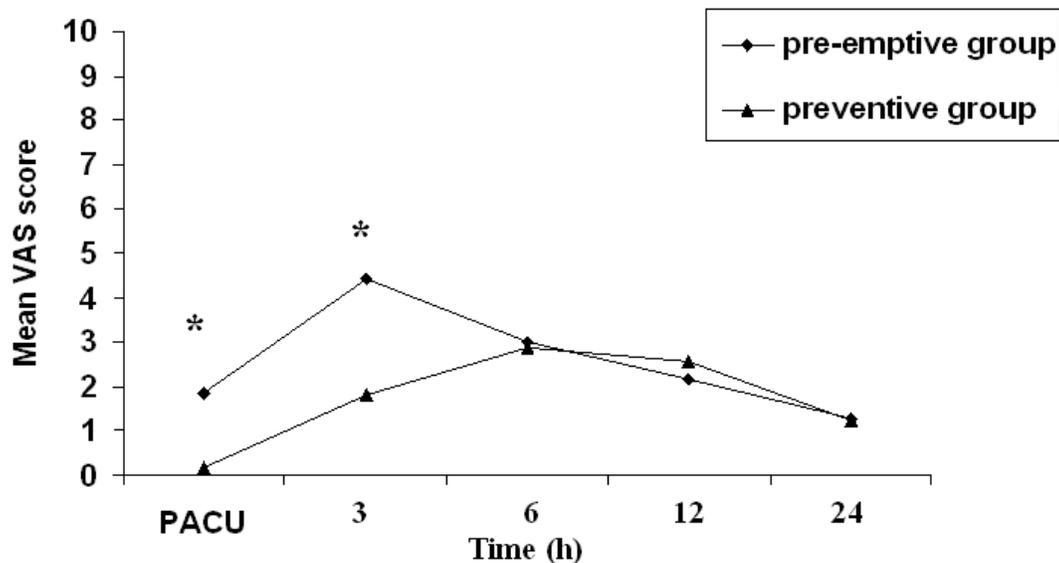


Figure 1. Visual analog scale (VAS) at each time interval of the study (* $p < 0.001$).

with bupivacaine blocks the sensory input of surgical stimuli (Wu et al., 2000). Fentanyl activates the opioid receptors and suppresses the initial response of dorsal horn nociceptive neurons and C-fiber stimulation (Akural et al., 2002). One possible explanation for our findings is that administration of opioids as premedication or during induction and maintenance of anesthesia may provide pre-emptive analgesia (Bong CL et al., 2005).

There are a number of limitations to this study: First, the study limited assessment of postoperative analgesia to the first 24 postoperative hours. Secondly, the study was not large enough to assess safety. Thirdly, we did not control group (epidural injection of placebo as pre-emptive and preventive), and the highest reported pain score was below 5 in this study (Table 2).

Conclusion

A preventive epidural analgesia with bupivacaine and fentanyl before the end of operation provides an improved postoperative analgesia in comparison to pre-emptive epidural injection of same agents with no side effects in patients undergoing major gynecologic surgery. Further trials are necessary to evaluate preventive analgesia on chronic postoperative pain.

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Full Length Research Paper

Bapedi phytomedicine and their use in the treatment of sexually transmitted infections in Limpopo Province, South Africa

Sebua Silas Semanya^{1*}, Marthienus Johannes Potgieter¹ and Lourens Johannes Christoffel Erasmus²

¹Department of Biodiversity, School of Molecular and Life Sciences, University of Limpopo, Private Bag X1106, Sovenga 0727, South Africa.

²Department of Physiology and Environmental Health, School of Molecular and Life Sciences, University of Limpopo, Private Bag X1106, Sovenga 0727, South Africa.

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Thirty four traditional healers from 17 municipalities, covering three districts of the Limpopo Province, were interviewed during the first half of 2011. Forty seven plant species belonging to 32 families, mostly from the Asteraceae (9%), Asphodelaceae, Fabaceae and Hyacinthaceae (6% for each) were used to treat sexually transmitted infections (STIs) such as gonorrhoea, HIV/AIDS, nta (unspecified venereal disease - Bapedi terminology) and syphilis. Eighty seven percent of the species were used to treat a single STI, with the remainder being used to treat two STIs. Double-used species include: *Aloe marlothii* (gonorrhoea and chlamydia), *Callilepis salicifolia* (gonorrhoea and HIV/AIDS), *Cucumis myriocarpus* (gonorrhoea and syphilis), *Drimia elata* (gonorrhoea and HIV/AIDS), *Hypoxis hemerocallidea* (gonorrhoea and HIV/AIDS) and *Ziziphus mucronata* (gonorrhoea and nta). Diagnosis of STIs by Bapedi traditional healers is based primarily on the presentation of symptoms and certain behavioural traits, which are not always accurate indicators. The present study concludes that Bapedi traditional healers' knowledge can lead to useful medicinal plants to manage and treat STIs. Furthermore, given the necessary health information and support, these healers could play an important role in the management and treatment of STIs in the Limpopo Province.

Key words: Traditional healers, ethnobotany, medicinal plants, sexually transmitted infections

INTRODUCTION

Sexually transmitted infections (STIs) are a major public health concern in developing countries. According to World Health Organization (1991), *Treponema pallidum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Trichomonas vaginalis* are the parasitic pathogens responsible for most STIs. These infections respond well to treatment with antibiotics. However, globally, there are over 25 STIs, some of which have serious and permanent health problems when left untreated, while many facilitate the spread of HIV/AIDS infections

(Paavonen, 2004).

In southern Africa, transmission rates are reaching epidemic proportions where STIs are currently one of the highest in the world (Van Vuuren and Naidoo, 2010). In South Africa, 26% of all deaths during the year 2000 were as a result of STIs (Johnson et al., 2000). Sexually transmitted infections are one of the most common reasons for people to visit traditional healers in South Africa. According to Msiska et al. (1997) rural patients are more dependent on traditional medicine from healers for STIs because of hesitancy to relate this form of illness to unknown doctors and being examined by a member of the opposite sex in western treatments. A survey by Peltzer (2003) found that among rural adult South

*Corresponding author. E-mail: Sebuasemenya@gmail.com.

Africans who had STIs in the past 12 months, 36% did consult a traditional healer for treatment.

These healers do not have access to laboratory services and rely on the presence of symptoms and certain behavioural traits to assist them in their diagnosis of STIs (Kambizi and Afolayan, 2001). In cases of symptomatic presentation the occurrence of one or more of the following forms part of the WHO syndromic management guidelines: Abnormal urethral discharge, dysuria or ulcers in the genital area (Johnson et al., 2011). These guidelines aim to treat STI patients according to their symptoms, are in line with the approach followed by traditional healers.

Traditional healers use medicinal plants as their primary source of medicine to treat STIs. Significant literature exists in support of herbal remedies being used to treat STIs by traditional healers of different cultures in Africa (Ndubani and Hojer, 1999; Kambizi and Afolayan, 2001; Chigora et al., 2007; Ssegawa and Kasenene, 2007; Kamatenesi-Mugisha et al., 2008; Kayode and Kayode, 2008; Njoroge and Bussmann, 2009; Hossan et al., 2010; Chinsembu and Hedimbi, 2010; Namukobe et al., 2011; Maroyi, 2011; Muthee et al., 2011). South Africa is no exception and studies such as Samie et al. (2005); Tshikalange et al. (2005); Amusan et al. (2005); Mulaudzi et al. (2011); De Wet et al. (2012) highlight this. The extensive documentation of the plant use by a significant number of cultures around the world has led to extensive knowledge of the used plants' chemistry and pharmacological effects (Alam et al., 2012; Asgarpanah and Ramezanloo, 2012; Nasri et al., 2012). It is thus unfortunate that one of the great ethnic groups in South Africa, the Bapedi, has received no attention regarding their *materia medica* for STIs. The aim of this study was to document medicinal plants used by Bapedi traditional healers to treat STIs in the Limpopo Province, South Africa.

MATERIALS AND METHODS

The study area and population

The present study was carried out in 17 local municipalities (Table 1) of the Limpopo Province, covering the three of the five districts (Capricorn, Sekhukhune and Waterberg) that constitute the Limpopo province (Figure 1). The vegetation in these districts was classified by Acocks (1988) as arid-semi savannas. It is characterized by a mixture of trees, shrubs and grasses (Mucina and Rutherford, 2006). This type of vegetation has provided a diverse flora with rich medicinal plants that the people of the study areas have always used to treat many illnesses.

The surveyed districts are inhabited by Black people mostly from the Bapedi, Vhavenda and VhaTsonga ethnic groups, as well as coloured (mixed-race group) and white people. The Bapedi ethnic group constitutes the largest cultural group in the Limpopo Province (South Africa), comprising 57% of the total provincial population (Limpopo Provincial Government, 2012). This ethnic group use herbal medications either alone or in combination with orthodox medicines for the treatment of several diseases (Semenya et al., 2012). Majority of the Bapedi people in the studied districts are rural

dwellers, hence the use of plants for the treatment of common diseases, such as STIs which is very prevalent.

Ethnobotanical survey

A reconnaissance survey was done in each local municipality to: (i) obtain permission to conduct this study within their area of jurisdiction, and (ii) to meet with the traditional healers to request them to participate in the study. Information was collected from January 2011 to July 2011. Semi-structured interviewees, observation and guided field walks with traditional healers were employed to obtain ethnobotanical data.

Semi-structured questionnaires were completed by 34 traditional healers from 17 local municipalities. In each local municipality two traditional healers were randomly selected and the objective of the study was explained in Sepedi, the local language. Interviews were designed to gather data on the plants used to treat STIs, methods of preparation, administration of medicine and diagnoses of STIs. Field observations were made on the morphological features and habitats of each medicinal plant species in the field. Based on ethnobotanical information provided by traditional healers, specimens were collected, numbered, pressed and dried for identification at the University of Limpopo's Larry Leach Herbarium.

Data analysis

The recorded data were organised and analysed for descriptive statistical patterns with Microsoft Excel spreadsheet software. Descriptive statistics, such as percentages and frequencies, have been used to analyse the data obtained from the questionnaires.

RESULTS

Sexually transmitted infection identification

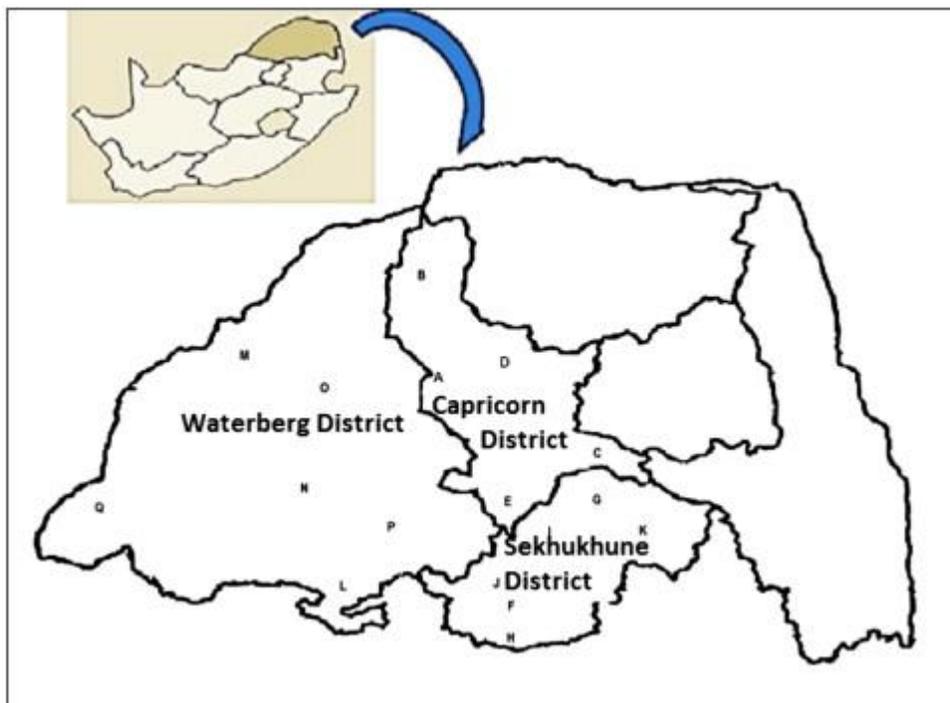
Five seemingly different STIs are treated by Bapedi traditional healers. These include gonorrhoea, chlamydia, HIV/AIDS, *nta* and syphilis. Not all of these STIs are treated by all the healers. In fact none of the traditional healers from a single municipality indicated that they treat all five-listed STIs. For example, two traditional healers from the Elias Motsoaledi municipality treat four of the five STIs, the exclusion being syphilis. Traditional healers from the Capricorn district treated only gonorrhoea and chlamydia; whilst those from the Sekhukhune district treat all the infections among them, and in the Waterberg district the focus was on chlamydia, gonorrhoea and HIV/AIDS.

Among the three districts general consensus regarding the presentation and identification of gonorrhoea was reached. All traditional healers agreed that behaviour such as unprotected sexual intercourse with many partners or an infected partner will result in being infected. The only symptomatic presentation used during the diagnosis was the presence of a smelly urethral discharge.

None of the healers from the Capricorn district treated HIV/AIDS. Therefore information regarding this disease was obtained only from Sekhukhune and Waterberg districts. Only one traditional healer (Modimolle municipa-

Table 1. Districts and local municipalities included in this study.

Capricorn district		Sekhukhune district		Waterberg district	
Aganang	A	Elias Motsoaledi	F	Bela-Bela	L
Blouberg	B	Fetakgomo	G	Lephalale	M
Lepelle-Nkumpi	C	Groblersdal	H	Modimolle	N
Molemole	D	Makhuduthamaga	I	Mogalakwena	O
Polokwane	E	Marble Hall	J	Mookgophong	P
		Tubatse	K	Thabazimbi	Q

**Figure 1.** Study area: Capricorn, Waterberg and Sekhukhune districts, Limpopo Province, South Africa. A to Q designates the involved municipalities.

lity) indicated exposure to contaminated blood as a source of infection. The remainder identified sexual intercourse with the partner of a person who died from HIV/AIDS as the leading cause of contracting this disease. Overwhelming support was observed for the occurrence of sudden and dramatic weight loss, which was, according to the healers, positively associated with HIV/AIDS.

Chlamydia was only treated by traditional healers from five local municipalities (Blouberg, Lepelle-Nkumpi, Polokwane, Elias Motsoaledi and Lephalale). General agreement has it that sexual intercourse with either a menstruating woman or a woman pregnant with another man's child will result in contracting this disease. These patients have an abnormal gait and an inability to urinate.

Information regarding the identification of *nta* is lacking as only one traditional healer from the Elias Motsoaledi municipality treats it. Unprotected sexual intercourse with

many partners was given as the reason for infection. A similar situation occurred regarding syphilis. Only one traditional healer from the Groblersdal municipality treats this disease. Once again unprotected sexual intercourse with many partners was a key factor in identification of the disease. However, this behavioural trait was complemented by the presence of a measles-like rash on the genitals.

Plants used to treat sexually transmitted infections

Bapedi traditional healers used 47 species of plants to treat chlamydia, gonorrhoea, HIV/AIDS, *nta* and syphilis (Table 2). These species belong to 43 genera and 32 families. The most representative families were Asteraceae (9%) followed by the Asphodelaceae, Fabaceae and Hyacinthaceae (6% each).

Table 2. Species and parts: Extract preparation, administration and dosages used by Bapedi traditional healers to treat sexually transmitted infections.

Species name	Voucher numbers	Botanical Family	Vernacular name	Used part/s	Preparation, dosage and administration	STIs treated	Citation No. (%)
<i>Aloe arborescens</i> Mill.	SS 59	Asphodelaceae	Kgopha-ya-fase	Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Aloe falcata</i> Baker	SS 330	Asphodelaceae	Kgopha	Root	Mixed with <i>E. crispera</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
				Root	Boiled singly for 20 minutes or mixed with <i>D. sylvatica</i> (bulb) and boiled for 20 minutes. One tin cup of either extract taken orally. Thrice a day	Gonorrhoea	
<i>Aloe marlothii</i> A. Berger subsp. <i>marlothii</i>	SS 80	Asphodelaceae	Kgopha-ya-go-ema	Root & leaf	Mixed and boiled for 15 minutes. One tin cup of the extract taken orally. Thrice a day		24%
				Leaf	Boiled for 10 minutes and one tin cup of warm extract is administered by healer (via a bulb syringe). Once a day	Chlamydia	
<i>Alternanthera pungens</i> Kunth	SS 402	Amaranthaceae	Mosweetswe	Tuber	Macerated in cow's milk for 24 hours and one tin cup of the decoction taken orally. Thrice a day	Gonorrhoea	3%
<i>Boscia albitrunca</i> (Burch.) Gilg & Gilg-Ben.	SS 300	Capparaceae	Mohlophi	Root	Mixed with <i>E. elephantina</i> (root), <i>P. ciliatus</i> (root) and <i>P. africanum</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Burkea africana</i> Hook.	SS 60	Leguminosae	Monatlo	Root	Mixed with <i>C. verum</i> (root), <i>H. hemerocallidea</i> (tuber) and <i>G. aspera</i> (entire plant). Boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Caesalpinia decapetala</i> (Roth) Alston.	SS 74	Fabaceae	Mokgabane	Root	Boiled for 10 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	3%
<i>Callilepis salicifolia</i> Oliv.	SS 62	Asteraceae	Phelana	Tuber	Boiled for 5 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	6%
				Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	
<i>Carica papaya</i> L.	SS 70	Caricaceae	Mophopho "wapoo"	Root	Mixed with <i>C. myriocarpus</i> (tuber) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Gonorrhoea	3%
<i>Catharanthus roseus</i> (L.) G. Don	SS 33	Apocynaceae	Lepolomo-le-le-pinki-la <i>drop</i>	Root	Boiled for 5-20 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	82%
<i>Cinnamomum verum</i> J. Presl	SS 337	Lauraceae	Mokwere-kwere-o-mogolo	Root	Mixed with <i>B. africana</i> (root), <i>H. hemerocallidea</i> (tuber) and <i>G. aspera</i> (entire plant). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Citrullus lanatus</i> (Thunb.) Matsum. & Nakai	SS 09	Cucurbitaceae	Morotse	Root	Mixed with <i>D. viscosa</i> (root) and <i>E. crispera</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Cotyledon orbiculata</i> L.	SS 37	Grassulaceae	Tsebe ya kolobe	Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day.	Gonorrhoea	3%
					Macerated in warm water for 24 hours. One tin cup of decoction taken orally. Thrice a day		
<i>Cucumis myriocarpus</i> subsp. <i>leptodermis</i>	SS 35	Cucurbitaceae	Magapyana	Tuber	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	Syphilis	6%
					Mixed with <i>C. papaya</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Gonorrhoea	
<i>Dioscorea sylvatica</i> var. <i>brevipes</i> .	SS 11	Dioscoreaceae	Monamela	Bulb	Mixed with <i>A. marlothii</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Gonorrhoea	3%
<i>Dodonaea viscosa</i> var. <i>angustifolia</i>	SS 117	Sapindaceae	Mofenshe	Root	Mixed with <i>C. lanatus</i> (root), and <i>E. crispera</i> (root). Boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
					Boiled for 10 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	
<i>Drimys elata</i> Jacq.	SS 18	Hyacinthaceae	Sekanama	Bulb	Mixed with <i>E. transvaalense</i> , <i>E. elephantina</i> (root), <i>S. birrea</i> (bark), <i>Z. capense</i> (root) and <i>S. viminale</i> (twigs). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	6%
<i>Elaeodendron transvaalense</i> (Burt Davy) Rott. Archer	SS 32	Celastraceae	Monamane	Root	Mixed with <i>D. elata</i> (bulb), <i>E. elephantina</i> (root), <i>S. birrea</i> (bark), <i>Z. capense</i> (root) and <i>S. viminale</i> (twigs). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%

Table 2. Contd.

<i>Elephantorrhiza elephantina</i> (Burch.) Skeels	SS 100	Leguminosae	Mosehlana/ moshisane	Root	Mixed with <i>P. africanum</i> (bark) and boiled for 20 minutes One tin cup of the extract taken orally. Thrice a day Mixed with <i>D. elata</i> (bulb), <i>S. birrea</i> (bark), <i>E. transvaalense</i> (root), <i>Z. capense</i> (root) and <i>S. viminale</i> (twigs). Boiled for 20 minutes One tin cup of the extract taken orally. Thrice a day Mixed with <i>B. albitrunca</i> (root), <i>P. ciliatus</i> (root) and <i>P. africanum</i> (root). Boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day.	HIV/AIDS	9%
<i>Euclea crispa</i> subsp. <i>crispa</i>	SS 57	Ebenaceae	Mokwerekwere	Root	Mixed with <i>A. falcata</i> (root) and boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day Mixed with <i>C. lanatus</i> (root) and <i>D. viscosa</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	6%
<i>Eucomis pallidiflora</i> subsp. <i>pole-evansii</i>	SS 355	Hyacinthaceae	Mathuba-difala	Entire plant	Mixed with <i>Z. mucronata</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	Chlamydia	3%
<i>Euphorbia maleolens</i> E. Phillips	SS 34	Euphorbiaceae	Rofa-bja-Tau	Entire plant	Mixed with <i>Triumfetta</i> spp. (root) and <i>Z. humile</i> (root). Pounded and five teaspoons taken orally with soft porridge. Thrice a day Mixed with <i>M. flabellifolius</i> (entire plant) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	HIV/AIDS	12%
<i>Geigeria aspera</i> Harv. var. <i>aspera</i>	SS 310	Asteraceae	Makgonatsohle	Entire plant	Mixed with <i>B. africana</i> (root), <i>C. verum</i> (root), <i>H. hemerocallidea</i> (tuber) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Gethyllis namaquensis</i> (Schonland) Oberm.	SS 83	Amaryllidaceae	Naka tsa tholo	Bulb	Macerate in warm water for 24 hours. One tin cup of the extract taken orally. Thrice a day	Chlamydia	3%
<i>Helichrysum caespitium</i> (DC.) Harv.	SS 78	Asteraceae	Bokgatha/Mabjana/Mmeetse	Entire plant	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day Mixed with <i>S. italica</i> (root) and pounded. Five teaspoons taken orally with a cup of warm water. Thrice a day	Gonorrhoea	3%
<i>Hypoxis hemerocallidea</i> (Fisch.) Mey. & Avé-Lall	SS 115	Hypoxidaceae	Titikwane/ sesogadi	Tuber	Pounded and five teaspoons taken orally with soft porridge. Thrice day Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day Mixed with <i>B. africana</i> (root), <i>C. verum</i> (root) and <i>G. aspera</i> (entire plant). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	12%
<i>Hypoxis obtusa</i> Burch. ex Ker Gawl.	SS 336	Hypoxidaceae	Monna maledu	Tuber	Mixed with <i>Z. mucronata</i> (root) and pounded. Five teaspoons taken orally in a cup of warm water. Thrice a day	Chlamydia	3%
<i>Ipomoea obscura</i> var. <i>obscura</i>	SS 200	Convolvulaceae	Kgomodimaswi	Root	Boiled for 5-20 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	1%
<i>Jatropha zeyheri</i> Sond.	SS 120	Euphorbiaceae	Unknown	Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	3%
<i>Kleinia longiflora</i> DC.	SS 217	Asteraceae	Lekgabolo/ motlalamaswi	Root	Boiled for 20 minutes and one tin cup of warm extract is administered by healer via bulb syringe. Once a day	Chlamydia	3%
<i>Myrothamnus flabellifolius</i> Welw	SS 111	Myrothamnaceae	Boka	Entire plant	Mixed with <i>E. maleolens</i> (entire plant) and pounded. Five teaspoons orally with soft porridge. Thrice daily for a week Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Opuntia ficus-indica</i> Mill.	SS 90	Cactaceae	Motloro	Root	Mixed with <i>Z. macronata</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Gonorrhoea	6%
<i>Pelargonium</i> spp.	SS 04	Geraniaceae	Selumi	Root	Boiled for 20 minutes and undisclosed volume of the extract taken orally. Thrice a day Pounded and six teaspoons taken orally with either warm water or porridge. Thrice a day	HIV/AIDS	3%

Table 2. Contd.

<i>Peltophorum africanum</i> Sond.	SS 13	Caesalpinaceae	Mosehla	Root	Mixed with <i>B. albitrunca</i> (root), <i>E. elephantina</i> (root) and <i>P. ciliatus</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	6%
				Bark	Mixed with <i>E. elephantina</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day		
<i>Plectranthus ciliatus</i> E. Mey. ex. Benth.	SS 322	Lamiaceae	Sehla sa pelo	Root	Mixed with <i>B. albitrunca</i> (root), <i>E. elephantina</i> (root), and <i>P. africanum</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Protea caffra</i> subsp. <i>caffra</i>	SS 341	Proteaceae	Unknown	Seeds	Pounded and six teaspoons taken orally in a cup of warm water. Thrice a day for a week.	Chlamydia	3%
<i>Sansevieria hyacinthoides</i> (L.) Druce	SS 199	Dracaenaceae	Makgotse	Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Sarcostemma viminale</i> subsp. <i>orangeanum</i>	SS 106	Apocynaceae	Mokwere-kwere- o-mogolo	Twigs	Mixed with <i>D. elata</i> (bulb), <i>E. elephantina</i> (root), <i>S. birrea</i> (bark), <i>E. transvaalense</i> (root) and <i>Z. capense</i> (root). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Sclerocarya birrea</i> sub sp. <i>birrea</i>	SS 01	Anacardiaceae	Morula	Bark	Mixed with <i>D. elata</i> (bulb), <i>E. elephantina</i> (root), <i>E. transvaalense</i> (root), <i>Z. capense</i> (root) and <i>S. viminale</i> (twigs). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
<i>Searsia lancea</i> (L.F.) F.A. Barkley	SS 227	Hyacinthaceae	Mokalabata/ Motshakhutshakhu	Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	Nta	3%
				Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day		
<i>Senna italica</i> subsp. <i>arachoides</i>	SS 321	Caesalpinaceae	Mankgane/sebetsane	Root	Mixed with <i>H. hemerocallidea</i> (tuber) and pounded. Five teaspoons taken orally with a cup of warm water. Thrice a day	Gonorrhoea	6%
<i>Solanum panduriforme</i> E. Mey.	SS 85	Solanaceae	Mothola-ye- serolwane	Fruits	Chopped and macerated in warm water for 24 hours. One tin cup of decoction administered by healer. Twice a day	Gonorrhoea	6%
<i>Tribulus terrestris</i> L.	SS 409	Zygophyllaceae	Mosehlo	Entire plant	Mixed with <i>Z. mucronata</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Chlamydia	3%
<i>Triumfetta</i> spp.	SS 64	Tilliaceae	Unknown	Root	Mixed with <i>E. maleolens</i> (entire plant) and <i>Z. humile</i> (root). Pounded and five teaspoons taken orally with soft porridge. Thrice a day	HIV/AIDS	3%
				Root	Mixed with <i>H. obtusa</i> (tuber) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day		
				Root	Mixed with <i>T. terrestris</i> (entire plant) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day		
<i>Ziziphus mucronata</i> Wild.	SS 12	Rhamnaceae	Mokgalo	Root	Mixed with <i>E. pallidiflora</i> (bulb) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day	Chlamydia	18%
				Root	Mixed with <i>O. ficus-indica</i> (root) and boiled for 20 minutes. One tin cup of the extract taken orally. Thrice a day		
				Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	Gonorrhoea	
<i>Zanthoxylum capense</i> (Thunb.) Harv.	SS 511	Rutaceae	Senokomaropa	Root	Mixed with <i>D. elata</i> (bulb), <i>E. elephantina</i> (root), <i>S. birrea</i> (bark), <i>E. transvaalense</i> (root) and <i>S. viminale</i> (twigs). Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day	HIV/AIDS	3%
				Root	Boiled for 20 minutes and one tin cup of the extract taken orally. Thrice a day		
<i>Zanthoxylum humile</i> (E.A.Bruce) P.G. Waterman	SS 19	Rutaceae	Monokwane	Root	Mixed with <i>E. maleolens</i> (entire plant) and <i>Triumfetta</i> spp. (root). Pounded and five teaspoons taken orally with soft porridge. Thrice a day	HIV/AIDS	6%

Table 2 indicate that 25 plant species are used to treat HIV/AIDS, 18 to treat gonorrhoea, eight to treat chlamydia, and one to treat nta and syphilis each. Twenty eight species were documented in the Waterberg district, 17 in the Sekhukhune

district, and 12 in the Capricorn district. Eighty seven percent of the 47 recorded species are used to treat a single STI (Table 2), with just 13% used to treat two STIs, these include *Aloe marlothii* (gonorrhoea and chlamydia), *Callilepis*

salicifolia (gonorrhoea and HIV/AIDS), *Cucumis myriocarpus* subsp. *leptodermis* (gonorrhoea and syphilis), *Drimia elata* (gonorrhoea and HIV/AIDS), *Hypoxis hemerocallidea* (gonorrhoea and HIV/AIDS) and *Ziziphus mucronata* (gonorrhoea

and *nta*). With the exception of *Catharanthus roseus* (82%), used to treat gonorrhoea, no other species were used with the same consistency by traditional healers in the three districts.

Plant parts used in remedy preparation

Roots (58%) were mostly used to prepare remedy for STIs, followed by entire plant (12%), tuber (10%), bulb (6%), leaf and bark (4% each), seed, twig and fruit (2% each). When herbs are used to prepare remedies it is mostly their tubers or roots; however, in some cases the entire plant can be used. Roots are also the preferred plant part when shrubs are used to prepare herbal remedies. In rare cases the entire plant are used. It is notable that the roots are mostly used when plant material is collected from trees, with very little focus on bark, leaves, seeds or twigs. Bulbs and tubers are mostly obtained from perennial geophytes.

Single vs. multiple plant extracts

Twenty nine single plant extract and 40 multi plant extract preparations were recorded (Table 2). Nine plant species were used in single and multi-extract preparations. Among the multi extracts, 67% of healers employ two species, 13% three species, 13% four species and 7% six species. It is noteworthy that all extracts exceeding two species are used to treat HIV/AIDS. Twenty five of the 40 multi extract preparations are used to treat HIV/AIDS, seven to treat gonorrhoea and chlamydia.

Preparation, administration and dosage of remedy

Various preparation methods, such as boiling, pounding and maceration are used by Bapedi traditional healers. There is a clear preference for boiling, as almost 80% of all preparations are boiled. The boiling of plant materials ranges from 5 to 20 minutes (depending on an individual healer).

Often the traditional healers opted to pound (14%) the plant material, whereas only a small number used maceration (6%). These healers use either stones or crushing metals. Maceration is normally done over a period of 24 hours. The maceration medium might differ, as some healers use warm water whereas other healers use cow's milk at room temperature.

This study clearly illustrate that oral self-administration (96%) is the method of choice. However, sometimes especially with rectal administration (enema) (4%), via bulb syringe, the healers did the administration. Extracts are normally administered as a fluid, but in the event of pounded material either a fluid (water) or soft porridge can be used. Extracts are normally stored in 2 L plastic

containers, and then consumed using a tin cup. In general one cup three times per day for a period of one week was adhered to. Powered medicines are stored in 400 gram containers, wrap in newspaper, cloth or plastic bags.

DISCUSSION

Sexually transmitted infection identification

As early as 1966, Bryant in his book on the *materia medica* of the Zulu tribe recorded an important phenomenon. He concluded that indigenous knowledge systems functioned on the premise that the symptoms equalled the ailment, and therefore focussed on symptomatic treatment rather than on the root cause of the symptoms. This is currently still the *status quo* as traditional healers treat symptoms, because asymptomatic people do not come for consultation.

With the exception of healers in two municipalities (Tubatse and Bela-Bela), all other healers treat gonorrhoea. Although 10 (33%) traditional healers indicated that they use unprotected sexual intercourse with an infected person in combination with a smelly urethral discharge to identify gonorrhoea. Most traditional healers (73%) rated unprotected sexual intercourse with multiple partners in combination with a smelly urethral discharge of greater importance in the identification process. The use of an abnormal urethral discharge concurs with the genital symptoms reported by Darj et al. (2010). Their study, focusing on the presentation of infected females at rural and urban clinics in Uganda, indicated that an abnormal vaginal discharge was the most prevalent symptom (75% urban vs. 84% rural), followed by genital itching and sores.

It is not surprising that unprotected sexual intercourse, either with an infected person or with multiple partners, formed part of the identification process as many initiatives in the media promote the use of condoms as a protective means. Unfortunately this study did not address the consistency of condom use or how many of the patients had multiple partners or an infected partner, as these aspects fell outside the scope. Sexual partnerships do have an impact on the spread of STIs such as gonorrhoea, syphilis (Kretzschmar and Morris, 1996) and HIV/AIDS (Morris and Kretzschmar, 1997). Seventeen healers from 10 local municipalities treated HIV/AIDS. This in itself was surprising as one would have expected that of all the STIs treated, HIV/AIDS would have been the top priority as it is very prevalent in the studied areas (Igumbor et al., 2003). HIV/AIDS produces gradual effects on the human body's immune responses resulting in the development of cancers and opportunistic infections (Vermani and Garg, 2002). The list of symptoms associated with this disease is exhaustive, but the more prominent ones are persistent fever, night sweat,

wasting syndrome, headache, skin rashes, diarrhoea, thrush, Kaposi's sarcoma, Candida esophagitis (Kapusnik-Uner, 1996) and disseminated atypical mycobacterial infection (Murray and Pizzorno, 1999). Typically its identification, in this study was based on a combination of behavioural traits and accompanying symptoms. The behavioural aspects were straight forward and most traditional healers agreed that unprotected sexual intercourse with an infected person or the partner of a person who died of HIV/AIDS would increase the likelihood of being infected. The diagnostic criteria were a huge disappointment as weight loss (wasting syndrome) was the only consistent symptom used. This disappointment stems from the fact that so many symptoms exist that it is difficult to believe that the traditional healers would base the diagnosis of this dreadful disease on a single symptom, and the fact that weight loss can result from any number of conditions, including, but not limited to HIV/AIDS. One traditional healer from Modimolle municipality had a different approach to the identification of HIV/AIDS infection. He listed exposure to contaminated blood as the cause and excluded sexual activities as contributing factors. Wasting syndrome did not feature among the symptoms; however, his list included coloured ligaments (green), prolonged flu-like symptoms and a feeling of dizziness on hot days. It seems reasonable to say that the coloured ligaments and dizziness does not make sense, and that the prolonged flu-like symptoms can at least partially be accepted as one of the diagnostic criteria.

Chlamydia was less often treated and only five of the 17 municipalities had traditional healers who treated it. Why most of these traditional healers (3/5 municipalities) reside in the Capricorn district is as yet not clear, and needs to be elucidated. Seven of the nine traditional healers who treated this disease mentioned that their patients had an abnormal gait ("stretch legs when walking") as well as an inability to urinate. Exposure to blood seems to play an important role in contracting this disease, as unprotected sexual intercourse with menstruating partners or one who just terminated her pregnancy was clearly indicated as a risk factor. This can lead to urogenital infections, which in turn can, to an extent, explain the inability to urinate. The phrase "stretch legs when walking" seems to be significant as 78% of the traditional healers referred to it exactly like this. The phenomenon itself needs further investigation.

Nta and syphilis were exclusively (indicating its prevalence in this district) treated by traditional healers from the Sekhukhune district. *Nta* was treated by traditional healers from the Elias Motsoaledi municipality, who used unprotected sexual intercourse with many partners as a means of identifying this ailment. No accompanying symptoms were recorded. The use of unprotected sexual intercourse for identification purposes is very vague as most of the other listed ailments also include it. Similar to this is a single traditional healer from the Groblersdal

municipality who used unprotected sexual intercourse with many partners in his identification of syphilis. In addition to this it was mentioned that patients had many measles on their genitals. It is very difficult to say with certainty what is meant with "measles". The fact that it seems to be confined to the genital area creates more confusion. The only reasonable explanation is syphilis, as a skin rash does appear during the secondary stage (Jones and Lopez, 2006). This skin rash appears all over the body, is painless and does not itch. As a result of this, there is a possibility that both the traditional healers and patient could have overlooked it, simply because traditional healers, in the case of an STI, would not necessarily associate a rash on other body parts with an STI, and the patient, due to a lack of discomfort, might not mention it.

Plant species used to treat sexually transmitted infections

Bapedi traditional healers used 47 species of plants to treat chlamydia, gonorrhoea, HIV/AIDS, *nta* and syphilis. The presence of such a large number of plant species and their associated ethno medicinal knowledge indicates that the study area has a higher diversity of medicinal plants and that indigenous knowledge regarding STIs of traditional healers in this area compare favourably with findings of De Wet et al. (2012) in northern Maputaland, KwaZulu-Natal Province (South Africa).

The dominant families in this study were: Asteraceae (9%), Asphodelaceae, Fabaceae and Hyacinthaceae (6% each). Although not reported to exclusively treat STIs, these families are consistently recorded as mostly used in different ethno medicinal inventories. Species from the Asteraceae and Asphodelaceae families were dominant in a study conducted in the Agter-Hantam, Northern Cape Province (De Beer and Van Wyk, 2011). The Hyacinthaceae were also reported to be one of the most used plant families in the Eastern Cape Province (Koduru et al., 2007). However, a number of studies (Kambizi and Afolayan, 2001; Hossan et al., 2010) focussing on STIs reported the dominance of Fabaceae. These studies concluded that since the Fabaceae provided the highest number of species, it might be an important family for STIs and medicinal plants in general. The preference of the Asphodelaceae and Asteraceae families in this study could be attributed to their wide distribution range, large number of taxa and plant numbers (Thomas et al., 2009). According to Jones (1998) the wide use of Asteraceae in traditional medicine is linked to the wide range of biologically active compounds it contains. Heinrich et al. (1998) concluded that the widespread use of species from this family might be linked to the fact that it is one of the largest families in the plant kingdom.

With the exclusion of *nta* and syphilis, Bapedi traditional healers treated all STIs with more than one species.

For instance HIV/AIDS was treated with 25 species, gonorrhoea with 18 species and chlamydia with eight. This ability to use many different species to treat a specific STI creates functional redundancy and facilitates resilience by increasing the likelihood for substitution if a particular plant is unavailable. Nevertheless, the high number plant species used by Bapedi traditional healers to treat HIV/AIDS was expected as currently there is no cure for this dreadful disease. These healers perhaps have been using plants for the symptoms but not the disease itself because it was unknown.

The majority of the plant species in this study was documented in the Waterberg district (28 spp.) and Sekhukhune district (17 spp.). Only 12 species was recorded in the Capricorn district. The degree of use could be linked to their distribution, abundance and/or intra cultural differences; an aspect that warrants further investigation.

Eighty seven percent of the 47 recorded species are used to treat a single STI (Table 2). The dominant use of a single species by Bapedi traditional healers perhaps has its advantages from a conservation point of view. This is because although the indigenous species are under threat, it is at least not under threat from being multi-used as well. However, Hossan et al. (2010) noted that using a variety of species against a particular ailment suggest that the disease is quite prevalent. In the current study 13% of species were used to treat two STIs, this includes *A. marlothii* (gonorrhoea and chlamydia), *C. salicifolia* (gonorrhoea and HIV/AIDS), *C. myriocarpus* (gonorrhoea and syphilis), *D. elata* (gonorrhoea and HIV/AIDS), *H. hemerocallidea* (gonorrhoea and HIV/AIDS) and *Z. mucronata* (gonorrhoea and nta).

With the exception of *C. roseus*, used to treat gonorrhoea, no other species is used with the same consistency by traditional healers in the three districts. The reason for this is currently unknown; however, Van Wyk and Wink (2004) noted that one of the recognised evidences of efficacy and safety of an indigenous remedy is its widespread use for treating an ailment. Therefore, it is acceptable to postulate that *C. roseus* might be widely preferred by Bapedi traditional healers due to its efficacy against gonorrhoea. The use of this species to treat unspecified venereal diseases was previous reported for the Venda region, Limpopo Province, South Africa (Mabogo, 1990) and unspecified areas in Southern and Eastern Africa (Watt and Breyer-Brandwijk, 1962). Therefore, it might be possible that in the Venda region and Southern and Eastern Africa *C. roseus* is used for gonorrhoea. This is because venereal disease is a collective term for STIs.

Some medicinal species used by Bapedi traditional healers to treat STIs have been validated through scientific research or through their extensive use by various cultures in South Africa and other parts of Africa. For instance, the use of *T. terrestris* to treat chlamydia by Bapedi traditional healers is similar to that reported by

Mabogo (1990) in the Venda region. However, there is a difference between Vha-Venda and Bapedi with regard to the parts used. Bapedi healers prefer using the whole plant (flowers, fruit, stem, leaf and root), while Vha-Venda healers only use the leaves (Mabogo, 1990). Cultural and indigenous knowledge differences between Bapedi and Vha-Venda concerning the use of plant parts might have contributed to the observed variations.

Furthermore, Bapedi traditional healers use the root of *C. papaya* to treat gonorrhoea. A similar finding was previously reported for the Venda region (Arnold and Gulumian, 1984), and the northern Maputaland, KwaZulu-Natal Province of South Africa (De Wet et al., 2012). However, there are clear differences between Bapedi, Vha-Venda healers and lay people regarding the plant parts used. Bapedi and Vha-Venda prefer to orally prescribe extracts prepared from root, while lay people prefer to use extracts made from the leaves. Therefore, it can be argued that knowledge of its medicinal use varies according to geographical location. This is because both Bapedi and Vha-Venda healers inhabit the same area (Limpopo Province), while lay people (KwaZulu-Natal Province) are located some distance from both the Bapedi and Vha-Venda. In other parts of Africa leaves of *C. papaya* are commonly the preferred part to treat unspecified STIs (Abbiw, 1990; Ndubani and Hojer, 1999). These studies verify the use of this species by Bapedi traditional healers in the treatment of STIs. The use of *T. terrestris* by Bapedi traditional healers to treat STIs corresponds with findings of Mabogo (1990). This similarity is of significance, because identical species-use by different cultures may be a reliable indication of curative properties. *T. terrestris* demonstrated antibacterial activity against *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Al-Bayati and Al-Mola, 2008). The use of *H. hemerocallidea* by Bapedi traditional healers to treat HIV/AIDS and gonorrhoea correspond with findings of Puranwasi (2006) and De Wet et al. (2012) who reported its use to treat HIV/AIDS by the Zulu people in northern Maputaland. Singh (1999) furthermore reported the extensive use of this species by Zulu traditional healers in the treatment of urinary infections caused by STIs. It is also interesting to note that pharmacological activities of extracts from this species resulted in the extraction of β -sitosterol and β -sitosterol glycoside, which showed a significant decrease in plasma viral loads and stabilized CD-4⁺ cell counts over a period of 40 months in HIV positive patients (Bouc et al., 1999). This finding supports the use of *H. hemerocallidea* by both Bapedi and Zulu healers to treat HIV/AIDS, as well as other South Africans (Babb et al., 2007) who use this species to manage this virus. Conservation measures should be taken as this species is nationally threatened (SANBI, 2001).

Ziziphus mucronata is used in this study to treat both chlamydia and gonorrhoea. Hutchings et al. (1996)

reported its use by Zulu healers to treat gonorrhoea only. No South African literature could be located to support its use by Bapedi healers to treat chlamydia. Available South African literature indicates its use to treat infertility and nerve pains by Vha-Venda healers (Mabogo, 1990), diarrhoea by Xhosa healers (Appidi et al., 2008) and as medicine to bring a good relationship with ancestors by Zulu healers (Ndawonde, 2006). In other parts of Africa, *Z. mucronata* is widely used to treat oral infections (Gundidza, 1986; Runyoro et al., 2006; Tapsoba and Deschamps, 2006).

To the best of our knowledge *C. decapetala* (gonorrhoea), *E. elephantina* (HIV/AIDS), *Z. mucronata* (chlamydia), *A. pungens* (gonorrhoea), *B. africana* (HIV/AIDS), *C. verum* (HIV/AIDS), *C. lanatus* (HIV/AIDS), *E. crispa* (HIV/AIDS), *E. maleolens* (HIV/AIDS), *G. aspera* (HIV/AIDS), *P. ciliatus* (HIV/AIDS), *P. caffra* (chlamydia), *S. lancea* (Nta), *S. viminale* (HIV/AIDS), *Z. capense* (HIV/AIDS) and *Z. humile* (HIV/AIDS) are reported for the first time in the treatment of the investigated STIs. This survey has made a major contribution in the plant species used traditionally for the treatment of STIs in South Africa and worldwide. It also offers considerable opportunities for further scientific research.

Plant parts used in remedy preparation

Data from this study illustrates a preference for the use of roots (58%) and entire plant (12%). This finding is almost similar to that reported by De Wet et al. (2012) in northern Maputaland. They noted the dominance of roots (25%), followed by leaves and whole plant (18%) in the treatment of STIs. However, Hossan et al. (2010) reported the dominance of root and leaves in their study which was conducted in Bangladesh. The wide use of the roots by Bapedi traditional healers to prepare medicine is based on the perception that more healing power is stored in this part (Semenya et al., 2012). Furthermore, their extensive use of entire plants (12%) was because they want to utilize all plant parts (roots parts, leaf and flower) concurrently. However, wide utilization of both roots and entire plant use has serious consequences from both ecological point of view and from the survival of the medicinal plant species as was observed in by Birhanu (2002) in Jabitehaan Wereda, West Gojam. Therefore, research should also be undertaken, to establish if substitute parts (such as leaves) have the same efficacy as other parts of the plant. It is reasonable to state that the limited use of seeds and fruits (2% for each) by Bapedi healers might be due to their seasonal availability.

Single vs. multiple plant extracts

Forty preparations were drawn from mixtures of different

species for the treatment of a single STI. For instance the root of *Z. mucronata* were mixed with either a bulb of *E. pallidiflora* or a tuber of *H. obtusa* to treat chlamydia, or a root of *O. ficus-indica* to treat gonorrhoea. The practice of combining different species to treat a single STI was also reported by De Wet et al. (2012) for Zulu lay people. They found that 33 species are used in 23 different combinations of two or more species per herbal remedy for the treatment of a single STI. For example leaves of *C. papaya* were mixed with *Senecio serratuloides* (leaf) and a tuber of *H. hemerocallidea* to treat gonorrhoea. In the current study the combination of a root of *C. papaya* with a tuber of *C. myriocarpus* is a remedy for gonorrhoea. Bapedi healers indicated that they combine species to re-enforce the medicines and increase its efficacy. This observation is in agreement with the study by Mabogo (1990) for Vha-Venda traditional healers. It is interesting to note that pharmacological studies support this claim (Chow et al., 2003). This was further scientifically validated by Otieno et al. (2008), they evaluated this practice by mixing root extracts of *Catha edulis*, *Eucomis natalensis*, *Harrisoni abyssinica* and *Ximenia caffra* against single extracts of the same species. Multi-species extracts inhibited all tested bacterial species, while single extracts inhibited only three of them. Eight out of ten multi-species extracts were bactericidal, while only two out of four single extracts were bactericidal. Therefore it is reasonable to state that the Bapedi practice of combining medicinal species to treat a single STI might be effective.

Twenty nine single extract were used to treat one or more STIs. For instance, *A. marlothii* is used to treat gonorrhoea, HIV/AIDS, and chlamydia by Bapedi healers in various surveyed areas, while species such as *H. obtusa* (chlamydia), *S. hyacinthoides* (HIV/AIDS) and *Pelargonium* species (HIV/AIDS) were exclusively used for a single STI (Table 2). The use of single species in the preparation of remedies was also reported by Fernandes et al. (2008) for the Venda region. For instance, *C. roseus*, *P. africanum* and *S. panduriforme* were amongst the species used by Venda healers to treat unspecified venereal diseases and infectious diseases (Fernandes et al., 2008). The preference of a single species by Bapedi healers in the preparation of extracts might be linked to their local availability. In the Southern Tigray, Northern Ethiopia, Giday and Gobana (2003) postulated that the wide utilization of a single plant species in the preparation of indigenous remedies by healers is ascribed to the differences in the socio-cultural landscapes, indigenous knowledge on synergetic effect of different medicinal plants and vegetation types. Saikia et al. (2006) reported that use of a single species in the preparation of an extract could be of great interest for the development of novel drugs as the exploration of therapeutic activity-bearing ingredients may be easier. However, the use of a single species by Bapedi healers in the preparation of extracts has both advantages and disadvantages from a conservation point of view. As

noted earlier when used to treat a single STI it reduces harvesting pressure of being multi-used, likewise the opposite increases harvesting, thereby posing a threat.

Preparation, administration and dosage of remedy

Various preparation methods, such as boiling, pounding and maceration are used by Bapedi traditional healers. These are the most common methods of medicinal preparation used for STIs in South Africa (De Wet et al., 2012) and other African countries (Njoroge and Bussmann, 2009). Eighty percent of the plant material in this study was boiled in water. De Wet et al. (2012) and Njoroge and Bussmann (2009) also noted the preference for boiling material. This might be due to the simplicity of preparation. Some Bapedi healers (14%) opted to pound dried plant materials. However, the low occurrence of pounding of medicinal material by Bapedi healers was expected as producing powders is a labour intensive process; the herbs must be cut into very small pieces, dried sufficiently to make them brittle, and then crushed intensively enough to reduce them to a reasonably fine powder. In central zone of Tigray, Northern Ethiopia, Yirga (2010) noted that the preference of pounding plant materials is driven by the scarcity of the plant in nature, and that healers preserve the plants that they could not easily find in communal areas. The same can be true with some of the threatened and declining (SANBI, 2001) species used by Bapedi healers. These species include *H. hemerocalidea* (declining) and *E. maleolens* (least concern), which were occasionally processed by pounding. The limited use of maceration, normally a 24-hour procedure, as a preparation method by Bapedi healers might be due to its long preparation time.

Findings of this study illustrate that oral self-administration (96%) was the method of choice. Studies such as Kambizi and Afolayan (2001) and Chigora et al. (2007) also noted that most medicines used in the treatment of STIs are prescribed orally. The preference of this method by Bapedi traditional healers might be due to the fact that medication in liquid form is already dissolved, so it can readily be absorbed by the human body. Occasionally, Bapedi healers administered medication rectally (6%) using a bulb-syringe; in such cases the traditional healers did the administration. The limited use of this method by Bapedi healers came as no surprise as they mentioned that it is very dangerous and mostly used by more experienced traditional healers. They agreed that incorrect dosages (too much) can be fatal.

Extracts are normally stored in 2 L plastic containers, and then consumed using a tin cup. In general one cup three times per day for a period of one week was adhered to; this is in line with the standard prescriptions in western medicine. However, traditional dosage remedies of Vha-Venda healers depend on the physical appearance of the patient and experience of individual healers (Mabogo, 1990). This finding was also reported in

different parts of Ethiopia by studies such as Addis et al. (2001) and Teklehaymanot et al. (2008). The lack of use of standard/measured doses, and the large volumes of the doses used are difficult to manage. This may be dangerous as some of the species could have a high degree of toxicity, and overdose might cause serious health problems. It is interesting to note that Bapedi traditional remedies have precision with regard to dosage. This is because most of the remedies are prepared using similar species, method of preparation, administration and dosage (Table 2).

Conclusion

The present study revealed that Bapedi traditional healers possess a good knowledge of STIs identification. However, their diagnosis of STIs is based primarily on the presentation of symptoms, which is not always accurate. This is because some of the patients might have symptoms similar to STIs but are not necessarily infected; consequently traditional healers might prescribe incorrect and ineffective medication. However, given proper guidance and education, traditional healers could serve as an important source of information and can be incorporated in community-based STI prevention. The large number of species employed to treat STIs clearly reflects the diversity of treatment protocols used by Bapedi healers. In the treatment of the more prominent STIs, a number of alternative species can be used, which in itself will ensure that treatment options will always be available. Further studies to determine the efficacy of some of the recorded species against the reported STIs is strongly recommended.

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Full Length Research Paper

Effect of chemotherapy with cisplatin and rapamycin on HeLa cells *in vitro*

Lu Han^{1*}, Jie-ling Wu² and Li-xiao Yang³

¹Dalian Obstetrics and Gynecology Hospital, Dalian 116033, China.

²Dalian Blood Center, Dalian 116001, China.

³Maternal and Child Health Hospital, Fangshan district, Beijing 102488, China.

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The aim of this study was to evaluate the effect of combination of rapamycin (RPM) with cisplatin on the proliferation of cervical cancer HeLa cells *in vitro*, as well as the expression of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor (VEGF). HeLa cells were treated with RPM and cisplatin, respectively or through combining them for 72 h. There were four groups in this experiment, namely, RPM, cisplatin, RPM combined with cisplatin, and control group (culture medium only). The expression of mRNA and the protein of genes HIF-1 α and VEGF were detected using reverse transcription-polymerase chain reaction (RT-PCR) and western blot respectively. A down-regulation of the mRNA and protein expression of HIF-1 α and VEGF was observed in HeLa cells corresponding to the rapamycin group, cisplatin group, and RPM combined with cisplatin group compared with the control group ($p < 0.05$). The mRNA and protein expression of HIF-1 α and VEGF were significantly down-regulated in the combined group. No significant difference was found between the RPM group and cisplatin group ($p < 0.05$). The mRNA and protein expression of both HIF-1 α and VEGF were significantly down-regulated by the combination of RPM with cisplatin

Key words: HeLa cells, Rapamycin, cisplatin, hypoxia-inducible factor-1 α , vascular endothelial growth factor.

INTRODUCTION

The prevalence of cervical cancer is the highest among malignancies, which involve the female genital tract. Although great progress has been achieved in its early detection, diagnosis, and treatment, the therapeutic outcomes of advanced cervical cancer remain unsatisfactory. Traditional surgical methods are mostly used for patients suffering in the early stage of the disease. Radiation therapy is adopted for the most intermediate and advanced patients, but it is helpless for recurring tumors. Neoadjuvant chemotherapy is mainly used for recurrent and advanced patients. Today, cisplatin is considered the first line of chemotherapy for cervical

cancer. However, discontinuation of chemotherapy in advanced patients usually occurs due to obvious side effect and drug resistance (Hidalgo and Rowinsky, 2000). According to recent studies, rapamycin (RPM) and its derivatives can have an anti-tumor effect on numerous types of malignancies (Mondesire et al., 2004; Aleskog et al., 2008). Moreover, such a lethal effect only occurs in tumor cells rather than normal cells. Thus, nowadays, RPM has become a new type of safe, nephrotoxicity-free, and efficacious tumor inhibitor. Furthermore, the combination of RPM and cisplatin may become a new treatment protocol for cervical cancer. Such a combination can probably reduce the dose of cisplatin used in treatment, thereby alleviating cisplatin-induced toxic and side effects. The existence of hypoxic cells in cervical cancer tissue and the high expression of hypoxia-inducible factor-1 α (HIF-1 α) serve as important

*Corresponding author. E-mail: luhancn@126.com. Tel: 86 013940801858. Fax: 86 0411 84439973.

causes for various treatment failures (Bachtiary et al., 2003; Burri et al., 2003). HIF-1 α can regulate the expression of different target genes, including coding vascular endothelial growth factor (VEGF), maintain the energy metabolism of cancer cells, affect neovascular formation, and promote the proliferation and metastasis of cervical cancer (Blagosklonny, 2001). Therefore, the antagonistic activity of HIF-1 α may be a potential target for cancer therapy. Recent studies have shown that mammalian target of RPM (mTOR) inhibitor can block HIF-1 α expression in the transcription and translation levels, resulting in an anti-tumor effect (Hudson et al., 2002; Jiang and Feng, 2006). In the current study, subtoxic doses of RPM and cisplatin were combined to evaluate the inhibitory effect of such treatment on HeLa cell line *in vitro* by determination of the expression levels of HIF-1 α and VEGF. The mechanism of tumor vascularization inhibition via inhibiting the expression of HIF-1 α and VEGF was further explored to provide a theoretical basis for new combination chemotherapy for cervical carcinoma.

MATERIALS AND METHODS

Cell cultures

HeLa human cervical cells were provided by the Department of Histology and Embryology of Dalian Medical University. They were cultivated in RPMI-1640 DMEM combination, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/L streptomycin, in 5% CO₂ at 37°C. The growth of cells was then recorded. When 70% to 80% cells reached adherent growth, the cells were digested with trypsin and then pre-cultivated. For further study, the cells in the logarithmic growth phase were used for further study.

Drug intervention

The cells were released by using trypsin treatment, and then counted a day before the drug intervention. When drugs were used, cells were transferred into the 96-well plates (concentration: 2×10^6 /ml) to reach 70 to 80% density. After adherent growth, drugs of different concentrations were added, and then the cells incubated in 5% CO₂ at 37°C for 24 h.

There were four groups in this experiment, namely, control group (culture medium only), RPM group (20 nmol/ml), cisplatin group (0.5 mg/ml), RPM (20 nmol/ml), and combined cisplatin group (0.5 mg/ml).

Reverse transcription-polymerase chain reaction (RT-PCR)

Trizol method was used for extraction of total cell RNA for 48 h after drug intervention in all groups previously described. Samples in amounts of 0.25 μ g RNA were treated with reverse transcriptase. HIF-1 α and VEGF mRNAs were amplified by RT-PCR. As an internal control, β -actin mRNA was also similarly amplified. The HIF-1 α up-stream primer sequence was 5'-AACAAAAACACAGCGAAGC-3', the downstream primer sequence was 5'-ATAGTGAATGTGGCCTGTG-3', and the product length was 124 bp. The VEGF upstream primer sequence was AGGGCAGAACATCACGAAG-3', the downstream primer sequence

was 5'-ACTCCAGGCCCTCGTCATTG-3', and the product length was 182 bp, respectively. Analogically, the β -actin upstream primer sequence was 5'-TCCTTCTGCATCCTGTGGCA-3', the downstream primer sequence was 5'-CAAGAGATGCCACGGCTGCT-3', and the product length was 275 bp. Primer synthesis was accomplished by TaKaRa Biotechnology Co., Ltd. PCR procedure was performed as follows: 35 cycles at 50°C for 30 min, 94°C for 2 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. RT-PCR products were detected by 2% agarose electrophoresis and recorded using UVP Gel Imaging System. Data were analyzed with Labwork 4.6 image acquisition and the relative expression of two genes using HIF-1 α / β -actin and VEGF/ β -actin were shown by analysis software.

Western blot assay

Total protein was extracted from HeLa cells from each group, and its concentration was detected by using the bicinchoninic acid (BCA) protein assay kit. The protein levels were assayed by the standard curve. The mixture (80 μ g total protein, 5 \times sample buffer and additional water to reach to 20 L) was boiled for 5 min. Proteins were separated after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), moved to the polyvinylidene fluoride (PVDF) membrane, and then immersed in the transference fluid for 30 min. The transference time was 120 min for HIF-1 α , 45 min for VEGF, and 60 min for β -actin, respectively. Then, the PVDF membrane was placed in skimmed milk powder for 2 h at room temperature. It was subsequently placed in mouse anti-HIF-1 α monoclonal antibody (1:100), mouse anti-human VEGF monoclonal antibody (1:200), and rabbit anti- β -actin antibody (1:2000) at 4°C overnight. The membrane was washed 3 times for 10 min with cold phosphate buffered saline with Tween-20 (PBST containing 1% Tween-20). Then, it was incubated with secondary antibody for 1 h at 37°C. The membrane was washed 3 times for 10 min with cold PBST (containing 1% Tween-20). The membrane was detected by electrochemiluminescence (ECL) and X-ray colour reaction, and then washed. The gray value was detected with the UVP Gel Imaging System.

RESULTS

HIF-1 α and VEGF mRNA expressions

The results from RT-PCR indicated that mRNA expressions of HIF-1 α and VEGF significantly decreased in RPM group, cisplatin group, and RPM combined with cisplatin group compared with the control group, and the difference was statistically significant ($P < 0.05$). The integral optical density (IOD) value of each band was recorded using UVP Gel Imaging System (Figures 1 and 2).

The data analysis indicated that mRNA expressions of HIF-1 α and VEGF in the cervical cancer HeLa cells were significantly down-regulated in RPM group, cisplatin group, and RPM combined with cisplatin group compared with the control group, and the difference was statistically significant ($P < 0.05$). The mRNA expressions of HIF-1 α and VEGF were significantly down-regulated in the combined group compared with RPM and cisplatin, and the difference was statistically significant ($P < 0.05$). There was no significant difference in the mRNA expressions of HIF-1 α and VEGF between RPM and cisplatin

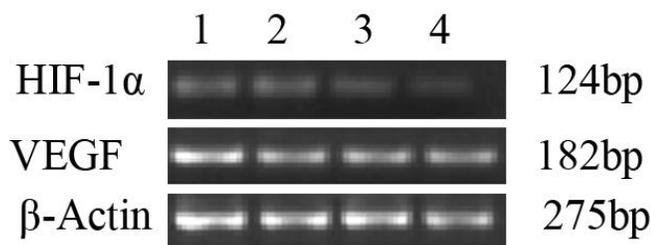


Figure 1. The amplification band of HIF-1 α mRNA and VEGF mRNA. 1, control group; 2, RPM group; 3, cisplatin group; 4, combination group.

groups ($P > 0.05$) (Table 1).

HIF-1 α and VEGF protein expression

As per the results of western blot assay, the protein bands were clear in each group, but they were weaker in the combination group. The IOD value of each band was recorded by using UVP Gel Imaging System (Figures 3 and 4).

The data analysis indicated that the levels of both HIF-1 α and VEGF protein expressions in cervical cancer HeLa cells were significantly down-regulated in RPM group, cisplatin group, and RPM combined with cisplatin group in comparison to the control group ($p < 0.05$). Similarly, the protein expressions levels of HIF-1 α and VEGF were significantly decreased in the combined group in comparison to the RPM group or cisplatin ($p < 0.05$). There was no significant difference in the levels of protein expressions of HIF-1 α and VEGF between RPM and cisplatin groups ($p > 0.05$) (Table 2).

DISCUSSION

VEGF is believed to play an important role in tumor angiogenesis and in the increased incidence of tumor metastasis by releasing tumor cells into the vasculature, promoting vascular endothelial proliferation, increasing capillary permeability, and modifying the extracellular matrix (Minet et al., 2000). Many researchers have shown a close relationship between the high expression of VEGF and its receptor in gynecologic malignant tumor tissues closely related to tumor invasion and metastasis (Sivridis et al., 2002). The high expression of HIF-1 α and VEGF in the cancer tissues of patients with epithelial ovarian cancer, endometrial cancer, and cervical is closely related with the tumor prognosis (Sivridis et al., 2002; Sonode et al., 2003; Wong et al., 2003). Angiogenesis in cancer masses has been established to be induced mainly by up-regulation of VEGF and HIF-1 α expression, and hence, higher transcriptional activities (Carmeliet et al., 1996; Bos et al., 2001). Therefore, the inhibition of the expression of HIF-1 α and VEGF may be

a new tumor treatment strategy through the anti-angiogenesis mechanism.

As closely related with the tumor, the development has been characterized by Akt/mTOR signal pathway, mainly by cell cycle acceleration, apoptosis reduction, and promoting of cell migration. RPM, a single specific inhibitor of Akt/mTOR, signal pathway, is primarily used as an immunosuppressive agent in organ transplantation postoperatively. Recent reports have indicated that RPM can inhibit proliferation of various cancer cells, such as leukemia, breast cancer, and liver cancer cells (Mondesire et al., 2004; Récher et al., 2005; Semela et al., 2007). Its anti-tumor effect works by blocking cell cycles, but it also acts by inducing cancer cell apoptosis and autophagy, resulting in cancer cell death (Brazelton and Morris, 1996; Faivre et al., 2006). It has also been found to reduce cancer angiogenesis by decreasing HIF-1 α and VEGF, activation of endothelial cell proliferation and migration, as well as by increasing thrombosis in tumor neovascularization (Young and Jan, 2006). Hudson et al. (2002) have reported that RPM can inhibit the expression of HIF-1 α and its transcriptional activation effect on VEGF in prostate cancer cells *in vitro*.

Cisplatin, one of the first-line chemotherapy drugs, is mainly used for chemo radiotherapy and for advanced or recurrent cervical cancer patients. However, its non-specific side effects and drug resistance commonly limit its clinical use in advance cervical cancer patients. Yuan et al. (2003) showed that the increased activity of Akt/mTOR signal pathway in ovarian cancer and breast cancer results in cisplatin resistance. Micheal et al. (2003) and Liu et al. (2007) have drawn the same conclusion based on their research on ovarian cancer and lung cancer, respectively, suggesting that Akt/mTOR inhibitor RPM can reverse cisplatin resistance. In this study, cervical cancer HeLa cells were treated with RPM alone or combined with cisplatin *in vitro* to further detect mRNA and protein expression of HIF-1 α and VEGF using RT-PCR and western blot assay. Our findings demonstrated that HIF-1 α and VEGF expression levels were decreased in a subtoxic dose (20 mg/ml).

Mondesire et al. (2004) in the RPM group, subtoxic dose (0.5 mg/ml) (Jiang et al., 2001) in the cisplatin group, and significantly in the combination group. The inhibitory effect of RPM combined with cisplatin on HIF-1 α and VEGF expression was higher than their effect when they were used separately, suggesting that the combined usage might reduce the dosage of chemotherapy drugs.

BaeJung et al. (2009) have reported a synergistic effect of RPM and cisplatin combination on endometrial cancer cells. Wu et al. (2005) have proposed that RPM can increase the sensitivity of drug-resistant lung cancer cell lines on cisplatin, leading to cell apoptosis of resistant lines due to a synergistic effect. The synergistic effect could be explained with the inhibitory Akt/mTOR signal pathway, reversal cisplatin resistance, but also with cell apoptosis despite RPM-induced cell cycle blockage, cancer cell

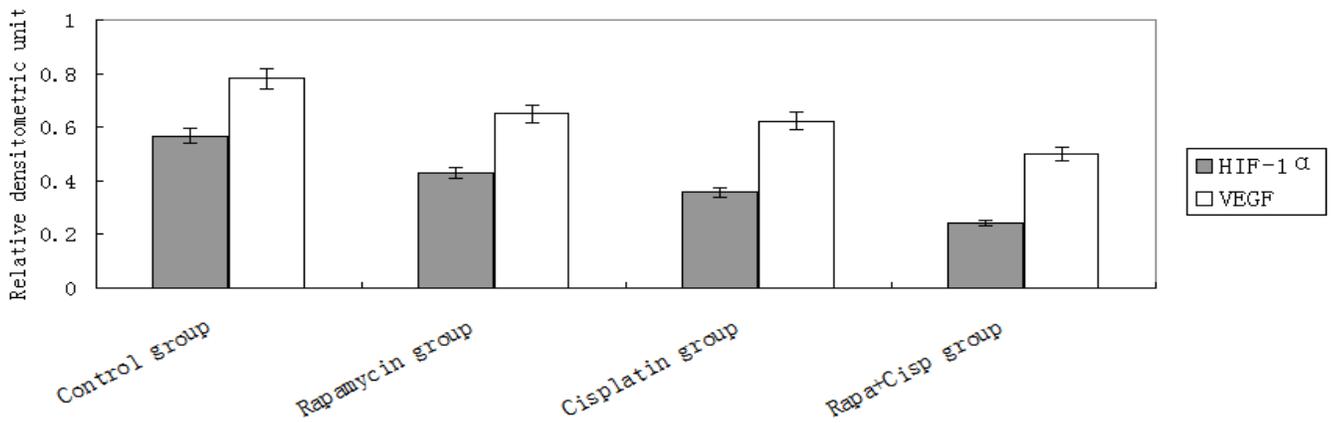


Figure 2. The relative density analysis of mRNA expression of HIF-1α and VEGF detected by RT-PCR.

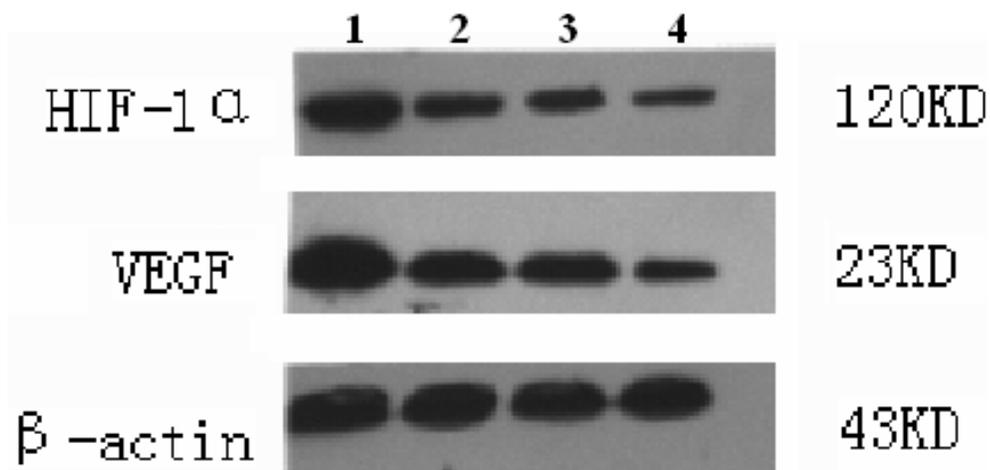


Figure 3. Protein electrophoretogram of HIF-1α and VEGF detected by western blot assay. 1, Control group; 2, RPM group; 3, cisplatin group; 4, combination group.

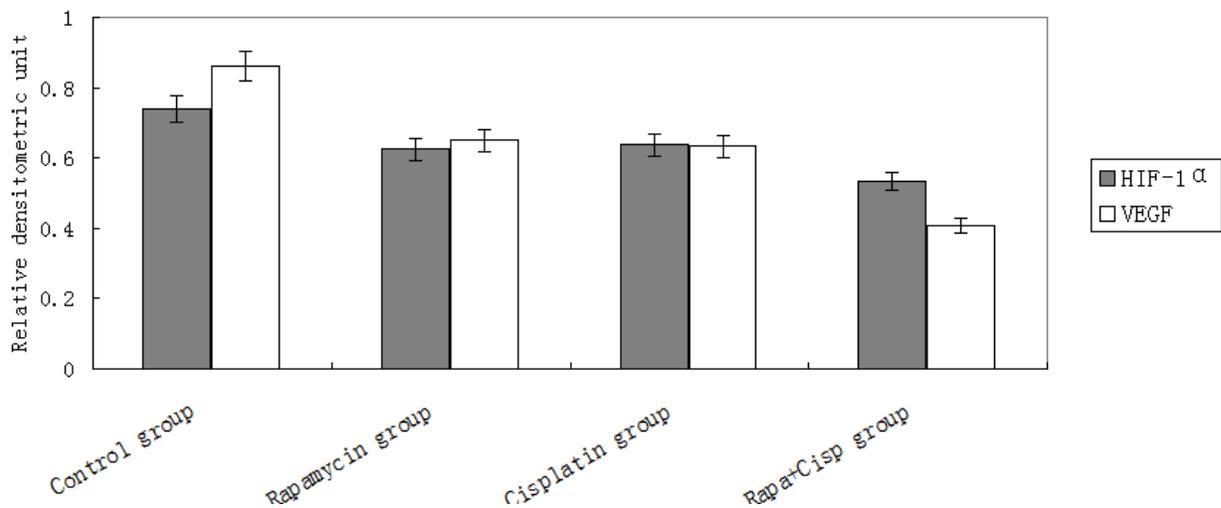


Figure 4. The relative density analysis of protein expression of HIF-1α and VEGF detected by western blot assay.

Table 1. The effects of RPM and cisplatin used alone or in combination on the mRNA expression in HeLa cells.

Group	HIF-1 α mRNA	VEGF mRNA
Control	0.567 \pm 0.084	0.781 \pm 0.139
Rapamycin	0.428 \pm 0.068*	0.651 \pm 0.112*
Cisplatin	0.357 \pm 0.051*	0.623 \pm 0.125*
Combination	0.242 \pm 0.048* Δ	0.498 \pm 0.093* Δ

*p < 0.05, versus Control group; Δ p < 0.05, versus RPM and cisplatin groups.

Table 2. The effect of every group on expression levels of HIF-1 α and VEGF protein in the HeLa cells.

Group	HIF-1 α IOD	VEGF IOD
Control	0.739 \pm 0.159	0.861 \pm 0.161
Rapamycin	0.625 \pm 0.132*	0.650 \pm 0.114*
Cisplatin	0.635 \pm 0.120*	0.623 \pm 0.102*
Combination	0.514 \pm 0.092* Δ	0.409 \pm 0.082* Δ

*p < 0.05, versus control group; Δ p < 0.05, versus RPM and cisplatin groups.

apoptosis and autophagy, as well as with reduced cancer angiogenesis by decreasing HIF-1 α and VEGF. Further research and studies on the synergistic anti-tumor effect of RPM and cisplatin are necessary.

A possibility of RPM to kill cancer cells with high selectivity without attacking normal cells (Podsypanina et al., 2001) has also been suggested. The synergistic effect between RPM and chemotherapy drugs can reduce drug dose, thus, decreasing the side effect of traditional chemotherapy and enhancing the patient's quality of life. In a study on the influence of the combination of RPM and taxol on subcutaneous xenograft ovarian cancer in nude mice, Jiang and Feng (2006) have found decreased micro vessel density and lower expression levels of both HIF-1 α and VEGF. Similarly, the combination of RPM and imatinib in chronic myeloid leukemia has enhanced survival in nude mice (Mohi et al., 2004). In this way, the combination of RPM and anti-tumor drugs would probably provide a novel anti-tumor option for the treatment of malignancies.

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Full Length Research Paper

Electrocatalytic determination of anti-hyperthyroid drug, methimazole, using a modified carbon-paste electrode

Fahimeh Jalali^{1*}, Loghman Miri¹ and Mahmoud Roushani²

¹Department of Chemistry, Razi University, Kermanshah, 67346, Iran.

²Department of Chemistry, Faculty of Science, Ilam University, Ilam, Iran.

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The preparation and electrochemical behavior of a carbon-paste electrode modified with an oxovanadium (IV) Schiff-base complex was studied. The modified electrode showed efficient catalysis in the electrochemical oxidation of methimazole (MMZ), an anti-hyperthyroid drug. The catalytic effect was observed for V^{III} oxidation peak which was produced via disproportionation of V^{IV} in acidic solution. The diffusion coefficient (D) of MMZ and the rate constant of catalytic reaction (k) were estimated by chronoamperometry. Determination of MMZ was carried out using differential pulse voltametry. A linear dynamic range of 9×10^{-7} to 1×10^{-4} M with a detection limit of 8×10^{-7} M and a limit of quantitation of 2.6×10^{-6} M was resulted for the drug. The relative standard deviation for determination of 20 μ M of the drug was found to be 1.7%. The method was used for the quantitation of MMZ in pharmaceutical formulations successfully.

Key words: Methimazole, determination, tablets, modified-carbon paste electrode.

INTRODUCTION

Methimazole, MMZ (1-methyl-2-mercaptoimidazole, tapazole), is an orally taken drug used in the therapy of hyperthyroidism (over activity of the thyroid gland). The chemical structure of the drug is shown in Scheme 1. Its action is to slow down iodide integration into tyrosine and thus, inhibits the production of thyroid hormones (Edward, 1992). MMZ is used as a drug to manage hyperthyroidism associated with Graves' disease, but it has side effects such as possible decrease of white blood cells (Kendall-Taylor, 1984). The drug has also been employed to promote growth in animals for human consumption. It has been reported that MMZ may also cause side effects such as nephritis, liver cirrhosis, skin irritation, allergies and pharyngitis with fever (Edward, 1992).

Several analytical methods have been described for the determination of MMZ, such as high-performance liquid

chromatography (Blanchflower et al., 1997; Kusmierek and Bald, 2007), gas chromatography-mass spectrometry (Zhang et al., 2005), flow-injection analysis with chemiluminescence detection (Economou et al., 2004), spectrophotometry (Jovanovic et al., 1992), fluorescence probe method (Dong et al., 2009) and gold nanoparticle-catalyzed chemiluminescence reaction (Sheng et al., 2011). Electrochemical methods, due to their advantages such as high sensitivity, high speed, and low-cost have been used in pharmaceutical analysis for a long time. Analysis of MMZ has been done by using modified electrodes such as glassy carbon electrode modified with multi-walled carbon nanotubes (Xi et al., 2010), an electrode modified with acetylene black/chitosan film (Yazhen, 2011), and a carbon paste electrode modified with a cobalt Schiff base complex (Shahrokhian and Ghalkhani, 2008).

Compared to conventional electrodes, chemically modified electrodes offer unique well-recognized advantages in electrocatalysis, especially in situations where the target analyte requires high over-potential, or

*Corresponding author. E-mail: fahimehjalali@yahoo.com.

the sensitivity is low. This characteristic of chemically modified electrodes arises from the combination of conventional electrochemical techniques with the chemical, structural and other specific properties of the modifying layer(s).

Among the modified electrodes those which include inorganic complexes have been largely used in the area of electrocatalysis (Galal, 1998; Wu et al., 1996). Owing to the reversible redox activity and catalytic properties of vanadium-salen complexes, they have been used in electrocatalytic oxidation of dipyrone (Teixeira et al., 2004) and L-dopa (Teixeira et al., 2004). In the present work, the preparation and electrochemical properties of a carbon paste electrode modified with an oxovanadium (IV) Schiff base complex (VOx) for voltammetric determination of MMZ is discussed. Carbon-paste is a well-known substrate for incorporation of modifiers, as a simple and easy renewing material to construct a modified electrode (Kalcher et al., 1995).

The modified electrode showed good sensitivity in the determination of MMZ, and a long life time without significant decrease in its response. It was successfully applied to the determination of MMZ in tablets.

MATERIALS AND METHODS

Chemicals

The VOx (Scheme 1) was synthesized in Yasuj University, Yasuj, Iran. The procedures for preparation and structural identification of VOx were described in literature (Kianfar et al., 2011; Liu and Anson., 2001). Briefly, the vanadyl complex was synthesized by refluxing a methanolic solution of the Schiff base ligand and vanadylacetylacetonate. The reaction was continued for 2 h until a green precipitate was obtained. It was filtered, washed with methanol and dried in vacuum.

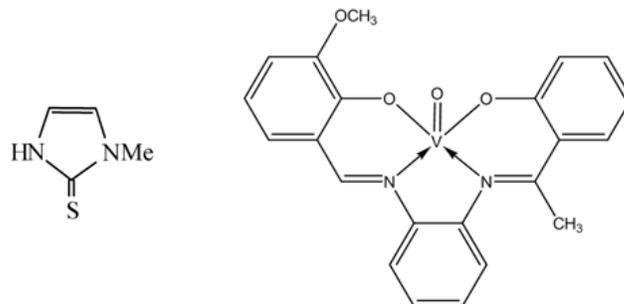
Pure powder of MMZ was a gift from Osvah Pharmaceutical Company (Tehran, Iran). Sodium hydrogenphosphate and hydrochloric acid (both from Fluka) were used to prepare the buffer solution. All other reagents were of analytical grade from Merck (Darmstadt, Germany) and were used without further purification. Doubly distilled water was used for preparation of aqueous solutions. A standard MMZ stock solution (1.0×10^{-3} M) was prepared from the pure powder and stored at 4°C. The working solutions were freshly prepared by serial dilution of the stock solution with 0.1 M phosphate buffer saline (PBS, pH 3).

Apparatus

The electrochemical measurements were performed using a Potentiostat/Galvanostat μ -Autolab system (Utrecht, The Netherlands). A three-electrode system, including a carbon paste working electrode, a platinum wire as the counter and a saturated calomel reference electrode (SCE) was employed. A Jenway 3345 pH/mV meter using a combined glass electrode was used for pH measurements.

Electrode preparation

The general procedure to prepare the modified carbon paste electrode was to mix VOx complex (0.2 g) with graphite powder (0.8 g)



Scheme 1. Chemical structures of MMZ (left) and the oxovanadium Schiff base complex (right).

and mineral oil (0.2 g). After thorough hand mixing using a mortar and pestle to obtain a very fine paste, the mixture was packed homogeneously in a glass tube. A copper wire was inserted into the carbon paste to implement the electrical conduction. The surface of the modified electrode (VOx/CPE) was finally smoothed manually on a clean filter paper. An unmodified carbon paste electrode was prepared using the same procedure in the absence of VOx complex.

Preparation of real samples

Ten tablets of MMZ (5 mg/tablet) were weighed and ground to a homogeneous powder. A proper portion of the fine powder (equivalent to 5 mg of MMZ) was carefully weighed and mixed with about 20 ml of PBS buffer (pH 3; 0.1 M). The suspension was stirred and heated for 10 min. and then filtered to remove insoluble residue. The clear solution obtained was diluted to 50 ml in a volumetric flask using the same buffer solution.

General voltammetric procedure

10 ml of the buffer solution (PBS; pH 3; 0.1 M) was transferred to the voltammetric cell. The electrodes were immersed in the solution and after setting the potential range and scan rate, the cyclic voltammogram was recorded. Then a proper portion of MMZ solution was added to the cell and the stated procedure was repeated.

Evaluation of limit of detection (LOD) and limit of quantitation (LOQ)

In order to evaluate LOD and LOQ, the voltammetric signal (anodic peak current) was obtained for the buffer solution repeatedly ($n = 10$) and the mean value (y_b) and standard deviation (s_b) of the results were obtained. LOD was calculated as a concentration of MMZ which its voltammetric signal was $y_b + 3s_b$. In the case of LOQ, the voltammetric signal should be $y_b + 10s_b$.

RESULTS AND DISCUSSION

Electrochemical behaviour of VOx complex

In order to study electrochemical behaviour of the vanadium complex and due to its insolubility in aqueous solution, the complex was dissolved in acetonitrile. Cyclic

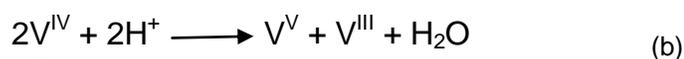
voltammograms of VOx solution in acetonitrile were recorded at different scan rates (5 to 100 mV s⁻¹). The potential range of the working electrode was from 0.0 to 1.0 V against SCE. As shown in Figure 1, a well resolved pair of anodic and cathodic peaks appeared with a peak separation of about 0.07 V and E_{1/2} [(E_{pc} + E_{pa})/2] of 0.06 V. The ratio of cathodic peak current (I_{pc}) to anodic peak current (I_{pa}) was about one in the range of scan rates used. These observations confirm a reversible electron transfer mechanism between two oxidation states of vanadium in the complex (Kianfar et al., 2011; Liu and Anson, 2001):



As shown in Figure 1, the effect of increasing scan rate is a gradual increase of both anodic and cathodic currents without potential shift which indicates a simple electron transfer mechanism. The anodic peak current increases linearly with the square root of scan rate (Inset), which shows the diffusional nature of the currents (Bard and Faulkner, 2001).

A carbon-paste was modified with VOx (VOx/CPE) and used as the working electrode in cyclic voltammetry. Figure 2 shows the result in aqueous solution (PBS, pH 3). The anodic peak (A₁) and its cathodic counterpart (C₁) observed at the surface of carbon-paste electrode are similar to those observed in acetonitrile solution which correspond to the V^V/V^{IV} couple. Another redox couple (A₂ and C₂) is observable at higher potentials (about 0.8 V) which is due to electron transfer of V^{III} / V^{IV} couple (Liu and Anson, 2000).

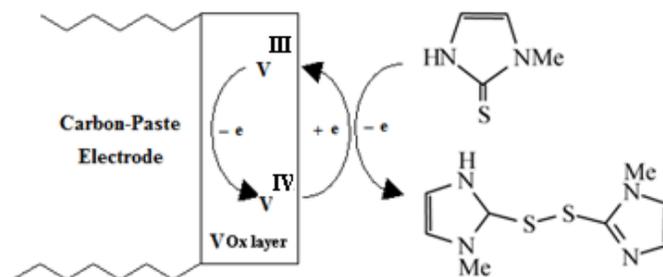
Vanadium (III) is formed in acidic solutions from V^{IV} through a disproportionation mechanism as shown in reaction b which is oxidized at the electrode surface:



Both reactions occurred at the surface of the electrode, while Reaction b occurred between the vanadium complex and the aqueous solution, the electron transfer Reaction c was conducted through the electrode. Due to the necessity of acidic conditions for disproportionation, the second pair (A₂ and C₂) was not observed in acetonitrile solution.

Electrocatalytic effect of VOx on the oxidation of MMZ

Figure 3 is a comparison between cyclic voltammograms of MMZ at the surface of an unmodified-carbon-paste electrode and VOx/CPE. As is obvious, in the presence of MMZ, the oxidation peak of V^{III} significantly increased which may be attributed to the catalytic effect on the oxidation of MMZ to the disulfide product (Aragoni et al.,



Scheme 2. Representation of electrocatalytic oxidation of MMZ.

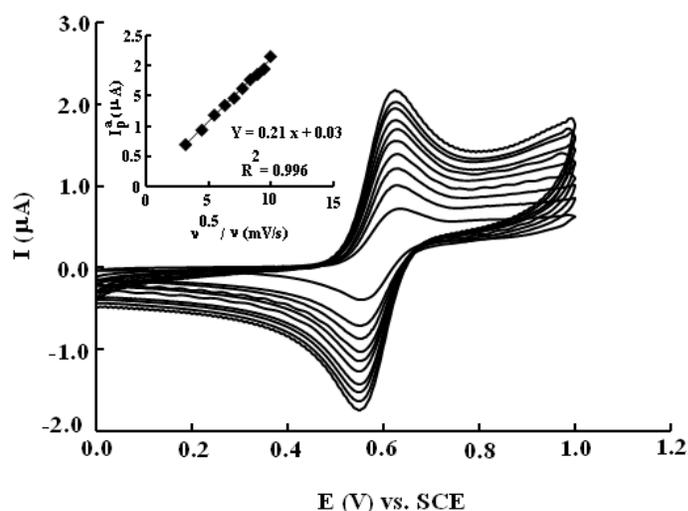


Figure 1. Cyclic voltammograms of VOx complex (1.0×10^{-4} M) in acetonitrile on a glassy carbon electrode at various scan rates: from inner to outer, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mV s⁻¹. Supporting electrolyte: tetraethylammonium perchlorate (0.1 M).

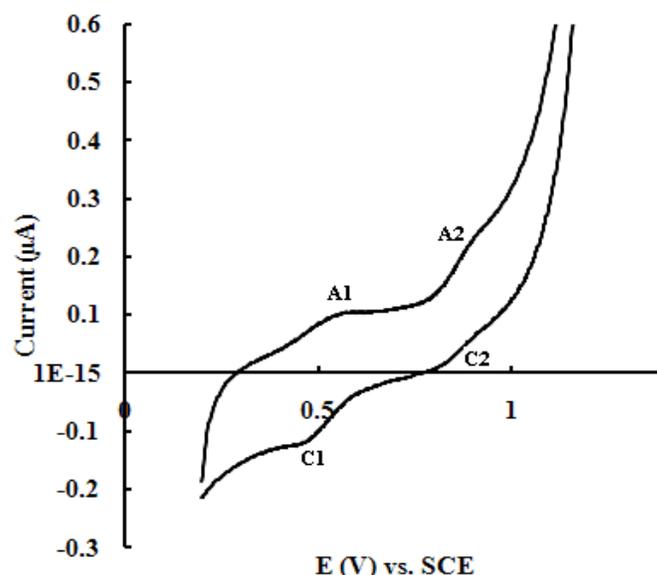


Figure 2. Cyclic voltammogram of VOx/CPE in a PBS (pH 3, 0.1 M). Scan rate, 50 mV s⁻¹.

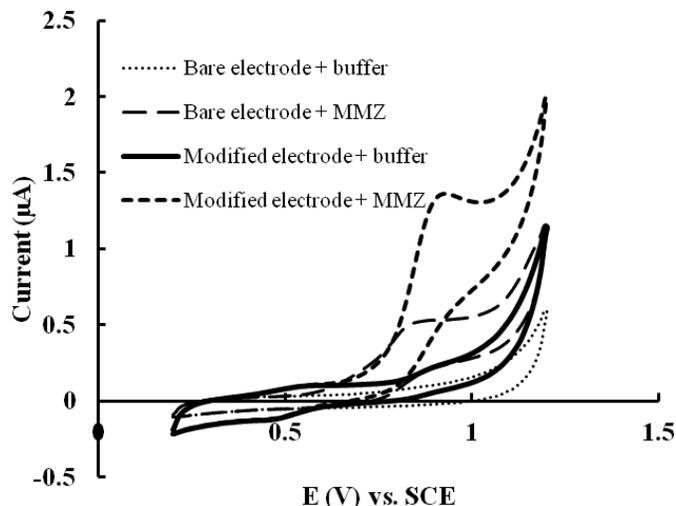


Figure 3. Cyclic voltammograms at a bare carbon-paste electrode (a) in the absence, (b) in the presence of 40 μM MMZ; and at VOx/CPE (c) in the absence and (d) in the presence of 40 μM MMZ; supporting electrolyte, PBS (pH 3.0, 0.1 M); scan rate, 50 mV s^{-1} .

2002). Scheme 2 shows the whole process at the surface of modified electrode in which a dimer is formed between two molecules of MMZ as the oxidation product.

Although MMZ was oxidizable at the surface of unmodified electrode (Figure 3) but the anodic current was much weaker than the electrocatalytic current at VOx/CPE.

The effect of pH of the solution on electrocatalytic peak current was studied. The anodic current decreased with increasing pH from 3.0 to 7.0 because protons are involved in the disproportionation reaction (Reaction b) to produce V^{III} . A pH of 3.0 was selected for the electrocatalytic oxidation of MMZ.

The effect of different scan rates (10 to 200 mV s^{-1}) on the electrocatalytic current was studied in PBS (pH 3.0) solution (Figure 4A). The results showed that the peak current increased linearly with the square root of scan rate (Figure 4B), which demonstrates that the anodic current is controlled by diffusion of MMZ to the electrode surface (Bard and Faulkner, 2001).

In order to obtain the diffusion coefficient of MMZ to the surface of VOx/CPE, static chronoamperometry was used. Figure 5A shows the chronoamperograms for four different concentrations of MMZ. Diffusion coefficient was obtained from the slopes of the linear plots of currents (I) against $t^{-1/2}$ (Figure 5B) according to Cottrell equation:

$$I = nFAD^{1/2}C^* / \pi^{1/2}t^{-1/2} \quad (1)$$

Where D and C^* are the diffusion coefficient and the bulk concentration of MMZ, respectively. A is the geometric area of the carbon-paste electrode, t is the time elapsed,

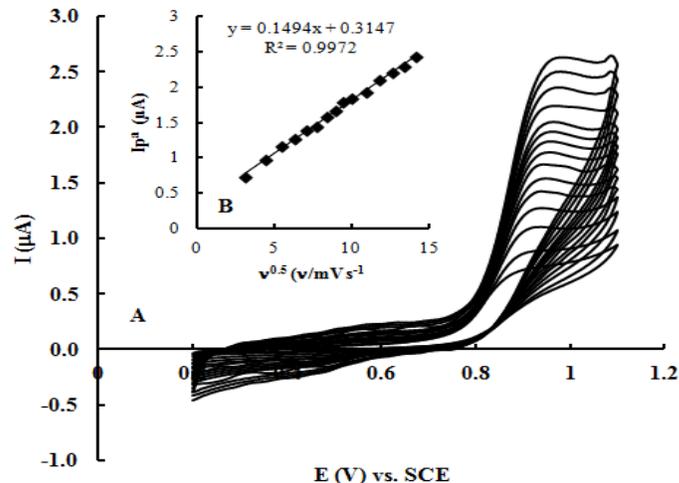


Figure 4. (A) Cyclic voltammograms at different scan rates using VOx/CPE as the working electrode. [MMZ] = 40 μM ; supporting electrolyte, phosphate buffer (pH 3.0, 0.1 M); Scan rates from down to top: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180 and 200 mV s^{-1} . (B), variation of the catalytic current versus the square root of scan rate.

and n is the number of electrons in the electrode process which is 1 in this case.

An average amount of $1.24 \times 10^{-4} \text{ cm}^2 \text{ s}^{-1}$ for D was obtained (Figure 5C). The rate constant for the chemical reaction between MMZ and redox sites in VOx/CPE, k , can be evaluated by chronoamperometry according to equation 2 (Galus, 1976):

$$I_C / I_L = \pi^{1/2} (kC^* t)^{1/2} \quad (2)$$

Where I_C is the catalytic current in the presence of MMZ, I_L is the limited current in the absence of MMZ, and k is the catalytic rate constant ($\text{cm}^3 \text{ mol}^{-1} \text{ s}^{-1}$). The calculated value of k was $1.13 \times 10^7 \text{ cm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ using the slope of the plot of I_C / I_L against $t^{1/2}$ (Figure 5D). The large value of k indicates a fast electron transfer reaction between MMZ and V^{III} ions.

Analytical aspects

Determination of MMZ

It was observed that the amount of catalytic current in cyclic voltammetry was proportional to the MMZ concentration. Since differential pulse voltammetry (DPV) has a higher sensitivity, it was used for the determination of MMZ. Figure 6A shows DPVs for various concentrations of MMZ on the surface of VOx/CPE.

The voltammetric calibration curve for the determination of MMZ, shown in Figure 6B, has a linear range from 0.9 to 100 μM with a LOD of 0.8 μM and LOQ

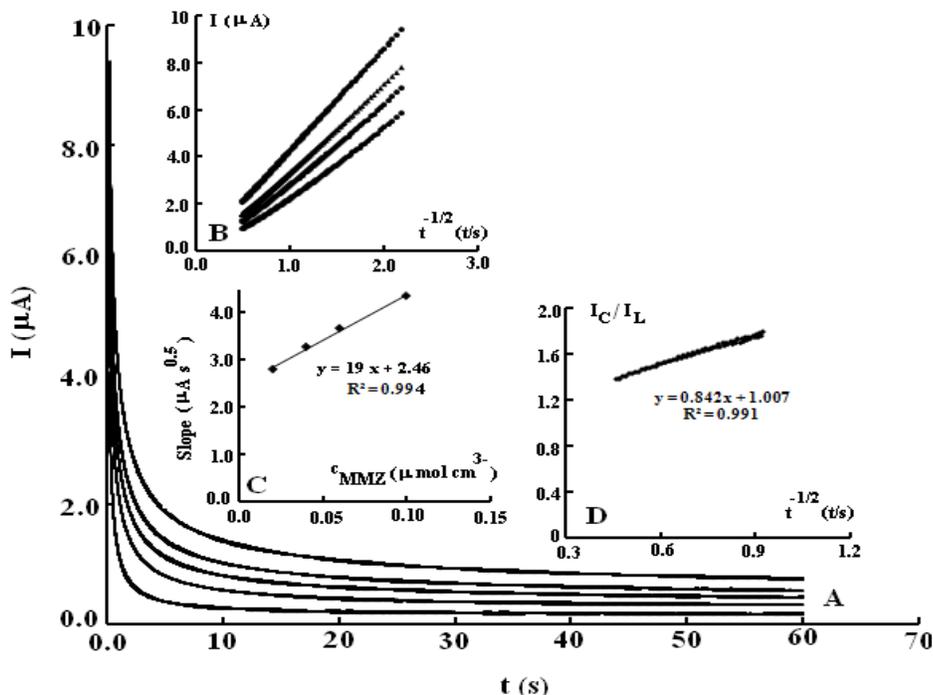


Figure 5. (A) Chronoamperograms obtained for VOx/CPE in the absence and presence of 20, 40, 60 and 100 μM MMZ in 0.1 M phosphate buffer. B) Cottrell plots for 20, 40, 60, and 100 μM MMZ. C) Dependence of slopes of Cottrell plots on concentration of MMZ. D) I_C/I_L versus $t^{-1/2}$.

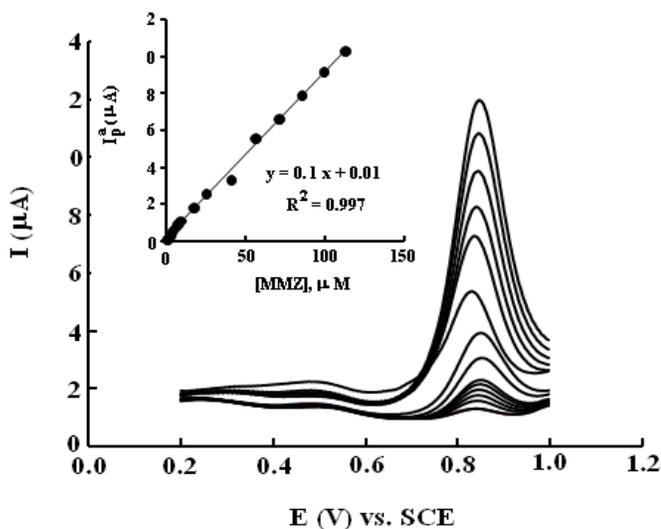


Figure 6. A) Differential pulse voltammograms for different concentrations of MMZ at VOx/ CPE. [methiamazole]: 0.9, 2.7, 4.4, 6.0, 7.6, 9.2, 17.4, 25.4, 41.1, 56.3, 71.1, 85.4, 99.2 and 112 μM in 0.1 M PBS (pH 3.0). B) Plot of electrocatalytic peak currents versus MMZ concentration.

of 2.63 μM . The slope and the correlation coefficient (r^2) for the least squares line were 0.1 $\mu\text{A}/\mu\text{M}$ and 0.997, respectively. The relative standard deviation (RSD) for four replicate measurements of 20 μM of MMZ under

optimum conditions was 1.76%. The results are comparable (Shahrokhian and Ghalkhani, 2008) or better than (Aslanoglu and Peker, 2003) the literature reports on the electrochemical determination of MMZ. The electrode could be used several times without significant loss of current response. Before each experiment the electrode was simply washed with distilled water.

Interferences

Under optimum conditions, the interference effect of some organic compounds which may be present in real samples of MMZ was studied. A concentration of 5 μM of MMZ was used and the suspicious compound was added. The tolerance limit of interferent was defined as an amount of it that retains the electrocatalytic current between 95 to 105% of its initial amount in the absence of interferent. The results show that 150-fold of amino acids asparagine, D, L-alanine, L-histidine and 40-fold glucose in the solution had almost no influence on the determination of MMZ.

Determination of MMZ in pharmaceutical preparations

In order to demonstrate the capability of VOx/CPE to the electrocatalytic quantitation of MMZ in real samples,

Table 1. Determination of MMZ in tablet samples.

Sample	MMZ (mol L ⁻¹)	Found (mol L ⁻¹)	Recovery %
1	3.8×10^{-6}	3.6×10^{-6}	95
2	5.7×10^{-6}	5.3×10^{-6}	93

tablets of MMZ (5 mg MMZ per tablet) were used as real samples. The calibration curve was plotted using the standard MMZ solutions and the amount of the drug in tablet was calculated by using the regression line equation. The results for the analysis of two different aliquots of the tablet solution are shown in Table 1. As it is shown, the recoveries are quite satisfactory and the modified electrode VOx/CPE can be successfully applied in the analysis of trace amounts of MMZ in pharmaceutical preparations.

Conclusions

In the present article, a VOx was used in the bulk-modification of a carbon-paste electrode. Cyclic voltammetry in PBS (pH 3) showed two redox couples for the vanadium complex, that is, V^{IV}/V^V and V^{III}/V^{IV} couples, in which V^{III} was produced by a disproportionation chemical reaction in acidic solution. The modifier showed an excellent electrocatalytic activity towards the oxidation of MMZ through the second redox couple, that is, V^{III}/V^{IV}. The diffusion coefficient of MMZ and the rate constant of the catalytic chemical reaction were calculated using chronoamperometry. DPV was used in the determination of MMZ in pharmaceutical preparations. The results were quite satisfactory. Ease of preparation of the modified electrode and the simple renewal procedure of it, as well as good reproducibility of the voltammetric responses make it an efficient sensor for the detection of trace amounts of MMZ in pharmaceutical samples.

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Full Length Research Paper

Neuropharmacological studies on ethyl acetate fraction of *Securinega virosa* root bark extract

Magaji Mohammed Garba^{1*}, Yaro Abdullahi Hamza², Musa Aliyu Muhammad³, Anuka Joseph Akpojo¹, Abdu-Aguye Ibrahim¹ and Hussaini Isa Marte^{1,4}

¹Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. ²Department of Pharmacology, Faculty of Medicine, Bayero University, Kano, Nigeria.

³Department of Pharmaceutical and Medicinal Chemistry, Ahmadu Bello University, Zaria, Nigeria.

⁴Department of Pharmacology, Faculty of Pharmacy, University of Maiduguri, Maiduguri, Nigeria.

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This study was conducted to evaluate the neuropharmacological activities of the ethyl acetate fraction of methanol root bark extract of *Securinega virosa* using *in vivo* models in laboratory animals. The fraction (125, 250 and 500 mg/kg) did not protect the animals against tonic hind limb extension induced by electroshock but produced a dose-dependently protection of animals against clonic spasm induced by pentylenetetrazole, with the highest protection of 66.67% produced by the highest dose tested. The fraction significantly ($P < 0.01$) and dose-dependently decreased the mean latency to sleep and increased mean sleep duration in mice treated with ketamine. However, it did not significantly increase the number of foot slips in the beam walking assay. These findings suggest that the ethyl acetate fraction of *Securinega virosa* root bark contains bioactive principle (s) that possesses sedative and anticonvulsant activities.

Key words: *Securinega virosa*, traditional medicine, epilepsy, sedative, electroshock, pentylenetetrazole, ketamine.

INTRODUCTION

Securinega virosa Roxb (Ex. Willd) Baill. family: Euphorbiaceae; is a commonly used medicinal plant which has enjoyed wide patronage among traditional practitioners in West Africa. It is a dense, low branching, many branched shrub, sometimes a small spreading tree up to about 6 m high, although, more commonly 2 to 3 m, evergreen or deciduous. It is widely distributed throughout tropical Africa (Dalziel, 1936). The local names of *S. virosa* in Nigeria include "Tsuwaawun karee, Gussu, Gwiiwar karee" (Hausa), "Iranje" (Yoruba), "Njisi nta" (Ibo), "Shim shim" (Kanuri), "kartfi-kartfi" (Shuwa arabs) and "Camal,

cambe, came" (Fulani) (Neuwinger, 1996).

The root and leaf decoctions (separately) are drunk for fever in many parts of Africa including the south-western Nigeria. In Ivory Coast, the root is said to possess analgesic property and is used for labour and other pains (Neuwinger, 1996). In many parts of Africa including the north Eastern Nigeria, the root and leafy twig decoctions are used for the treatment of epilepsy. The root decoction is used as sedative in children to send them to sleep (Robert, 1961).

Previous studies in our laboratory showed that the crude methanol extract of *S. virosa* possesses anticonvulsant and sedative activities (Magaji et al., 2007, 2008). In an attempt to isolate and characterize the anticonvulsant and sedative principles of the root bark of the plant, the crude extract was successively partitioned

*Corresponding author. E-mail: magmas1@yahoo.com. Tel: +2348034685849.

into petroleum ether, chloroform, ethyl acetate and *n*-butanol. In this study, we report the anticonvulsant and sedative properties of the ethyl acetate fraction of *S. virosa* methanol root bark extract.

MATERIALS AND METHODS

Plant

The plant material was collected in February, 2009, in Basawa-Zaria, Sabon Gari local Government area of Kaduna State, Nigeria. The plant was identified by Messrs Umar Gallah and Musa Muhammad of the Herbarium section of the Department of Biological Sciences, Ahmadu Bello University Zaria-Nigeria, by comparing with existing voucher specimen (No 918).

Extraction and fractionation

The root bark of the plant was removed and dried under shade until constant weight was obtained. It was subsequently size-reduced to obtain the powdered root bark. The powdered root bark (1000 g) was extracted with 4 litres of methanol (70%) in a soxhlet apparatus for 72 h. The resultant extract was then concentrated *in vacuo* resulting into a brownish residue (9.5% yield), subsequently referred to as crude methanol root bark extract (CME). CME (50 g) was dissolved in water, filtered and the filtrate successively partitioned with petroleum ether, chloroform, ethyl acetate and *n*-butanol. The ethyl acetate fraction was concentrated *in vacuo* affording a light brownish residue (3.4% yields) subsequently referred to as ethyl acetate fraction (EAF).

Animals

Day old Rangers cockerels (34 ± 4 g) were obtained from the National Animal Production Research Institute (NAPRI), Shika, Kaduna state, Nigeria. Swiss albino mice of either sex (20 ± 2 g) were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria, Nigeria. Mice, housed in polypropylene cages at room temperature were maintained on standard rodent feed and water *ad libitum*. All experimental protocols were in accordance with the Ahmadu Bello University Research policy and ethic and regulations governing the care and use of experimental animals as contained in "Principles of laboratory animal care" (NIH Publication no. 85-23, revised 1985). The experiments were conducted in quiet laboratory between hours of 900 h to 1600 h.

Drugs/chemicals and treatment

EAF, normal saline, ketamine, diazepam, phenytoin and sodium valproate were administered via the intraperitoneal routes. Pentylentetrazole was given subcutaneously. All administrations were at volumes equivalent to 10 ml/kg.

Phytochemical screening

Crude methanol root bark extract and EAF were screened for the presence of alkaloids, tannins, saponins, flavonoids and cardiac glycosides using standard protocols previously described by Silva et al. (1998).

Acute toxicity study

Median lethal doses for crude methanol extract and EAF in mice were estimated using the intraperitoneal route. Briefly, the method was divided into two phases. In the initial phase, 3 groups of three mice each were treated with the fraction at doses of 10, 100 and 1000 mg/kg body weight *i.p.* and observed for signs of toxicity and death for 24 h. In the second phase, 3 groups each containing one mouse was treated with three more specific doses of the fraction (1600, 2900 and 5000 mg/kg) based on the outcome of the first phase. The lethal dose (LD₅₀) value was estimated by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived (Lorke, 1983).

Maximum electroshock-induced seizure in chicks

Tonic hind limb extensions in chicks were induced by passing alternating electrical current (200 Hz, 90 mA, 0.8 s, 0.8 ms) through corneal electrode (Swinyard and Kufferberg, 1985; Sayyah et al., 2002). Thirty minutes before electroshock, five groups of chicks (n = 10) were treated with normal saline, EAF (125, 250 and 500 mg/kg) or phenytoin (20 mg/kg). Ability to prevent tonic hind-limb extension or to prolong its latency was considered as an indication of anticonvulsant activity (Swinyard, 1969; Sayyah et al., 2002).

Pentylentetrazole-induced seizure in mice

Clonic seizures were induced in mice (n = 6) by treatment with 85 mg/kg (CD₉₇) pentylentetrazole (Swinyard et al., 1989). Thirty minutes before treatment with the convulsant, mice were treated with normal saline, EAF (125, 250 and 500 mg/kg) or sodium valproate (200 mg/kg). Absence of an episode of clonic spasm of at least 5 s duration indicated a protective effect. The latency to the clonic spasm for each unprotected animal was also noted.

Ketamine induced sleep test in mice

The method previously described by Mimura et al. (1990) was adopted. Thirty minutes post-treatment with normal saline, EAF (125, 250 and 500 mg/kg) or diazepam (0.5 mg/kg), animals (n = 6) were administered with ketamine (100 mg/kg). The time interval between ketamine administration and loss of righting reflex was considered as latency to sleep while the time from the loss to regaining of righting reflex as the duration of sleep (Bastidas Ramirez et al., 1998; Rabbani et al., 2003).

Beam walking assay in mice

Mice previously trained to walk along a horizontal ruler (80 × 3 cm) from a start platform to a goal box (a hamster house) were used for the study. Thirty minutes post-treatment with the normal saline, EAF or the positive control (diazepam, 0.5 mg/kg), each mouse was placed on the beam (60 cm long and 8 mm in diameter) at one end and allowed to walk to the goal box. Mice that fell were returned to the position they fell from, with a maximum time of 60 s allowed on beam. The number of foot slips (one or both hind limb slipping from the beam) was recorded with the aid of a tally counter (Stanley et al., 2005).

Statistical analysis

Results were expressed as mean ± standard error of mean.

Table 1. Phytochemical constituents present in the methanol root bark extract of *Securinega virosa* and its Ethyl acetate fractions.

Constituent	MRBE	EAF
Tannins	+	+
Flavonoids	+	+
Cardiac glycosides	+	+
Saponins	+	-
Alkaloids	+	-
Steroid/Terpenoids	+	-
Anthraquinone	-	-

CME: Methanol root bark extract of *Securinega virosa*; EAF: ethyl acetate fraction of methanol root bark extract of *Securinega virosa*; +: present; -: absent.

Statistical analysis was performed by analysis of variance (ANOVA); when a statistically significant result was obtained with ANOVA, a post hoc Dunnett's t-test was performed for multiple comparisons. Values of $P < 0.05$ were considered significant.

RESULTS

Acute toxicity study

The intraperitoneal median lethal dose of EAF in mice was found to be 2154 mg/kg while that of the crude methanol root bark extract was found to be 774.6 mg/kg.

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of flavonoids, tannins and cardiac glycosides in EAF. However, it was negative for saponins and alkaloids found to be present in the crude methanol root bark extract (Table 1).

Maximal electroshock test in chick and pentylenetetrazole-induced seizure in mice

EAF did not protect the animals against tonic hind limb extension induced by electroshock; neither did it reduce the recovery time in the unprotected animals. Conversely, phenytoin (20 mg/kg) protected 100% of the animals against convulsion. EAF produced a dose-dependent protection of animals against clonic spasm induced by pentylenetetrazole, with the highest protection of 66.67% produced by the highest dose tested; 500 mg/kg. EAF also produced a significant ($P < 0.05$) reduction in the mean onset of seizure in the unprotected animals compared with normal saline treated control (Table 2).

Ketamine induced sleep test in mice

EAF significantly ($P < 0.01$) and dose-dependently decreased the mean latency to sleep and increased mean sleep duration in mice treated with ketamine (Figure 1).

Beam walking assay in mice

EAF did not significantly affect the foot slips in mice. In contrast, diazepam (0.5 mg/kg) significantly increased the mean number of foot slips compared with normal saline treated control (Figure 2).

DISCUSSION

In the present study, we used maximal electroshock test and pentylenetetrazole induced seizure models to evaluate the anticonvulsant effects of ethyl acetate fraction of methanol root bark extract of *S. virosa*. Expectedly, the standard drugs used in both models; phenytoin and sodium valproate for maximal electroshock (MES) and pentylenetetrazole (PTZ)-induced seizure, respectively, offered 100% protection in the animals. However, the ethyl acetate fraction did not protect the animals in the MES model suggesting that its activities may not involve prevention of seizure spread from an epileptic foci; and it may not be beneficial in the management of generalized tonic-clonic seizure. The fraction protected 66.67% of the animals against PTZ and significantly increased the mean onset of seizure in unprotected animals. PTZ test identifies compounds that can raise seizure threshold in the brain (White et al., 1998) and drugs that reduce T-type Ca^{2+} currents, such as ethosuximide have been found to be protective against seizures induced by PTZ.

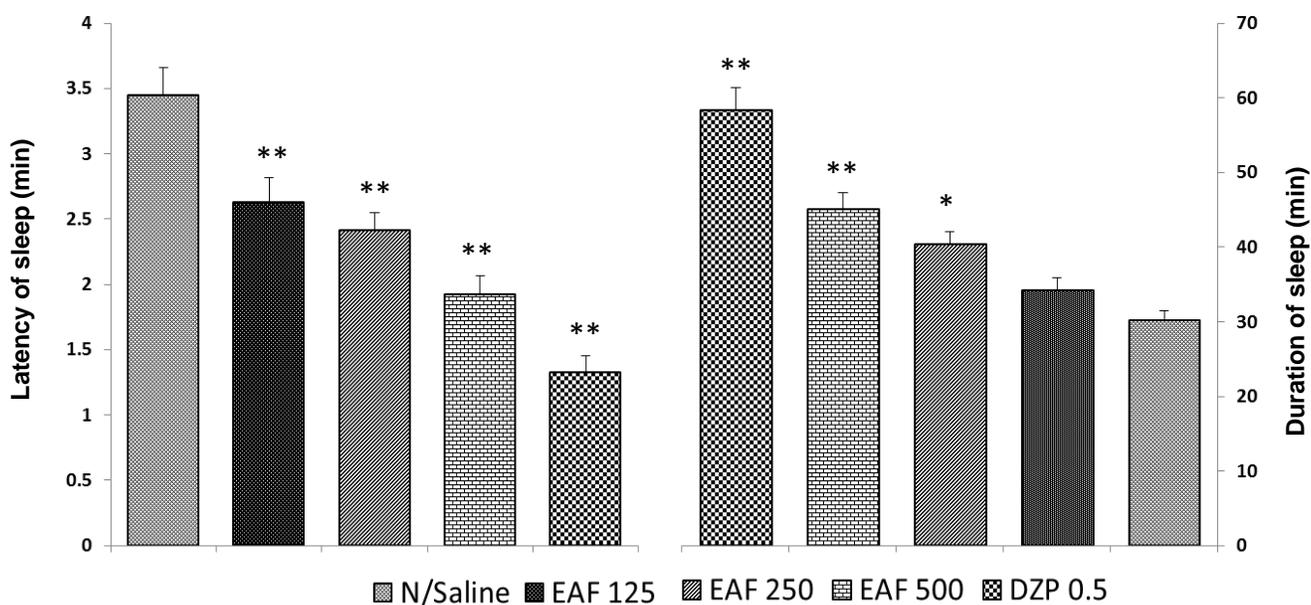
Previously, PTZ has been reported to interact with gamma aminobutyric acid (GABA) neurotransmitters and the GABA receptor complex (Loscher and Schmidt, 1988; De Deyn et al., 1992). Dopaminergic mechanism has also been implicated in PTZ-induced seizures. Drugs such as felbamate, which block glutamatergic excitation mediated by N-Methyl-D-aspartate (NMDA) receptors, have demonstrated activity against PTZ-induced seizures, suggesting the involvement of NMDA system in the initiation and propagation of PTZ-induced seizures (MacDonald and Kelly, 1993). It is therefore possible to suggest that the anti-PTZ activity of the fraction may involve one or more of these aforementioned mechanisms.

The ability of the fraction to reduce the latency to sleep and increase the total sleep time is an indication of its sleep inducing property. Similar influence in sleep indices were noticed with diazepam. However, greater reduction in the onset of sleep and increase in the total sleep time

Table 2. Effect of ethyl acetate fraction of methanol root bark extract of *Securinega virosa* against pentylenetetrazole-induced seizure and maximal electroshock tests.

Treatment (mg/kg)	MES		PTZ	
	Quantal protection against seizure	Mean recovery time (min)	Quantal protection against seizure	Mean onset of seizure (min)
N/Saline	0/10	5.86±1.58	0/6	3.51±0.43
EAF 125	0/10	7.22±0.88	2/6	8.99±2.85*
EAF 250	0/10	7.44±0.77	2/6	6.93±2.01*
EAF 500	0/10	8.00±0.85	4/6	7.12±1.87
PHT 20	10/10	-		
VPA 200			6/6	-

Protection against seizure expressed as quantal protection; Onset of seizure and recovery time were expressed as mean ± SEM; N/saline (normal saline); MES (maximal electroshock); PTZ (pentylenetetrazole); EAF (ethyl acetate fraction); VPA (sodium valproate); PHT (phenytoin); *P < 0.05; compared with N/Saline (10 ml/kg)-treated control.

**Figure 1.** Effects of ethyl acetate fraction (EAF) and diazepam (DZP) on ketamine-induced sleep in mice. Latency to sleep and duration of sleep were expressed as mean ± SEM; *P < 0.01; **P < 0.001; compared with N/Saline (10 ml/kg)-treated control.

produced by the standard agent (diazepam) is indicative of lesser sedative potential of the fraction.

The fraction did not significantly increase the number of slips in the beam walking assay for motor coordination, an indication that its effect may be centrally mediated and not due to peripheral muscular blockade (Perez et al., 1998). Diazepam significantly increased the number of slips. This result is consistent with previous data which indicated that benzodiazepines induce motor coordination deficit in the beam walking assay (Stanley et al., 2005; Danjuma et al., 2009).

Previous works have reported the anticonvulsant and sedative properties of flavonoids (Johnston, 2005; Yao et al., 2010; Hanrahan et al., 2011). Flavonoids, found to be present in the ethyl acetate fraction of methanol root bark extract of *S. virosa*, may therefore be responsible for the observed anticonvulsant and sleep promoting activities. Further purification and pharmacological investigations are needed to identify the active principle(s) responsible for the anticonvulsant and sedative properties of the ethyl acetate fraction of the *S. virosa* methanol root bark extract.

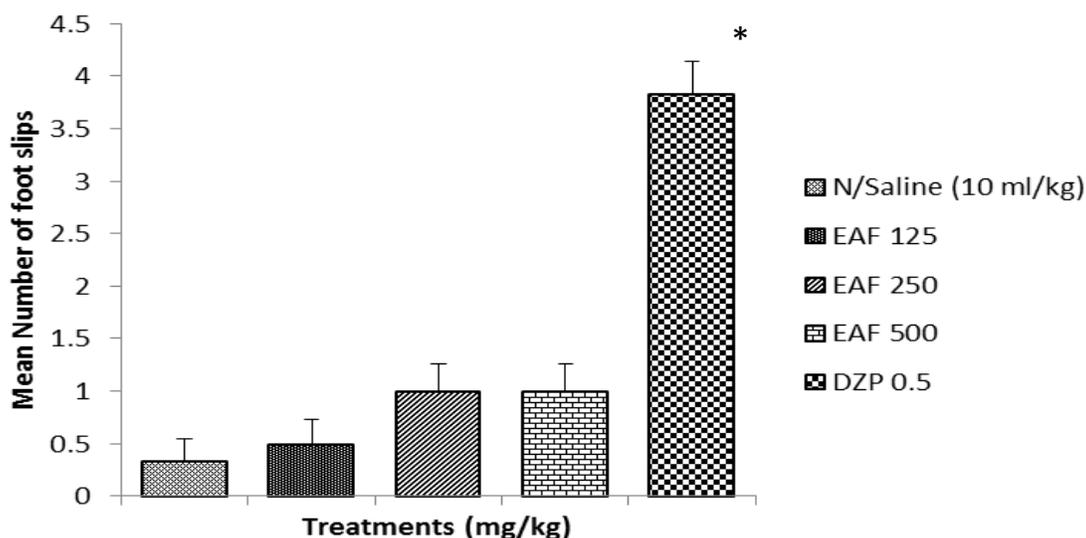


Figure 2. Effects of ethyl acetate fraction (EAF) and diazepam (DZP) on motor coordination in mice. Number of foot slips expressed as mean \pm SEM; * $P < 0.05$, compared with normal saline treated group.

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Full Length Research Paper

Ameliorative effects of rutin and ascorbic acid combination on hypercholesterolemia-induced hepatotoxicity in female rats

Abdulaziz M. Aleisa¹, Hatem M. Abuohashish¹, Mohammed M. Ahmed², Salim S. Al-Rejaie^{1,2*}, Osama A. Alkhamees³ and Abdulaziz S. Alroujaye³

¹Department of Pharmacology and Toxicology, College of Pharmacy, P. O. Box 55760, King Saud University, Riyadh 11544, Saudi Arabia.

²Experimental Animal Care Center, College of Pharmacy, P. O. Box 55760, King Saud University, Riyadh 11544, Saudi Arabia.

³Department of Pharmacology, College of Medicine, A1 Imam Mohammad Ibn Saud Islamic University, Riyadh P. O. Box 11623, Saudi Arabia.

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It has been shown that female rats are more responsive to dietary cholesterol challenge than male and also, hypercholesterolemia induction is easier in female rats. The present study was designed to investigate the effects of rutin (RT) and ascorbic acid (AA) combination on high cholesterol diet (HCD)-induced hepatic damage in female Wistar rats. Rats were randomly divided into four groups and fed by respective diets for 6 consecutive weeks. Hepatic enzymes activity and lipid profile were estimated in plasma samples. Nucleic acids, total proteins, malondialdehyde (MDA), glutathione (GSH), total cholesterol (TC) and triglycerides (TG) levels were measured in liver. Histopathological changes were observed in hepatic tissue. Enzymatic activity and lipid profile increased significantly in plasma of HCD fed rats, which was normalized in HCD+RT+AA group. In hepatic cells, total protein, nucleic acids and GSH levels were significantly decreased, while MDA, TC and TG levels were increased by HCD. These changes were significantly corrected in HCD+RT+AA group. The apparent protection was further confirmed by the histopathological screening. In conclusion, the study provides the presence of oxidative stress in hypercholesterolemic female rats and suggests beneficial effects of RT and AA combinations in combating the oxidative process in nonalcoholic fatty liver disease.

Key words: Rutin, ascorbic acid, hypercholesterolemia, oxidative stress.

INTRODUCTION

Hypercholesterolemia is considered as one of the most familiar metabolic disorders and it is closely associated with obesity, diabetes mellitus, and several other metabolic syndromes (Farrell et al., 2008; Postic and Girard, 2008; Trauner et al., 2010). Hypercholesterolemia can eventually lead to nonalcoholic fatty liver disease

(NAFLD) by depositing the lipids and triglycerides in liver which is usually progress to cirrhosis or even hepato cellular carcinoma (Kim et al., 2012; Lee et al., 2007). Experimentally induced hypercholesterolemia can impairs lipid metabolism leading to elevation of both blood and tissue lipid profile (Vasu et al., 2005). Moreover, studies demonstrated that even short exposure to high cholesterol diet (HCD) is capable of inducing hypercholesterolemia and is significantly associated with oxidative stress (Tomofuji et al., 2006).

Oxidative stress and generation of reactive oxygen

*Corresponding author. E-mail: rejaie@ksu.edu.sa. Tel: +96614677178. Fax: +96614677200.

species (ROS) are deemed to play a vital role in hepatocyte apoptosis and in the pathogenesis of NAFLD (Kojima et al., 2007; Trauner et al., 2010). Hypercholesterolemia was shown to impair oxidative stress biomarkers such as malondialdehyde (MDA) and superoxide dismutase (SOD) and also known to boost ROS production via different mechanisms and hence increase lipid peroxidation. Studies demonstrated that oxidative stress associated with hypercholesterolemia have harmful effect on different organs particularly heart, liver, and kidney. Moreover, generation of ROS have been implicated in the pathophysiology of various disease including; heart failure (Prasad et al., 1996), ischemic heart disease (Ferrari et al., 1998), hepatic injury (Jarrar et al., 2000), and chronic renal damage and failure (Baker et al., 1985; Galle, 2001). Therefore, it is necessary to search for effective approaches to control hypercholesterolemia and the associated fatty liver complication. Non pharmacological approaches for hypercholesterolemia include increased physical activity and weight reduction through lifestyle modification as well as dietary changes (Kim et al., 2012). Antioxidant supplementations may effectively suppress oxidative stress, which seems to be a useful therapy (Yang et al., 2012).

Rutin (RT), a quercetin-3-rutinosid or vitamin-P, is a well known flavonoidal glycoside and set as an affective phenolic compound. It is an antioxidant, which comprised of the flavanolquercetin and the disaccharide rutinose (Ihme et al., 1996; Lindahl and Tagesson, 1997). Phenolic compounds are mainly found in onions, apples, tea and red wine (Hertog et al., 1993; Khan et al., 2012). Various pharmacological properties were reported for rutin including antibacterial, antitumor, anti-inflammatory, anti-diarrheal, antiulcer, anti-mutagenic, vasodilator and immunomodulator (Janbaz et al., 2002). Moreover, rutin has inhibitory effects against membrane lipid peroxidation and generation of ROS like in other plant materials were reported (Lopez-Revuelta et al., 2006; Wang, 2012; Yin et al., 2012) and can suppress adipocyte differentiation from pre-adipocytes (Choi et al., 2006). In addition, rutin can also decrease the level of TBARS and increase the SOD activity suggesting a possible protective role in oxidative stress-mediated diseases (Park et al., 2002). On the other hand, ascorbic acid (AA; as a reduced form of vitamin C) is a famous effective antioxidant. Ascorbic acid is the most predominant form of vitamin C in the human body and is involved in tissue growth and repair. It is a water-soluble enzyme cofactor, abundantly present in different plants and animals. AA has a powerful antioxidant activity, which made it well known to protect tissues from oxidative injury via efficiently quenching the damaging free radicals produced by different biological processes (Heaney et al., 2008; Verrax and Calderon, 2008).

When multiple antioxidants are used in combination, they protect against vulnerability to other agents and

synergistically potentiate their antioxidant properties. These synergistically potentiated antioxidant effects of agents contribute to the improvement of cognitive function. Thus the present study was designed to investigate the additive hepatoprotective effects of RT and AA combination against hypercholesterolemia induced oxidative injury following HCD supplementation to female Wistar rats.

MATERIALS AND METHODS

Animals

Twenty four young female Wistar albino rats, roughly the same age of 7 weeks, weighing 80 to 100 g were supplied from the Experimental Animal Care Center (King Saud University, Riyadh, Saudi Arabia). The animals were acclimatized to laboratory conditions before the tests for ten days. They were fed on Purina rat chow diet (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and water *ad libitum* and were maintained under standard conditions of temperature ($22\pm 1^\circ\text{C}$), humidity (50 to 55%), and light (12 h light/dark cycles). All methods including euthanasia procedure were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Institute of Health (NIH Publications No. 80-23; 1996) and it was approved by the Ethics committee of Experimental Animal Care Center, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia).

Dietary protocol

Experimental diets were prepared in pellet form by adding 0.1% RT + 0.2% AA (RT+AA), 1% cholesterol + 0.5% cholic acid (HCD) or 0.1% RT + 0.2% AA (RT+AA) + 1% cholesterol + 0.5% cholic acid (HCD+RT+AA) in rat chow powder. The diets were prepared weekly and shade dried. Animals were randomly divided into four groups (six in each group); (1) Control (rat cow), (2) RT+AA, (3) HCD and (4) HCD+RT+AA. All animals were kept on free access to food and water during the whole experimental period for six weeks. At the end of the experiment, animals were sacrificed by decapitation and the trunk blood was collected in heparinized tubes. Liver tissues were dissected, weighed and immediately dipped in liquid nitrogen for one min and then preserved at -75°C (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, USA) till analysis. Plasma samples were collected after centrifugation at 4000 rpm for 15 min and stored in freezer at -20°C till analysis.

Blood chemistry

Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), TG, TC, high density lipoprotein-cholesterol (HCD-C) and low density lipoprotein-cholesterol (LDL-C) were estimated by using commercially available diagnostic kits (Human, Wiesbaden, Germany).

Estimation of nucleic acids and total protein levels in liver

Nucleic acids (DNA and RNA) were estimated in liver by the method described by Bregman (1983). In brief, hepatic tissues were homogenized in ice-cold distilled water, and then the homogenates

Table 1. Effects of rutin (RT) and ascorbic acid (AA) combination on plasma level of AST, ALT, ALP, TG, TC, HDL-C and LDL-C, in high-cholesterol diet (HCD) fed rats for six consecutive weeks.

Parameter	Control	RT + AA	HCD	HCD + RT + AA
AST (U/L)	39.96 ± 3.36	41.04 ± 7.20	49.59 ± 4.41 **	39.39 ± 4.56 #
ALT (U/L)	20.65 ± 1.59	21.85 ± 2.37	24.69 ± 3.29 *	21.29 ± 1.54 #
ALP (U/L)	270.65 ± 34.85	281.21 ± 30.55	408.04 ± 48.70 ***	330.84 ± 18.54 ###
TG (mg/dl)	65.62 ± 4.86	64.72 ± 9.62	156.80 ± 24.59 ***	120.93 ± 7.89 ###
TC (mg/dl)	70.06 ± 22.08	65.94 ± 8.54	139.39 ± 24.61 ***	99.88 ± 12.04 ##
HDL-C (mg/dl)	27.43±4.95	28.36±2.6	20.35±1.89 **	27.51±2.69 ##
LDL-C (mg/dl)	48.32±4.24	46.04±5.03	86.62±6.88 ***	56.65±4.58 ###

Data were expressed as mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. *P<0.05, **P<0.01 and ***P<0.001 HCD and RT+AA vs. Control; #P<0.05, ##P<0.01 and ###P<0.001 HCD+RT+AA vs. HCD.

were suspended in 10% ice-cold trichloroacetic acid (TCA). Pellets were extracted twice with 95% ethanol. For quantification of DNA levels, the nucleic acids extract was treated with diphenylamine reagent and the intensity of blue color was measured at 600 nm. RNA levels were estimated by treating the nucleic acids extract with orcinol reagent and the green color was recorded at 660 nm on spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The modified Lowry method by Schacterle and Pollack (1973) was used to estimate liver levels of total protein. Bovine plasma albumin was used as standard.

Estimation of MDA in liver

The method described by Ohkawa et al. (1979) was used to determine MDA concentrations in liver. Briefly, 200 mg of liver samples were homogenized in aqueous 0.15 M KCl solution to give 10% homogenate. 1 ml of homogenate was then mixed with one ml of 10% TCA and centrifuged at 3,000 rpm for 15 min. 1 ml of supernatant was suspended into 1 ml of 0.67% 2-thiobarbutaric acid. Sample tubes were then placed into a boiling water bath and kept for 15 min. Samples were allowed to cool down at room temperature followed by centrifugation at 3000 rpm for 15 min. The optical density of the clear pink supernatants was measured at 532 nm.

Estimation of GSH level in liver

The concentration of GSH was determined as described by Sedlak and Lindsay (1968). Briefly, 200 mg from liver samples were dissected out and homogenized in ice-cold 0.02 M ethylenediaminetetraacetic acid (EDTA). An aliquots of 0.5 mL of tissue homogenate was mixed with 0.2 M Tris buffer, pH 8.2 and 0.1 mL of 0.01 M Ellman's reagent, [5,5'-dithiobis-(2-nitro-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 g at room temperature for 15 min. The absorbance of the clear supernatants was measured using spectrophotometer at 412 nm in one centimeter quartz cells.

Estimation of lipid contents in liver

Total cholesterol and triglycerides levels in liver tissues were estimated by using Folch et al. (1957) method. In brief, liver tissues were homogenized in 0.15 mol/L of ice-cold KCl (10% w/w) and

lipids were extracted with chloroform:methanol (2:1). After the extraction and evaporation, tissue lipids were re-dissolved in isopropanol, and liver cholesterol and triglyceride levels were estimated enzymatically by commercially available kits (Human, Wiesbaden, Germany).

Histopathological evaluation

Randomly two rats from each group: control, HCD, and HCD+RT+AA were taken for histopathological examination. The cross-section from each liver was fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 3 µm, stained with Hematoxylin and Eosin (H & E) stain and placed in slides for light microscopic examination. Slides were evaluated by a histopathologist who was blinded to the treatment groups to avoid any kind of bias.

Statistical analysis

All data were expressed as mean ± Standard Deviation (SD) and statistically analyzed using one-way ANOVA followed by Student-Newman-Keuls multiple comparisons test. The differences were considered statistically significant at P<0.05. Graph Pad prism program (version 5) was used as analyzing software.

RESULTS

In HCD fed rats, mean liver weights were significantly increased as compared to control animals. Combined supplementation of RT and AA along with high cholesterol caused significant attenuation in liver weights compared to HCD group (Figure 1).

Plasma enzymatic activities of AST, ALT and ALP were significantly increased in HCD fed rats compared to control group. These activities were significantly inhibited in HCD+RT+AA group when compared to HCD fed animals (Table 1). Plasma lipid levels including TC, TG and LDL-C were significantly increased in HCD fed rats as compared to control group of animals. In contrast,

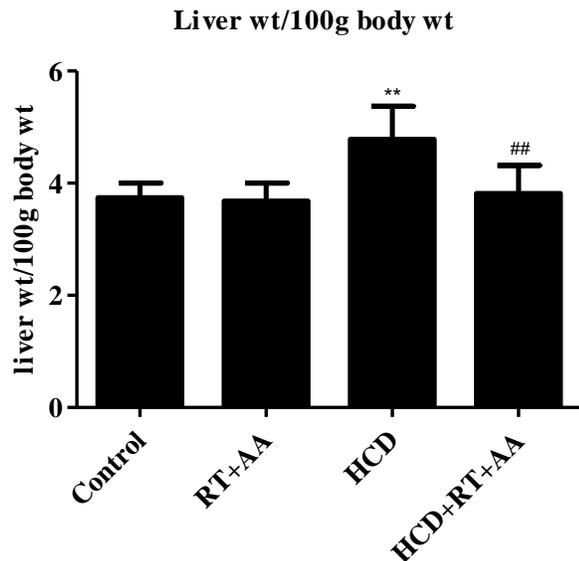


Figure 1. Effects of rutin (RT) and ascorbic acid (AA) combination on liver weight per 100 gram body weight in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. *P<0.01 HCD and RT+AA vs. Control group; ##P<0.01 HCD+RT+AA vs. HCD group.

plasma HDL-C levels were significantly decreased in HCD fed animals compared to controls. In HCD+RT+AA group, the mean levels of TC, TG and LDL-C were significantly decreased as compared to HCD fed rats respectively. This combined therapy of vitamins to HCD fed rats also significantly elevated the HCD-cholesterol levels compared to HCD group (Table 1).

In hepatic cells, DNA and RNA levels significantly decreased from 182.10 ± 8.91 to 157.53 ± 7.42 $\mu\text{g}/100$ mg and 612.88 ± 26.53 to 539.13 ± 26.45 $\mu\text{g}/100$ mg in HCD fed rats respectively. Similarly total protein levels also decreased from 15.26 ± 0.35 to 14.46 ± 0.31 mg/100 mg and it was found to be statistically significant. Feeding of animals with RT and AA in combination showed significant enhancement in the reduced levels of DNA, RNA and total protein when compared to HCD group respectively (Figure 2).

Oxidative markers showed significant changes in hepatic cells of HCD fed rats such as MDA levels increased and GSH levels decreased significantly while compared to control animals. The combined supplementation of RT and AA along with high cholesterol significantly brings back the MDA and GSH levels near to normal values (Figure 3).

Hepatic lipid compounds including TC and TG (mg/g tissue) levels were significantly increased in HCD fed rats compared to control animals. As similar to plasma lipid

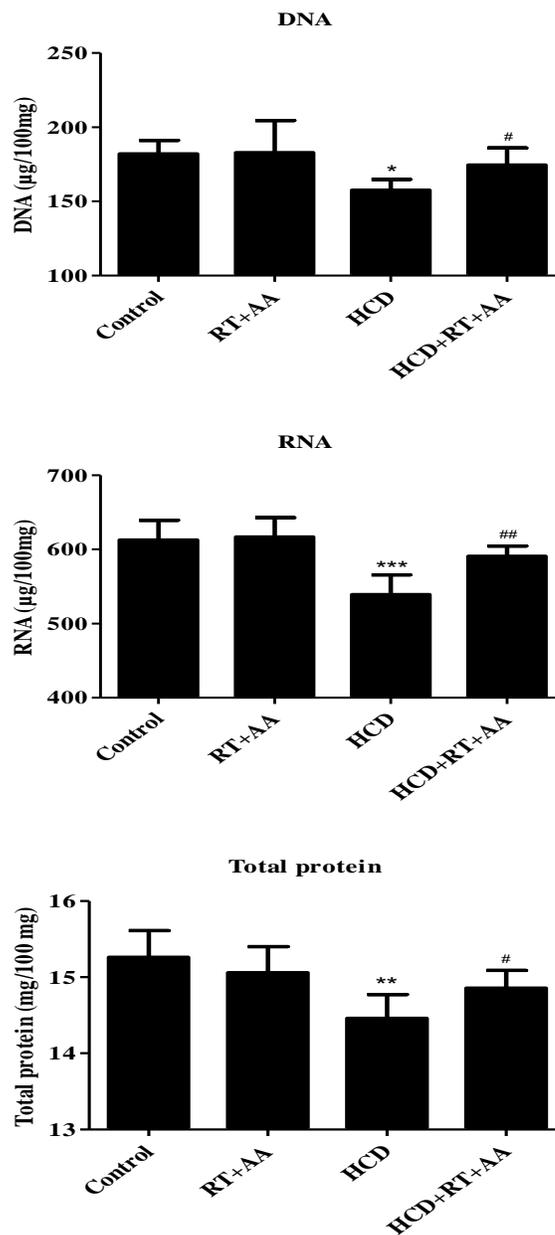


Figure 2. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic nucleic acids and total protein level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. *P<0.05, **P<0.01 and ***P<0.001 HCD and RT+AA vs. Control group; #P<0.05 and ##P<0.01 HCD+RT+AA vs. HCD group.

levels, RT and AA combined supplementation to HCD fed rats significantly protected the elevated levels of these lipids in hepatic cells as compared to HCD group (Figure 4).

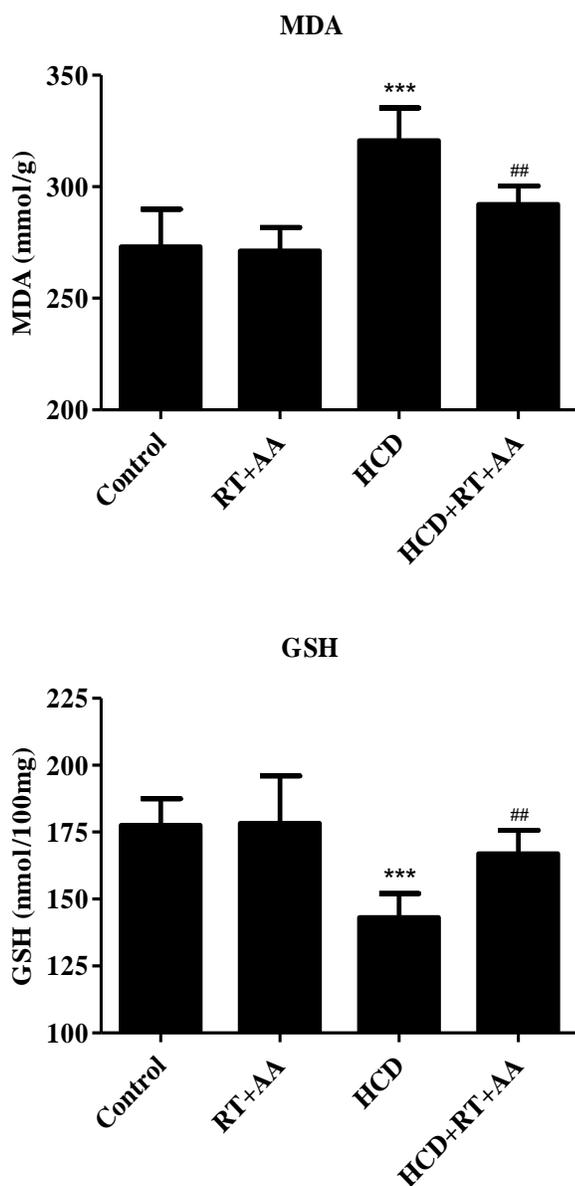


Figure 3. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic MDA and GSH level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. ^{***}P<0.001 HCD and RT+AA vs. Control group; ^{##}P<0.01 HCD+RT+AA vs. HCD group.

Histopathological changes in liver cross sections were presented in Figure 5. In control group, hepatocytes are looking benign and normal. Liver section from HCD group revealed scattered foci of steatohepatosis of liver, hepatocytes with swollen, epithelial cells associated with scattered foci of periportal to lobular inflammatory cell infiltrates. In conclusion, histopathological diagnosis

showed moderate degree of hepatotoxicity in HCD fed rats. Liver sections from HCD+RT+AA group showed benign looking hepatocytes separated by congested central veins. Lobular lymphocytic infiltrate were noticed in few areas with no regenerating nodules or fibrosis. Finally, the histopathological diagnosis was mild degree of hepatotoxicity in RT and AA supplemented along with HCD fed rats.

DISCUSSION

In this study, combined protective effects of RT and AA on hypercholesterolemia induced hepatotoxicity were investigated in female Wistar albino rats. Hypercholesterolemia was induced by following HCD supplementation for six consecutive weeks and that has conformed through the biochemical and histopathological changes. Numerous studies reported that HCD has the ability to induce hepatotoxicity and contribute in causing fatty liver (Hirako et al., 2011; Park et al., 2002; Wang et al., 2011). Furthermore, hypercholesterolemic diet supplementation causes oxidative damage in liver by increasing enzymes and lipid profile (Choe et al., 2001; Katsube et al., 2006). The major hypothetical view of this study is to project the efficiency of antioxidant vitamins combined therapy against hypercholesterolemia-induced hepatotoxicity in female rats.

The present data of liver weights are in agreement with these reports as weights were significantly increased by HCD supplementation compared to controls. Histopathological screening also revealed the changes induced in liver of HCD supplemented rats by showing fat accumulation and inflammatory infiltrates. Park and his colleagues (2002) demonstrated that, HCD induces hypercholesterolemia which may contribute to cause oxidative stress and ROS generation. In another study, Balkan et al. (2002) reported that HCD-induced elevation in hepatic and plasma levels of lipids as well as oxidative enzymes. Our present results justified these studies when the plasma liver enzymes (ALT and AST) and lipid profile (TC, TG and LDL-C) levels significantly increased in HCD fed animals. The significant reduction in the HCD induced elevation in liver enzymes by RT and AA combination indicated that the combined antioxidant therapy is effective against oxidative process. These findings are in agreement with earlier experimental studies, where rutin and ascorbic acid individually were found to have protective effects against the oxidative stress (Abhilash et al., 2012; Banerjee et al., 2009; Janbaz et al., 2002). In the present study, lipid peroxidation markers including MDA and GSH revealed hepatic oxidative damage in HCD fed rats by significantly increasing MDA levels and decreasing the GSH levels while compared to control animals. Such alterations in lipid peroxidation products were also observed in brain, kidney and erythrocytes

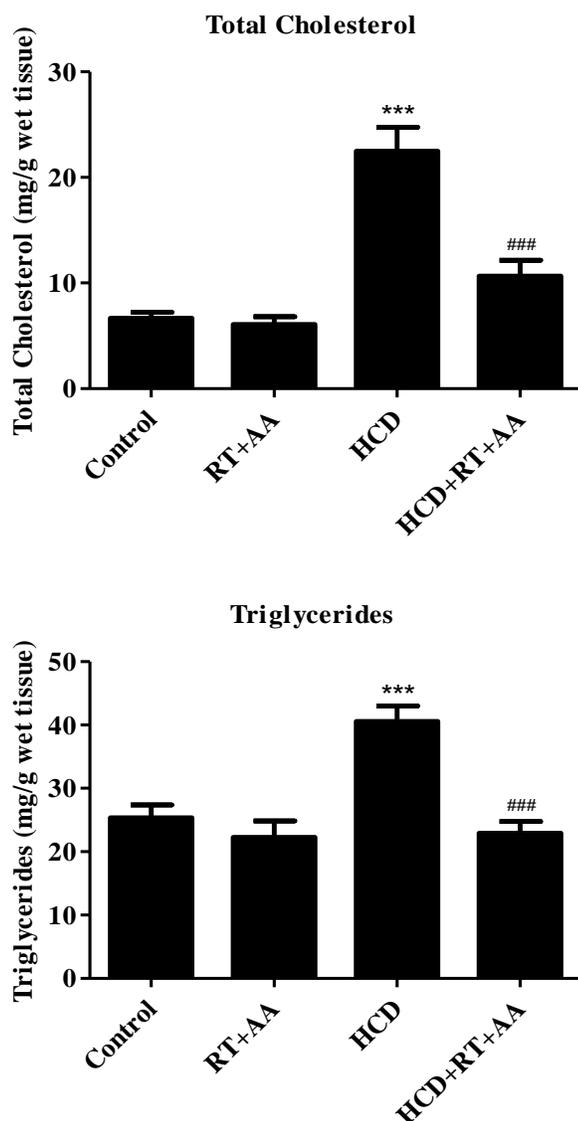


Figure 4. Effects of rutin (RT) and ascorbic acid (AA) combination on hepatic total cholesterol and triglycerides level in high-cholesterol diet (HCD) fed rats following 6 weeks of supplementation. Data were expressed as Mean±S.D and analyzed using one-way ANOVA followed by Student Newman-Keuls method as post hoc test. Six rats were used in each group. ^{***}P<0.001 all HCD and RT+AA vs. Control group; ^{###}P<0.001 HCD+RT+AA vs. HCD group.

of animals fed on HCD (Montilla et al., 2006). Moreover, HCD is reported to alter lipid composition in cell membranes and hence, the extracellular matrix to be more prone to free radical generation (Scheuer et al., 2000). Similar changes were observed in the present study, with significant increase at TC and TG levels of hepatic cells in HCD fed rats as compared to control animals. Furthermore, the present results showed the reduction in hepatic nucleic acids and total protein levels in HCD

in HCD fed animals providing an evidenced for intracellular cytotoxicity.

Rutin is one of the flavonoids glycoside known as vitamin-P, which is widely accepted as physiologic antioxidants. Flavonoids are now believed to have a strong potential to protect against the many degenerative diseases linked to free radical-related tissue damage due to their capacity to protect critical macromolecules, such as chromosomal DNA, structural proteins and enzymes and membrane lipids (Dreosti, 2000; Rice-Evans et al., 1996). Rutin has been reported to exhibit multiple pharmacological activities including anti-inflammatory, vasoactive and membrane lipid peroxidation inhibitory properties (Ihme et al., 1996; Lindahl and Tagesson, 1997; Lopez-Revuelta et al., 2006; Park et al., 2002). Vitamin C (ascorbic acid) is recognized for its effective ability to prevent and control various diseases including allergic rhinitis (Thornhill and Kelly, 2000), diabetes (Anderson et al., 2006), heart disease (Ling et al., 2002) and cancer (Enwonwu and Meeks, 1995). In the present study, combined supplementation of RT and AA significantly prevented hypercholesterolemia induced liver injury. We believe that these effects are through the additive hepatoprotective effects of both vitamins. These findings are in accordance with other investigations, where both RT and AA were found to prevent hepatotoxicity and hepatic injury in different animal models (Abhilash et al., 2012; Banerjee et al., 2009; Janbaz et al., 2002; Rana et al., 2010; Shenbagam and Nalini, 2011). Both RT and AA are well recognized to protect against free radicals induced tissue damage through several biological processes in many extracellular and intracellular reactions (Mahmoud, 2011; Ozkaya et al., 2011). Measurements of hepatic DNA and RNA levels showed that RT and AA can attenuate HCD induced cytotoxic damage in liver tissues of the animals. ROS induced-cytotoxicity harmfully affects unsaturated fatty acids, which has been implicated in the pathogenesis of various diseases (Mahmoud, 2011). The cytoprotective effects of RT, as one of the phenolic flavonoids, and AA are also well established (Negre-Salvayre et al., 1995, Passoni and Coelho, 2008). Thus their combined cytoprotective effects are deemed to be through their ability to reduce free radicals production, which is expected to powerfully protect cellular membranes and components. Similar protective effects of this combination have been seen against hypercholesterolemia-induced oxidative stress as they efficiently reduced HCD-induced elevation in hepatic level of lipid peroxidation marker, MDA and significantly enhanced the reduced GSH levels. The elevated levels of total cholesterol and triglycerides in hepatic cells were also protected significantly by the combined supplementation of RT and AA.

The influence of the flavonoids on the endogenous regulation of cholesterol biosynthesis has been discussed in previous studies (Attaway and Buslig, 1998; Borradaile

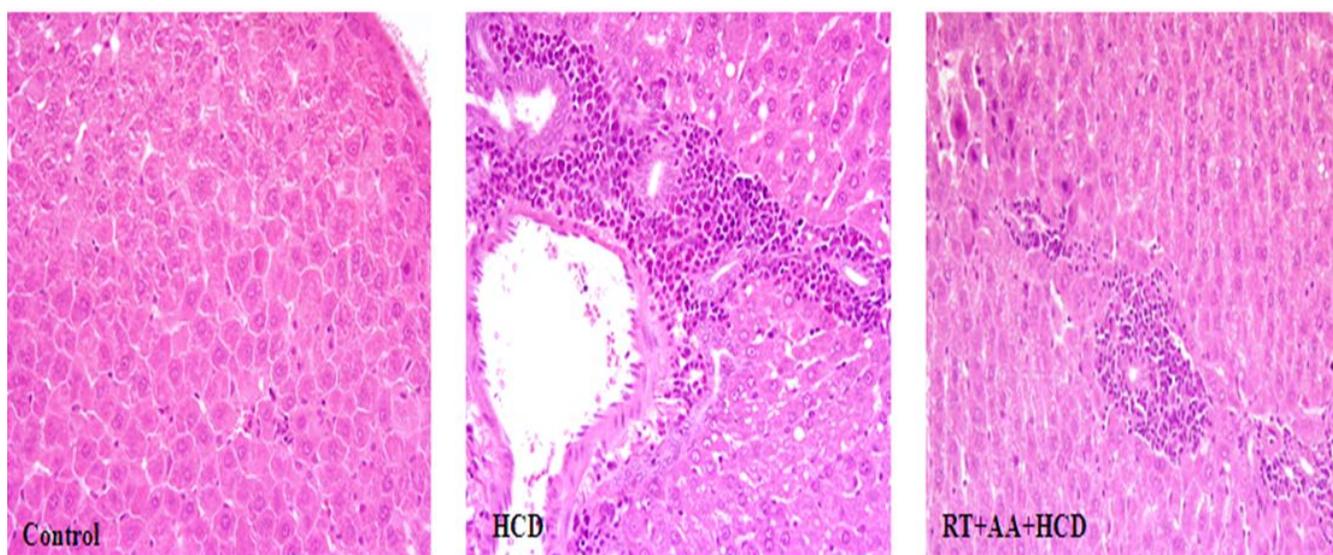


Figure 5. Histopathological sections from rats' liver showing: normal looking hepatocytes without steatosis or inflammatory cell infiltrates in control group; scattered foci of steatohepatosis of liver hepatocytes with swollen epithelial cells associated with scattered foci of periportal to lobular inflammatory cell infiltrates as well as few regenerative changes in HCD group; benign looking hepatocytes separated by congested central veins along with few inflammatory cell infiltrates in HCD+RT+AA group.

et al., 1999; Havsteen, 2002). Studies also suggested the potential improvement in the protective properties of dietary supplements and vitamins after their combination (Khan et al., 2012; Rozanowska et al., 2012). Qureshi et al. (2012), found that combining several dietary supplements can reduce cardiovascular risk factors in humans. Moreover, vitamin E was found to enhance protective effects of ascorbate on light-induced toxicity to retinal pigment epithelial cells (Rozanowska et al., 2012). According to the histopathological findings in the current study, supplementation of HCD and RT with AA significantly ameliorated hepatocellular ballooning and steatohepatosis.

In conclusion, our study demonstrated that oral co-administration of rutin and ascorbic acid attenuates hypercholesterolemia-induced hepatic toxicity in female rats by decreasing liver enzymatic and lipid peroxidative markers as well as increasing the antioxidative cascade.

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Full Length Research Paper

***In vivo* skin irritation potential of a cream containing *Moringa oleifera* leaf extract**

Atif Ali^{1*}, Naveed Akhtar¹, Ahmad Mahmood Mumtaz², Muhammad Shoaib Khan¹, Furqan Muhammad Iqbal³ and Syed Sauod Zaidi⁴

¹Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur, Pakistan

²College of Pharmacy, G. C. University, Faisalabad, Pakistan

³Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan

⁴South Dakota State University, Brookings SD, USA.

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The aim of the present study was to evaluate the skin irritation potential of a cream containing *Moringa oleifera* leaf extract. Skin irritation potential of a cream containing *M. oleifera* leaf extract (3%) versus base was investigated by performing an *in-vivo* visual scoring (skin irritation), patch test and erythema index for long term study by using non-invasive instrumental assessment Mexameter in 11 volunteers in a single blinded study. The active cream and base were applied twice daily to the face (cheeks) for a period of 12 weeks. The instrumental measurements were carried out under a draught-free room, with controlled temperature (18.0 to 20.6°C) and relative humidity (55 to 65%). No serious adverse effects were observed. The *M. oleifera* leaf extract cream was not-irritant according to 48 h semi-occluded patch test. There was a significant decrease in skin erythema and base showed insignificant results when applied ANOVA. The results suggested that the *M. oleifera* leaf extract cream was very well accepted by all volunteers and decreased erythema content. Additionally, product can be regarded as safe for topical application.

Key words: *Moringa oleifera*, extract, cream, skin irritation potential, mexameter.

INTRODUCTION

Natural remedies have been used widely in cosmetics and pharmaceuticals for improving skin appearance and skin conditions in which photo-toxicity, inflammation, psoriasis, alopecia areata and atopic dermatitis are the most prominent.

Herbal treatments applied topically have gained considerable attention due to their widespread use and ill-defined benefit/risk ratio (Aburjai and Natsheh, 2003). However, plant extracts also used in topical and cosmetic formulations as fragrance, colorants, anti-irritant and anti-aging etc. Natural products may induce allergic and irritant contact dermatitis and phyto-photo-dermatitis (Almeida et al., 2008).

Members of the Ranunculaceae, Euphorbiaceae and Asteraceae (Compositae), Umbellifereae, Rutaceae and

Moraceae, Lauraceae, Magnoliaceae and Jubulaceae plant families are especially involved in irritant contact dermatitis, phyto-photo-dermatitis and allergic contact dermatitis (Christopher, 1997; Rates, 2001). Moreover, UV irradiation is one of the major causes to make morphological and ultra-structural changes in human skin (Svobodova et al., 2006).

Phyto-photo-protectives which include antioxidants, phenolic acids, flavonoids and high molecular polyphenols have been incorporated in topical formulations against UV mediated oxidative damage and offers a simple approach to build up the endogenous protection systems which are omnipresent in plants. But it is most important point to explore those natural products that can aggravate skin adverse effects such as phyto-photo-dermatitis, allergic and irritant contact dermatitis (Almeida et al., 2008) and a number of skin diseases are believed to be associated with oxidative stress including psoriasis, acne and cutaneous vasculitis (Rates, 2001).

*Corresponding author. E-mail: ajmaline2000@gmail.com.

Evaluation of skin irritation potential is a foremost interest in safety measurement of cosmetic formulations, when long-term use of these formulations is expected. Non-invasive biophysical tools have been operated earlier to measure skin irritation potential of cosmetic formulations (Mahmood and Akhtar, 2012). *Moringa oleifera* (Moringaceae) pan-tropical species (Iqbal and Bhanger, 2006); bioactive compounds such as gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin; carotene, vitamin C, vitamin B, vitamin A, phenolics, carotenoids etc have been reported (Manguro and Lemmen, 2007; Singh et al., 2009). *M. oleifera* leaf extract have been identified as potent anti-oxidant (Iqbal and Bhanger, 2006). Leave are used as anti-inflammatory, purgative, applied as poultice to sores, rubbed on the temples for headaches, used for piles, fevers, sore throat, bronchitis, eye and ear infections, scurvy and catarrh (Anwar et al., 2007). The aim of the present study was to evaluate the skin irritation potential of an extract of *M. oleifera* leaves in the form of topical application.

MATERIALS AND METHODS

M. oleifera leaves were gathered during July 2010 in Dera Ghazi Khan, Pakistan and air dried at room temperature for a period of 4 weeks.

Identification of plant

The identification of the plant (*M. oleifera*) was executed at the Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Pakistan. The specimen (voucher Number: MO-LE-09-10-31) was placed in the Herbarium of The Islamia University Bahawalpur.

Abil EM 90 was procured from Franken Chemicals Germany, Paraffin oil from Merck Germany, Methanol and Phosphoric acid from BDH England. Deionized water was obtained in the Pharmaceutical Labs of Department of Pharmacy, The Islamia University of Bahawalpur, Pakistan.

Preparation of the active cream

An active cream was prepared by an anionic hydrophilic colloid (14% Paraffin oil), 2.5% Abil EM 90, 3% *M. oleifera* leaves aqueous methanolic extract, 0.2% phosphoric acid, 1% fragrance and rest of deionized water. Heated oily phase and aqueous phase were mixed using homogenizer (Euro-Star, IKAD 230, Germany) by addition of phosphoric acid, extract and fragrance. Base was prepared without extract. The same method was adopted to prepare the base without extract.

Subjects

Eleven subjects were selected with an age between 20 to 35 years. All subjects were healthy males with no known dermatological diseases or allergy to substance in active creams. Declaration of Helsinki was followed in this blind study. Informed consent was signed before start of this study from all volunteers. The exclusion criteria were as follows: presence of, any dermatitis and/or other

skin or allergic diseases, smokers and previous treatment of forearms' skin with cosmetic active creams such as sunscreens, moisturizers or anti-ageing cosmetics. During the test period, the subjects were allowed to wash normally, but were instructed not to use any other skin care products on their arms. The volunteers were asked not to apply any topical products on cheeks 24 h before the beginning and throughout the test period. Additionally, solar exposure and use of occlusive clothes on the test area were forbidden.

Instrumental assessment

Non-invasive bioengineering measurements were performed. The erythema measurements (EI) were performed with reflectance spectrophotometer, a Mexameter from Courage and Khazaka Electronics GmbH, Cologne Germany. The Mexameter was calibrated according to guidelines of manufactures. All measurements were made in a draught-free room, with controlled temperature (18.0 to 20.6°C) and relative humidity (50 to 65%).

Study protocol

Physical stability was evaluated by exposing the creams at 8, 25 and 40°C at 40°C with 75% RH (relative humidity) to storage for a period of two months. Physical characteristics of creams, that is, color, creaming, liquefaction, centrifugation and pH were noted at various intervals for a period of 2 months.

Skin compatibility by evaluation of primary skin irritation

For primary irritation potential of creams, patch tests were accomplished on both forearms of each volunteer on the first day of skin assessment. A 5 × 4 cm area was marked on the forearms. The patch (Bandage disc) for the left forearm was drenched with 1.0 g of base while the patch for right forearm was drenched with 1.0 g of active cream with surgical dressing after application on marked areas. The patches were removed after 48 h and the forearms were observed for any skin irritation by an experience dermatologist and also using Mexameter. The quantification of the skin irritation given through a numeric scale was used to quantify the skin irritation (visual scoring). The average irritant score of the active cream calculated from the average of the quotations obtained for each volunteer, allowing ranking from "non-irritant to very irritant".

The reactions were evaluated according the following arbitrary scale. No erythema: 0, Light erythema (hardly visible): 1, Clearly visible erythema: 2, Moderate erythema: 3, Serious erythema (dark red with possible formation of light eschars): 4, No edema: 0, Very light edema (hardly visible): 1, Light edema: 2, Moderate edema (about 1 mm raised skin): 3, Strong edema (extended swelling even beyond the application area): 4, index of average irritation was classified according amended Draize system: Non-irritating, 0.5 to 2.0: slightly irritating, 2.0 to 5.0: moderately irritating, 5.0 to 8.0 (highly irritating).

Erythema index in long term study

In vivo investigations have been carried out during the winter months (October to January). All instrumental measurements were done by the author according to manufacturer's instructions. Two weeks before study begin and during the treatment period, the volunteers permitted only the use of normal cleansing products. Each volunteer was then handed two creams, an active cream containing the extract of the plant and a base without the extract.

The volunteers were well-informed about the correct use of the creams. Measurements of skin erythema was done every second week up to the end of study period of three months. Approximately 500 mg of both active cream and base were instructed to apply to the cheeks twice daily (mornings, 7:00 to 9:00; evenings, 19:00 to 21:00) over a 12 weeks period at home by the volunteers. The area around the eyes was omitted. Before all measurements, volunteers remained in the room for at least 15 min in order to tolerate full skin adjustment to room temperature.

Efficacy perception – subjective analysis

To assess the effectiveness of the two creams, that is, base and active cream tested in this study, the volunteers were asked to answer a questionnaire consisting of seven parameters after three months from the beginning of the study. 1. Ease of application; 2. Spreadability; 3. Sense just after application; 4. Sense on long term; 5. Irritation; 6. Shine on skin; 7. Sense on softness.

Ethical standards

The approval of this study was taken from the Board of the Advanced Study and Research (BASAR), the Islamia University, Bahawalpur and the Institutional Ethical Committee, Faculty of Pharmacy and Alternative Medicine, The Islamia University, Bahawalpur.

Statistical analysis

Skin erythema contents after application of base and after application of active cream were compared at same time intervals (that is, 0 h readings of skin erythema after application of base were compared with 0 h readings of skin erythema after application of active cream, 48 h readings of skin erythema after application of base were compared with 48 h readings of skin erythema after application of active cream). Different parameters of sensory evaluation (that is, ease of application, spreadability, sense just after application, sense in long term, irritation, shine on skin and sense of softness) were compared for base and active cream; after their application on the cheeks of human volunteers. Paired sample t-test was applied to calculate for base and active cream. The erythema values of the right and left cheek of the volunteers were calculated at 0 h, 2nd, 4th, 6th, 8th, 10th and 12th week. SPSS 17.0 was used for data analysis on the computer by using the two-way ANOVA for variation between different time intervals and the paired sample t-test for the variation between the two active creams. The level of significance was 5%.

RESULTS AND DISCUSSION

Skin compatibility by evaluation of primary skin irritation

Patch testing after a single application is a widely used procedure to evaluate acute irritant reactions (Gaspar et al., 2008). It was found by performing patch testing on forearms of volunteers for 48 h for both the base and active cream that erythema level after application of base was slightly decreased while the erythema level after application of active cream was pronouncedly decreased

after 48 h (data not shown).

But with paired sample t-test, it was evident that the effects of active cream and base were insignificant regarding the skin Erythema even though the active cream decreased the skin erythema more than the base. Initially, evaluation of irritancy testing was based on visual scoring only. This type of evaluation, although subjective, can be a sensitive, reliable and reproducible method.

The possible irritating power of the *Moringa* leaf extract was evaluated according to single application, 48 h semi-occluded patch test. Neither erythema nor edema after the application was observed (Table 1). Finally, it was concluded that both the active cream and base produced no skin irritation after performing patch test of 48 h, so both emulsions can be used safely on human skin for *in-vivo* evaluation.

Erythema index for long term study

In this study, it was found that there were slight variations observed in erythema values of base till 12 weeks. However, in active cream, it was found that there was gradual decrease in erythema values to 12th weeks (Table 2 and Figure 1). With the help of ANOVA test, it was found that changes in erythema values produced by active cream were significant and base were insignificant with respect to time. When the paired sample t-test was applied, it was found that the base and active cream showed significant variations regarding erythema values except 2nd, 4th, and 6th weeks. Anti-oxidants and phenolic compounds have been used in dermatology to approach widely for skin disorders in recent few years (Nichols and Katiyar, 2010; Ali et al., 2012). The inflammatory reaction following acute UV irradiation and the degenerative progressions associated to chronic UV radiation skin exposure are largely mediated by the overproduction of ROS and by impairment of the antioxidant endogenous system (Almeida et al., 2008; Bissett, 2009). Most of the polyphenols play a vital role to protect skin against UV-induced disorders. UV-induced skin inflammation, oxidative stress and DNA damage with a focus on mechanisms underlying the photo-protective effects of these polyphenols (Nichols and Katiyar, 2010).

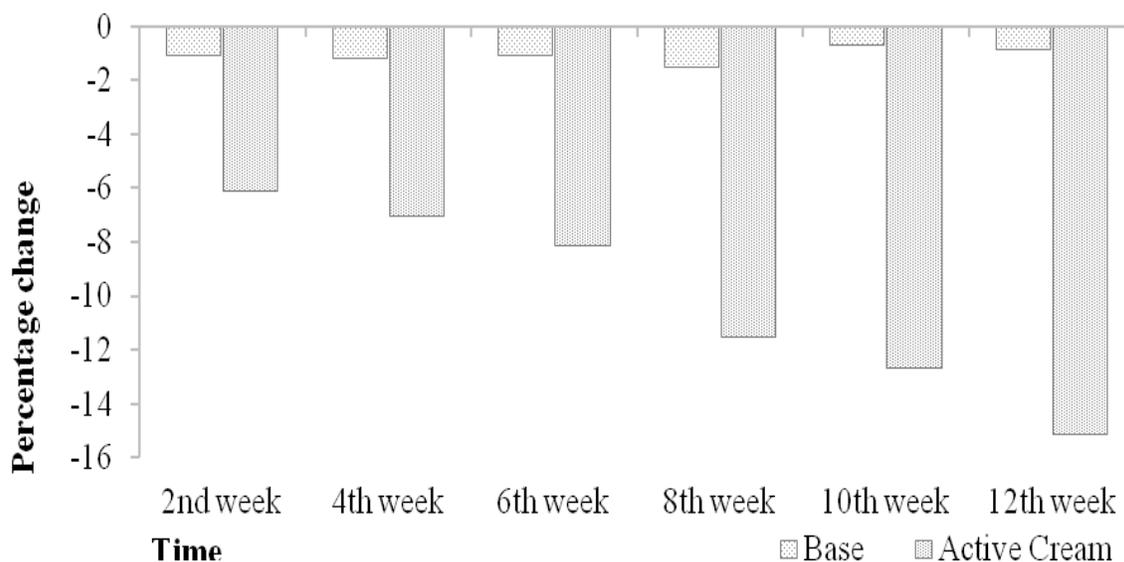
Phenolic antioxidants present in *Moringa* leaves reduce free-radical damage, thereby preventing impairment at the cellular level. They inhibit inflammation, which leads to collagen efficiency, and they offer protection against photo-damage and skin cancer. Several phenolic compounds were identified in *M. oleifera* leaf extract obtained under optimized conditions, namely, phenolic acids (gallic, chlorogenic, ellagic and ferulic acid) and flavonoids (myricetin, kaempferol, quercetin and rutin) (Sultana and Anwar, 2008). DPPH scavenging activity has been found 91% for these phenolic compounds, and thus their accepted input to the free radical scavenging activity of the whole extract. In fact, a preventive effect

Table 1. Values and classification of average irritation indexes.

Volunteer	Erythema	Edema	Total reading 48 h
1	0	0	0
2	0	0	0
3	0	0	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0
11	0	0	0
Total irritation			0
Irritation index			0.00
result			Non irritant

Table 2. Percentage of change in the erythema values of volunteers after the application of base and active cream.

Time	2 nd week	4 th week	6 th week	8 th week	10 th week	12 th week
Base	-1.05	-1.17	-1.06	-1.51	-0.67	-0.85
Active cream	-6.09	-7.05	-8.14	-11.5	-12.65	-15.13

**Figure 1.** Percentage of change in the erythema values of volunteers after the application of base and active cream.

against photo-oxidative stress induced by UVA radiation has been depicted for rutin. Despite the absence of reports of adverse effects of *M. oleifera* leaves or of the phenolic compounds found in its composition, safety cannot be buried and appropriate tolerance investigations

should be performed.

Efficacy perception – subjective analysis

Average points for each parameter were shown in Table 3

Table 3. Average values \pm SEM for panel test.

Variable	Average point for base \pm SEM	Average points for active cream \pm SEM
Ease of application	4.07 \pm 0.05	4.21 \pm 0.12
Spreadability	4.17 \pm 0.08	4.34 \pm 0.06
Sense just after application	3.95 \pm 0.07	3.03 \pm 0.08
Sense in long term	4.06 \pm 0.08	4.04 \pm 0.11
Irritation	0.00 \pm 0.00	0.00 \pm 0.000
Shine on skin	4.14 \pm 0.09	4.07 \pm 0.04
Sense of softness	4.45 \pm 0.08	4.60 \pm 0.09

for both base and active cream. From paired sample t-test, non-significant difference between the average points for base and active cream were observed which showed that there was no variation between base and active cream.

Conclusions

In conclusion, the optimized *M. oleifera* leaf extract presents attractive features that could be applicable for topical application in the prevention and treatment of oxidative stress-mediated diseases and photo-aging. Furthermore, the good skin tolerance found after a single application under patch test reinforces its accepted awareness as topical antioxidant, after inclusion in appropriate and secure topical bases.

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Full Length Research Paper

Isolation and identification of phytochemical constituents from the fruits of *Acanthopanax senticosus*

Jeong Min Lee¹, Dong Gu Lee¹, Ki Ho Lee¹, Seon Haeng Cho², Kung-Woo Nam³, and Sanghyun Lee^{1*}

¹Department of Integrative Plant Science, Chung-Ang University, Anseong 456-756, Republic of Korea.

²Gongju National University of Education, Gongju 314-711, Republic of Korea.

³Office of Industrial Cooperation, Soon Chun Hyang University, Asan 336-745, Republic of Korea.

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Phytochemical constituents were isolated from the fruits of *Acanthopanax senticosus* by repeated chromatography and prep-HPLC. Their structures were identified as β -sitosterol (1), daucosterol (2), *p*-hydroxybenzoic acid (3), vanillic acid (4), uracil (5), eleutheroside K (6), songoroside A (7), copteroside B (8) and *myo*-inositol (9) by spectroscopic analysis. Among them, *p*-hydroxybenzoic acid (3), eleutheroside K (6) and songoroside A (7) were isolated for the first time from the fruits of *A. senticosus*, and songoroside A (7) was isolated for the first time from *Acanthopanax* species.

Key words: *Acanthopanax senticosus*, Araliaceae, repeated chromatography, songoroside A.

INTRODUCTION

Acanthopanax senticosus (Araliaceae) is a deciduous perennial shrub species which is distributed in Korea, China, Japan and Russia. The herb grows in mixed and coniferous mountain forests forming low undergrowth or is found in groups in thickets. *A. senticosus* is broadly tolerant of soil type, growing in sandy, loamy, and heavy clay soils with acid, neutral, or alkaline chemistry, including soils of low nutritional value. *A. senticosus* grows to 2 ~ 3 m in height. The stem bark is gray-brown, the stem is long, covered with thin thorns, and bears five leaflets and umbel-shaped flowers that can be in July in most habitats (Perry and Metgen, 1980; Yook, 1990). The medicinal uses of *A. senticosus*, present in all parts of the plant include anti-bacterial, anti-cancer, anti-inflammatory, anti-gout, anti-hepatitis, anti-hyperglycemic, anti-leishmanicidal, anti-oxidant, anti-pyretic, choleric, hemostatic, anti-xanthine oxidase, immunostimulatory, hypo-cholesterolemic, radio-protectant (Davydov and

Krikorian, 2000), anti-microbial (Lee et al., 2004a; Kim et al., 2006) and inhibition against irradiation-induced injury in rats (Li and Zhou, 2007). The phytochemicals of *A. senticosus* are composed of lignans (eleutheroside E, syringaresinol, and sesamin) (Ryu et al., 2004), terpenoids (chiisanogenin, chiisanoside, isochiisanoside, and oleanolic acid) (Yook et al., 1991; Park et al., 2000; Lee et al., 2003), phenolic compounds (eleutheroside B, chlorogenic acid, and caffeic acid) (Bladt et al., 1990; Nishibe et al., 1990), coumarins (isofraxidin and isofraxidin-7-O- β -D-glucoside) (Wagner et al., 1982; Bai et al., 2011), and flavonoids (hyperin, rutin, quercetin, and quercitrin) (Chen et al., 2002; Lee et al., 2003; Xiaoguang et al., 2007).

There are many reports on the analysis of phytochemicals in *A. senticosus* (Row and Song, 2004; Lee et al., 2004b; Apers et al., 2005; Kim et al., 2006; Li et al., 2006; Ma et al., 2011). There have been many reports on the medicinal effects, isolation, and identification of compounds from the roots, stems and leaves of *A. senticosus*. However, there have been few investigations of the fruits of *A. senticosus*. Therefore, this research is focused on the isolation and identification of

*Corresponding author. E-mail: slee@cau.ac.kr. Tel/Fax: +82316704688, +82316764686.

compounds from *A. senticosus* fruits by repeated chromatography and recycling preparative high performance liquid chromatography (prep-HPLC).

MATERIALS AND METHODS

Plant materials

The fruits of *A. senticosus* (Araliaceae) were collected at Gongju and verified by Prof. Seon Haeng Cho, Gongju National University of Education, Republic of Korea. A voucher specimen (No. LEE 2008-01) was deposited at the Herbarium of Department of Integrative Plant Science, Chung-Ang University, Republic of Korea.

General experimental procedures

Electron ionization mass spectrometry (EI-MS) was measured with a Jeol JMS-600 W (Tokyo, Japan) mass spectrometer. ^1H - and ^{13}C -nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance 300 or 500 NMR (Rheinstetten, Germany) spectrometers in CDCl_3 , $\text{C}_5\text{D}_5\text{N}$, DMSO or CD_3OD using tetramethylsilane (TMS) as an internal standard. Chemical shifts were reported in parts per million (δ) and coupling constants (J) were expressed in Hertz (Hz). Thin layer chromatography (TLC) analysis was conducted with Kiesel gel 60 F254 (Art. 5715, Merck Co., Germany) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by spraying with 10% H_2SO_4 in MeOH. Repeated chromatography was conducted with a silica gel (200 to 400 mesh ASTM; Merck Co., Germany). All other chemicals and reagents were analytical grade. Prep-HPLC was conducted by a JAI LC-9104 (Tokyo, Japan) system equipped with an L-6050 pump and UV-3702 UV/VIS detector.

Extraction and isolation

The dried fruits of *A. senticosus* (3.0 kg) were ground into powder and extracted with methanol (MeOH, 10 L \times 3) under reflux. The resultant extracts were combined and concentrated under reduced pressure to afford 594.2 g of the residue. The MeOH extract (594.2 g) was suspended in water (H_2O) and then partitioned successively with equal volumes of chloroform (CHCl_3 , 34.6 g), ethyl acetate (EtOAc, 44.4 g) and *n*-butanol (*n*-BuOH, 87.0 g). A portion of the CHCl_3 fraction was chromatographed on a silica gel column eluted in a gradient of *n*-hexane - EtOAc (97:3 and 30:70) to afford compounds 1 and 2, respectively. A portion of the EtOAc fraction was chromatographed on a silica gel column eluted in a gradient of *n*-hexane and EtOAc (100% *n*-hexane up to 100% EtOAc) and EtOAc and MeOH (100% EtOAc up to 100% MeOH) to afford 9 subfractions (E_1 to E_9). Subfraction E_4 (*n*-hexane:EtOAc = 85:15) was analyzed by prep-HPLC using a JAIGEL-GS column with UV/VIS detection set at 240 nm and a mobile phase of CHCl_3 -MeOH- H_2O (80:20:2) at ambient temperature to afford compounds 3 (t_r 47 min) and 4 (t_r 58 min). Subfraction E_6 (*n*-hexane:EtOAc = 35:65) was rechromatographed on a silica gel (No. 7729) column eluted in a gradient of CHCl_3 and MeOH (90:10) to afford compound 5. Subfraction E_7 was rechromatographed on a silica gel (No. 7729) column eluted in a gradient of CHCl_3 and MeOH (80:20 and 70:30) to afford compounds 6 and 7, respectively. Subfraction E_8 (*n*-hexane:EtOAc = 5:95) was rechromatographed on a Sephadex LH-20 eluted in MeOH to obtain compound 8. A portion of the *n*-BuOH fraction was chromatographed on a silica gel column eluted in a gradient of CHCl_3 and MeOH (80:20) to afford compound 9. Compound 1 – white powder; EI-MS m/z : 414 [$\text{M}]^+$ (100.0), 396 (42.5), 381 (21.8), 329 (25.0), 303 (28.9), 289 (4.0), 273 (25.3), 255

(48.0), 231 (15.9), 213 (25.2), 159 (25.6), 145 (25.8); ^1H - and ^{13}C -NMR (300 MHz, CDCl_3): Table 1.

Compound 2 – white powder; FAB-MS m/z : 577 [$\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (300 MHz, $\text{C}_5\text{D}_5\text{N}$): Table 1.

Compound 3 – white powder; EI-MS m/z : 138 [$\text{M}]^+$ (92.9), 121 (100.0), 93 (23.7), 81 (3.8), 65 (12.6); ^1H - and ^{13}C -NMR (300 MHz, DMSO): Table 2.

Compound 4 – white powder; EI-MS m/z : 168 [$\text{M}]^+$ (100.0), 153 (33.1), 137 (93.5), 125 (10), 109 (21), 81 (9); ^1H - and ^{13}C -NMR (500 MHz, DMSO): Table 2.

Compound 5 – white powder; EI-MS m/z : 112 [$\text{M}]^+$ (100), 69 (52); ^1H -NMR (300 MHz, CD_3OD): δ 7.41 (1H, d, $J = 7.8$ Hz, H-6), 5.63 (1H, d, $J = 7.8$ Hz, H-5).

Compound 6 – amorphous powder; FAB-MS m/z : 735 [$\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$): Table 3.

Compound 7 – amorphous powder; FAB-MS m/z : 589 [$\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$): Table 3.

Compound 8 – amorphous powder; FAB-MS m/z : 649 [$\text{M} + \text{H}]^+$; ^1H - and ^{13}C -NMR (500 MHz, $\text{C}_5\text{D}_5\text{N}$): Table 3.

Compound 9 – brown powder; EI-MS m/z : 144 [$\text{M}-2\text{H}_2\text{O}]^+$ (11.6), 115 (1.7), 102 (32.1), 91 (9.4), 73 (100.0), 60 (27.1); ^1H -NMR (300 MHz, DMSO): δ 4.58 (1H, d, $J = 4.2$ Hz, OH), 4.52 (1H, d, $J = 4.2$ Hz, OH), 4.38 (H, d, $J = 5.7$ Hz, OH), 3.11 (2H, m, CH), 3.00 (1H, m, CH); ^{13}C -NMR (75 MHz, DMSO): δ 75.6 (C-2), 74.6 (C-5), 73.1 (C-1,3), 72.2 (C-4,6).

RESULTS AND DISCUSSION

A chromatographic separation of the MeOH extract of *A. senticosus* led to the isolation of compounds 1 to 9 (Figure 1). Compounds 1 and 2 were obtained as white powders from the CHCl_3 fractions. ^1H -NMR spectra of 1 and 2 showed the existence of a sterol skeleton and a molecular ion peak at m/z 414 [$\text{M}]^+$ in the EI-MS and 577 [$\text{M} + \text{H}]^+$ in the FAB-MS. Two angular methyl singlets of H-18 and -19 at δ 0.67 to 0.68 and 0.94 to 1.01 and three doublets of H-21, -26 and -27 at δ 0.92 to 1.00, 0.81 to 0.94 and 0.86 to 0.89 were observed, respectively. An olefinic proton signal of H-6 was observed at δ 5.35. ^{13}C -NMR spectra of compounds 1 and 2 showed 29 and 35 resonances, respectively. C-5 and -6 signals of compounds 1 and 2 were observed at δ 141.0 to 141.3 and 121.9 to 122.3, respectively. Compounds 1 and 2 had similar structural signals. The typical pattern of a glucose moiety was observed in the ^1H - and ^{13}C -NMR spectra in compound 2. The anomeric proton of compound 2 produced a peak at δ 5.09 (d, $J = 6.9$ Hz), and the glucose position was at C-3 (β -linkage) of the aglycone according to HMBC analysis.

Accordingly, the structures of compounds 1 and 2 were elucidated as β -sitosterol (stigmast-5-en-3-ol) and daucosterol (β -sitosterol-3-O- β -D-glucoside), respectively, by comparison of the spectral data, as described in the literature (Umlauf et al., 2004; Park et al., 2009; Yang et al., 2009; Lee et al., 2011; Zhang et al., 2011). In previous papers, β -sitosterol, the most common plant sterol has been reported to have anti-inflammatory, anti-tumor and anti-microbial activities (Park et al., 2001; Yuk et al., 2007; Xu et al., 2011). Daucosterol, a β -sitosterol glycoside induces a protective Th1 immune response

Table 1. ^1H - and ^{13}C -NMR spectral data for compounds 1 and 2.

No.	1		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	-	37.4	-	37.3
2	-	31.8	-	29.4
3	3.52 (m)	71.9	-	78.5
4	2.27 (m)	42.4	-	38.4
5	-	141.0	-	141.3
6	5.35 (m)	121.9	5.35 (m)	122.3
7	-	32.0	-	31.8
8	-	32.0	-	30.6
9	-	50.3	-	50.7
10	-	36.7	-	36.8
11	1.99 (m)	21.2	-	20.4
12	-	39.8	-	40.3
13	-	42.4	-	42.7
14	-	57.0	-	56.9
15	-	24.5	-	23.8
16	-	28.4	-	26.7
17	-	56.1	-	56.6
18	0.68 (s)	12.0	0.67 (s)	12.4
19	1.01 (s)	19.1	0.94 (s)	19.6
20	-	36.3	-	34.5
21	0.92 (d, 6.3)	18.9	1.00 (d, 6.3)	19.4
22	-	34.1	-	32.6
23	-	26.2	-	24.9
24	-	46.0	-	46.4
25	-	29.1	-	28.9
26	0.81 (d, 6.3)	19.1	0.94 (d, 5.4)	19.8
27	0.86 (d, 4.2)	19.6	0.89 (d, 6.3)	20.1
28	-	23.2	-	21.7
29	0.80 (t, 5.8)	12.1	0.87 (m)	12.5
Glc				
1			5.09 (d, 6.9)	103.0
2				75.8
3				79.0
4				72.1
5				78.9
6				63.2

Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz.

against disseminated candidiasis (Lee et al., 2007).

Compounds 3 and 4 were obtained as white powders from the EtOAc fraction and showed molecular ion peaks at m/z 138 $[\text{M}]^+$ and 168 $[\text{M}]^+$ in the EI-MS, respectively. The ^1H -NMR spectra of compounds 3 and 4 showed phenolic compound signals. The only differences between compounds 3 and 4 are the typical A_2B_2 and ABX types in the benzene ring. In the ^1H - and ^{13}C -NMR spectra of compound 3, two doublets of aromatic proton

signals at δ 7.78 ($J = 8.7$ Hz) and 6.81 ($J = 9.0$ Hz), four aromatic carbon signals at δ 115.2, 121.5, 131.6, 161.6, and one carboxyl carbon signal at δ 167.3 were observed. In addition, two doublets and one double doublet of aromatic proton signals at δ 7.35 ($J = 1.8, 8.0$ Hz), 7.06 ($J = 1.8$ Hz) and 6.61 ($J = 8.0$ Hz) were observed in compound 4.

Accordingly, the structures of compounds 3 and 4 were identified as *p*-hydroxybenzoic acid and vanillic acid,

Table 2. ^1H - and ^{13}C -NMR spectral data for compounds 3 and 4.

No.	3		4	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	-	121.5	-	123.8
2	7.78 d (8.7)	131.6	7.06 d (1.8)	114.3
3	6.81 d (9.0)	115.2	-	149.2
4	-	161.6	-	153.3
5	6.81 d (9.0)	115.2	6.61 d (8.0)	116.1
6	7.78 d (8.7)	131.6	7.35 dd (1.8, 8.0)	126.0
COOH	-	167.3	-	170.8
OMe			3.76 s	57.0

Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz.

Table 3. ^1H and ^{13}C -NMR spectral data of compounds 6 to 8.

No.	6		7		8	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		39.4		39.2		39.2
2		27.0		27.0		26.5
3	3.27 (dd, 4.2, 11.7)	89.3	3.31 (br d, 10.8)	89.5	3.31 (br d, 6.5)	79.9
4		40.0		40.0		44.0
5		56.4		56.3		48.0
6		19.0		19.0		18.8
7		33.7		33.7		33.4
8		40.2		40.2		40.3
9		48.5		48.5		48.6
10		37.5		37.5		37.4
11		24.2		24.3		24.4
12	5.50 (t-like, 3.2)	123.0	5.47 (t-like)	123.0	5.47 (t-like)	123.1
13		145.3		145.3		145.4
14		42.6		42.5		42.5
15		28.8		28.8		28.8
16		24.3		24.3		24.3
17		47.0		47.0		47.2
18		42.7		42.7		42.7
19		47.2		47.2		46.9
20		31.4		31.5		31.4
21		34.7		34.8		34.8
22		33.7		33.8		33.8
23	1.32 (s)	28.6	1.33 (s)	28.8		64.9
24	0.98 (s)	17.5	0.97 (s)	17.5	0.93 (s)	14.2
25	0.85 (s)	16.0	0.78 (s)	16.0	0.91 (s)	16.6
26	1.09 (s)	17.9	1.02 (s)	17.9	1.02 (s)	18.0
27	1.20 (s)	26.7	1.33 (s)	26.7	1.27 (s)	26.8
28		180.7		180.7		180.9
29	0.99 (s)	33.8	0.99 (s)	33.8	0.94 (s)	33.8
30	1.02 (s)	24.3	1.02 (s)	24.3	1.00 (s)	24.2

respectively, by comparison of the spectral data, as described in the literature (Shimizu et al., 1983; Pyo et al., 2002; González-Baró et al., 2008; Zhang et al., 2011;

Yuan et al., 2012). *p*-Hydroxybenzoic acid shows antioxidant activity on DPPH radical assay and the inhibition of lipoperoxidation (Yamaguchi et al., 2006). Vanillic acid is

Table 3. Contd.

No.	6		7		8	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
Ara						
1	4.93 (d, 5.3)	105.3				
2		76.4				
3		74.6				
4		72.9				
5		65.2				
Rha						
1	6.17 (br s)	102.3				
2		74.3				
3		73.1				
4		70.4				
5		69.2				
6	1.66 (d, 6.2)	19.0				
Xyl						
1			5.05 (d, 5.3)	107.3		
2				76.0		
3				78.9		
4				74.4		
5				68.0		
GluA						
1					5.23 (d, 7.8)	105.0
2						76.9
3						79.0
4						74.3
5						75.1
6						175.0

Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hertz.

a phenolic derivative of edible plants and fruits and has antibacterial and antimicrobial properties against *Listeria monocytogenes*, *Listeria innocua*, *Listeria grayi* and *Listeria seeligeri* (Rai and Maurya, 1966; Delaquis et al., 2005).

Compound 5 was obtained as white powder from the EtOAc fraction and showed a molecular ion peak at m/z 112 $[M]^+$ in the EI-MS. In the 1H -NMR spectrum of compound 5, two doublets of typical olefinic proton signals at δ 7.41 ($J = 7.8$ Hz) and 5.63 ($J = 7.8$ Hz) were observed. Accordingly, the structure of compound 5 was elucidated as uracil by comparison of the spectral data, as described in the literature (Lee et al., 2002). Uracil can be used to determine microbial contamination of tomatoes as its presence is an indication of lactic acid bacteria contamination in the fruit (Hidalgo et al., 2005). Compounds 6 to 8 were obtained as amorphous powders from the EtOAc fraction and showed molecular ion peaks

at m/z 735 $[M+H]^+$, 589 $[M+H]^+$ and 649 $[M+H]^+$ in the FAB-MS, respectively. The aglycone of compounds 6 and 7 was oleanolic acid, while that of compound 8 was hederagenin. In the 1H -NMR spectra of compounds 6 to 8, one olefinic proton signal at δ 5.47-5.50 (H-12) and one oxygen-bearing methine proton signal at δ 3.27-3.31 (H-3) were observed. Seven tertiary methyl group signals at δ 0.78 to 1.33 (each s, H-23, 24, 25, 26, 27, 29 and 30) were observed in compounds 6 and 7. In addition, six tertiary methyl groups signals at δ 0.91 to 1.27 (each s, H-24, 25, 26, 27, 29 and 30) were observed in compound 8.

In the ^{13}C -NMR spectra of compounds 6 to 8, two sp^2 carbons at δ 123.0 to 123.1 (C-12) and 145.3 to 145.4 (C-13) and one ester carboxyl group at δ 180.7 to 180.9 (C-28) were observed. The chemical shift of the oxygen-bearing carbon signal was observed at δ 89.5, 89.3 and 79.9 (C-3), suggesting that sugar moieties were attached.

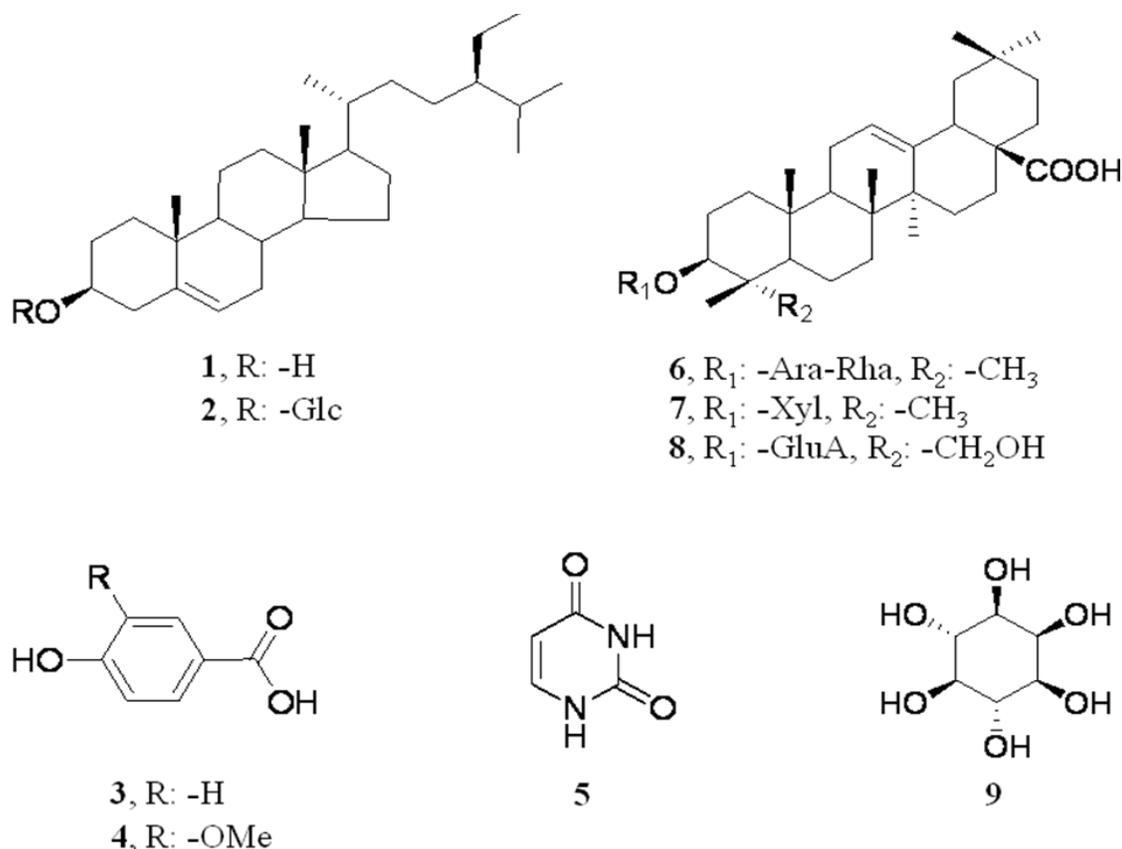


Figure 1. Structures of compounds 1 to 9.

In the $^1\text{H-NMR}$ spectrum of compound 6, anomeric protons of δ 4.93 (d, $J = 5.3$ Hz, H-1 of Ara) and 6.17 (br s, H-1 of Rha) were observed. Identification of the correlation between δ 4.93 (H-1 of Ara) and δ 89.3 (C-3), and δ 6.17 (H-1 of Rha) and δ 76.4 (Ara-2) by HMBC indicated that an α -L-rhamnosyl-(1 \rightarrow 2)- α -L-arabinoside moiety was linked to C-3 of the aglycone of compound 6. Compound 7 showed one anomeric proton signal at δ 5.05 (d, $J = 5.3$ Hz, H-1 of Xyl). Identification of the correlation between δ 5.05 (H-1 of Xyl) and δ 89.5 (C-3) by HMBC indicated that a β -D-xyloside moiety was linked to C-3 of the aglycone of compound 7. Compound 8 showed one anomeric proton signal at δ 5.23 (d, $J = 7.8$ Hz, H-1 of GluA). Identification of the correlation between δ 5.23 (H-1 of GluA) and δ 79.9 (C-3) by HMBC indicated that a β -D-glucuronic acid moiety was linked to C-3 of the aglycone of compound 8. Due to the upfield shift of C-3, aglycone C-23 was indicative of a substitution by CH_2OH . $^{13}\text{C-NMR}$ spectra of compounds 6, 7 and 8 showed 41, 35 and 36 resonances, respectively.

Accordingly, the structures of compounds 6 to 8 were identified as eleutheroside K, songoroside A and copteroside B, respectively, by comparison of the spectral data, as described in the literature (Saluja and Santani, 1986; Akimailiev et al., 1988; Shao et al., 1989; Majester-Savornin et al., 1991; Alabdul Magid et al., 2006).

Eleutheroside K, songoroside A and copteroside B have anti-leishmanial activity, anti-inflammatory effects and inhibitory activity toward pancreatic lipase (Dai et al., 1989; Delmas et al., 2000; Li et al., 2007).

Compound 9 was obtained as a brown powder from the *n*-BuOH fraction. Three hydroxyl proton signals at δ 4.58, 4.52, and 4.38 and two multiplets of CH proton signals at δ 3.11 and 3.00 were observed. The $^{13}\text{C-NMR}$ spectrum of compound 9 showed four ring carbon signals at δ 75.6, 74.6, 73.1 and 72.2. Accordingly, the structure of compound 9 was elucidated as *myo*-inositol by comparison of the spectral data, as described in the literature (Yasue et al., 1968). A previous placebo-controlled study has demonstrated that *myo*-inositol supplementation improves features of dysmetabolic syndrome in postmenopausal women, including triglycerides, HDL cholesterol and diastolic blood pressure (Giordano et al., 2011).

In conclusion, nine compounds, β -sitosterol (1), daucosterol (2), *p*-hydroxybenzoic acid (3), vanillic acid (4), uracil (5), eleutheroside K (6), songoroside A (7), copteroside B (8) and *myo*-inositol (9) were isolated from the fruits of *A. senticosus*. To the best of our knowledge, this is the first report on the isolation of *p*-hydroxybenzoic acid (3), eleutheroside K (6), and songoroside A (7) from the fruits of *A. senticosus*, and songoroside A (7) from

Acanthopanax species.

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Full Length Research Paper

Accelerated extraction of Xanthone from *Mangosteen pericarp* using ultrasonic technique

Nuttawan Yoswathana

Department of Chemical Engineering, Faculty of Engineering, Mahidol University, Nakhonpathom, 73170, Thailand.
E-mail: nuttawan.yos@mahidol.ac.th

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***Mangosteen pericarp* has been used for long time as traditional medicine. One of main active ingredient in *M. pericarp* is xanthone. Xanthone has remarkable effects on cardiovascular health, antiviral and anti-inflammatory. Ultrasonic Assisted Extraction (UAE) was employed to extract xanthone from dried *M. pericarp* and compared with soxhlet extraction and maceration. The UAE was applied by various temperature (33, 45 and 55°C), amplitude (25, 50 and 75%) and solvent (0, 50 and 95% ethanol) for 1 h extraction time. The conditions for the highest xanthone recovery were determined at temperature 33°C, and 50% ethanol at 50% amplitude, resulting 0.16 mg/g of dried *M. pericarp*. The Box-Behnken design was applied to investigate the optimum condition of UAE. The optimum conditions from Box-Behnken design to obtain the highest xanthone recovery were determined to be temperature 33°C, amplitude 75 and 80% ethanol. The results presented that for UAE in 0.5 h, soxhlet extraction in 2 h and maceration in 2 h, the extracted xanthenes were 0.1760, 0.1221 and 0.0565 mg/g of dried *M. pericarp* respectively.**

Key word: Ultrasound assisted extraction (UAE), *Mangosteen pericarp*, xanthone, maceration.

INTRODUCTION

Mangosteen (*Garcinia mangostana* L.) is known as “the queen of fruits” is in *Guttiferae* family. Mangosteen is grown in Thailand and Southeast Asian countries. The fruit pericarp of this plant has been used for long time as a traditional medicine for treatment of abdominal pain, diarrhea, dysentery, infected wound, suppuration and chronic ulcer. The major active substances in *Mangosteen pericarp* is xanthone. The xanthone has a remarkable effect on cardiovascular health, antioxidant, antibiotic, antiviral and anti-inflammatory (Suksamran et al., 2002; Sabphon et al., 2012). Due to its pharmacological activities, it is popularly applied to herbal cosmetics and pharmaceutical products. There are many ways to extract active substances such as xanthone from *M. pericarp*. The conventional extraction methods such as soxhlet or maceration are time-consuming methods or operate at elevated temperature that could thermally damage the active substances. In contrast, novel extraction technique such as ultrasonic operates at moderate or room temperature and promise much more yield compare to conventional extraction methods.

Ultrasonic extraction is proved to be economical and effective method. It is used as alternative extraction technique at the laboratory or industry scale due to shorter extraction time and higher extraction yield (Toma et al., 2001; Nasri et al., 2012). Ultrasonic has ability to penetrate the cellular wall, reduce the particle size, and increases the mass transfer between the cell walls and the outside because of the cavitation effect (Entezari et al., 2004; Asgarpanah and Ramezanloo, 2012).

In this study, the effect of ultrasonic on extraction of xanthone from *M. pericarp* was investigated and compared to conventional soxhlet and maceration methods. For design of experimental was response surface methodology using Box-Behnken method applied that could reduce the number of total experiments during ultrasonic extraction for three level of investigation (temperature, ultrasonic power and solvent). The Box-Behnken design has advantages that reduce experiments due to its requirement of only three levels and it is efficiency to prepare and explain when compare to the full factorial design and other methods (Ferreira et al., 2007).

Table 1. Box-Behnken design^a.

Experiment	Coded level		
	X ₁	X ₂	X ₃
	Ultrasonic temperature (°C)	Ultrasonic amplitude (%)	Solvent (% ethanol)
1	0 (45)	1 (75)	1 (95)
2	0 (45)	1 (75)	-1 (0)
3	0 (45)	-1 (25)	1 (95)
4	0 (45)	-1 (25)	-1 (0)
5	1 (55)	0 (50)	1 (95)
6	1 (55)	0 (50)	-1 (0)
7	-1 (33)	0 (50)	1 (95)
8	-1 (33)	0 (50)	-1 (0)
9	1 (55)	1 (75)	0 (50)
10	1 (55)	-1 (25)	0 (50)
11	-1 (33)	1 (75)	0 (50)
12	-1 (33)	-1 (25)	0 (50)
13	0 (45)	0 (50)	0 (50)
14	0 (45)	0 (50)	0 (50)
15	0 (45)	0 (50)	0 (50)

^a The values in parentheses mean practical levels.

MATERIALS AND METHODS

Dried mangosteen (*G. mangostana* L.) pericarps were obtained from Government Pharmaceutical Organization (GPO, Thailand). For lab study, the dried sample was crushed by hammer and grounded with grinder (5657 HAAN, Retsch, Germany) to obtain 3 mm particle size. The dried sample powder was packed in plastic bags and stored in darkness.

Chemicals and reagents

95% ethanol was purchased from Alcoh-A (Thailand). 99.9% methanol was purchased from Burdick & Jackson (Korea). Xanthone standard was purchased from Sigma Chemical Company (St. Louis, MO, USA).

Maceration

The experiment was performed in a 250 ml beaker filled with 5 g dried sample powder in 100 ml of 95% v/v ethanol. The extraction was carried out at room temperature for 0.5, 1 and 2 h without shaking.

Soxhlet extraction

A classical Soxhlet apparatus was employed in which 5 g of grounded sample was placed into cartridge with 250 ml of 95% v/v ethanol in round bottom flask. Extraction was carried out at a boiling point (78.1°C) of ethanol for 0.5, 1 and 2 h.

Ultrasonic assisted extraction

5 g of dried sample powder mixed with 100 ml solvent (0, 50 and 95% ethanol content) was applied for each ultrasonic extraction. The

extraction was performed in a 200 ml beaker with heating jacket. The temperature in heating jacket was adjusted using a water bath with accuracy of about $\pm 1^\circ\text{C}$ (ISOTEMP 2150, Fisher Scientific, US). The extraction process was performed using an ultrasonic probe (200 W max. power, HD 3200, 20 kHz, SONOPULS, Ultrasonic Homogenizers, Germany) at different ultrasonic conditions (temperature of 33 to 55°C, ultrasonic amplitude of 25 to 75% and ethanol concentration of 0 to 95%) for constant extraction time of 1 h. The extracts of soxhlet, maceration and ultrasonic was filtrated through filter paper no. 1 (Whatman, Germany) and removed solvent by using rotary evaporator (R-215, BUCHI Rotavapor, Switzerland) at 60°C under vacuum. 99% v/v methanol was added for adjust volume to 25 ml. After that, the extract was subjected to analysis of xanthone concentration.

The Box-Behnken design was applied to determine the response pattern of temperature (X₁), ultrasonic amplitude (X₂) and solvent (X₃), respectively, with three levels for each variable, while the dependent variable was the xanthone recovery. The symbols and levels are shown in Table 1. The whole design consisted of 15 experimental points, which were carried out in a randomized order, to maximize the effect of unexplained variability in the observed response due to extraneous factors.

Analytical method for xanthone recovery

The concentration of xanthenes was determined by UV-spectrophotometer (1001 Plus, Milton Roy Spectronic, USA). The absorbance of the solutions was measured at 517 nm. 99% v/v methanol was used as the blank.

Statistical analysis

All of the experiments were carried out in triplicate, and the average of the xanthone recovery was taken as a responsive value. ANOVA statistic analysis of variance was applied using Microsoft EXCEL 2010 to evaluate the data. Analyses of the variance were used to

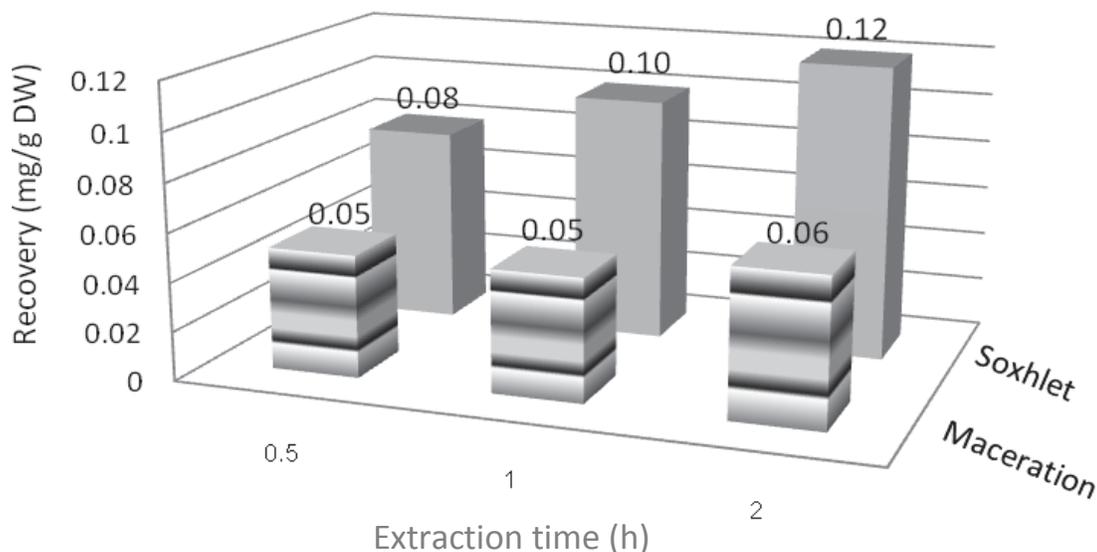


Figure 1. The xanthone recovery by maceration and soxhlet extraction.

determine the significant difference between results. Differences were considered significant when the probability of a type I error was less than 5% ($P < 0.05$).

RESULTS AND DISCUSSION

Soxhlet extraction

The effect of extraction time on xanthone yield is demonstrated in Figure 1. With increasing the extraction time increased the yield. Soxhlet extraction after 2 h resulted to a xanthone recovery up to 0.12 mg/g dried sample (Figure 1).

Maceration

In general was the xanthone yield during maceration lower than soxhlet extraction. Increasing the maceration time has slight effect on xanthone recovery. The amount of extracted xanthone during 2 h maceration was about 0.06 mg/g dried sample (Figure 1).

Ultrasonic extraction

Effect of ultrasonic temperature

The effect of temperature on the release of xanthonex extracted in 95% ethanol as a solvent at 50% ultrasonic amplitude is shown in Figure 2. It was found that the increase in temperature from 33 to 45°C enhanced the xanthone recovery and the increasing was leveled up when the temperature raised from 45 to 55°C. Temperature affected many physical properties such as

viscosity, diffusivity, solubility, vapor pressure and surface tension. The main effect of ultrasounds is cavitation. At high vapor pressure of the liquid, more bubbles will be created that enhance cavitation effect (Hemwimol et al., 2006).

Effect of ultrasonic amplitude

The effect of amplitude on the release of xanthonex extracted in 95% ethanol as solvent at 33°C ultrasonic temperature is shown in Figure 3. It was found that the increase in ultrasonic amplitude enhanced the xanthone recovery significantly. Amplitude is the objective measurement of the degree of change in atmospheric pressure caused by sound waves. In general, an increase in intensity will provide an increase in the cavitation effects (Mason, 1999).

Effect of solvent (% ethanol)

The ratio of water in ethanol solution had a significant effect on the extraction of xanthonex (Figure 4). It was found that water, as solvent (without ethanol) was not effective for xanthone extraction. This is maybe due to the differences in polarity between the water and xanthone. The polarity of xanthonex is much lower than water, so that it is not well soluble in water. 50% ethanol was found to be the more effective solvent. Water content in the applied solvent played an important role in the extraction. Ultrasound enlarged the pores of the cell walls so that the diffusion process and mass transfer were improved (Soares et al., 2006). The intensity of ultrasonic cavitation in the ethanol mixture in the presence of water

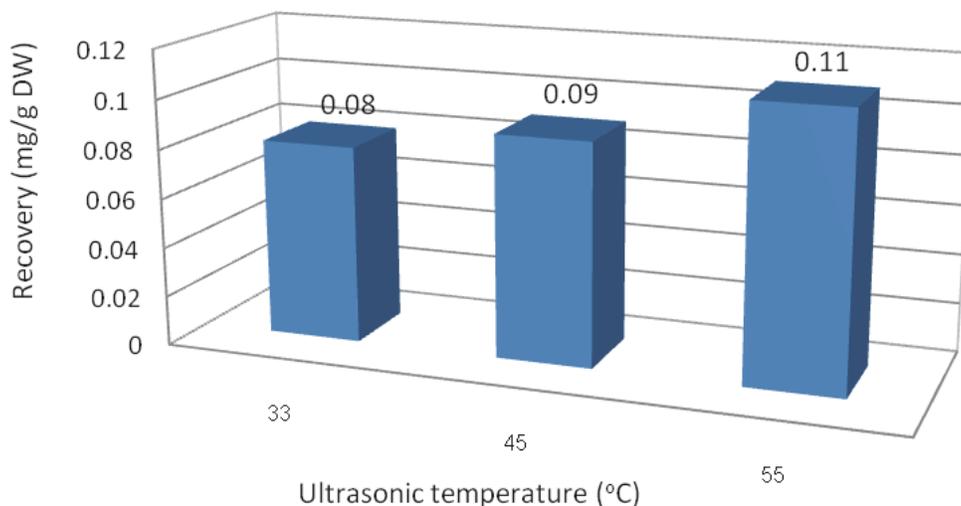


Figure 2. Effect of ultrasonic temperature on the ultrasonic assisted extraction of xanthone at 50% ultrasonic amplitude in 95% (v/v) ethanol.

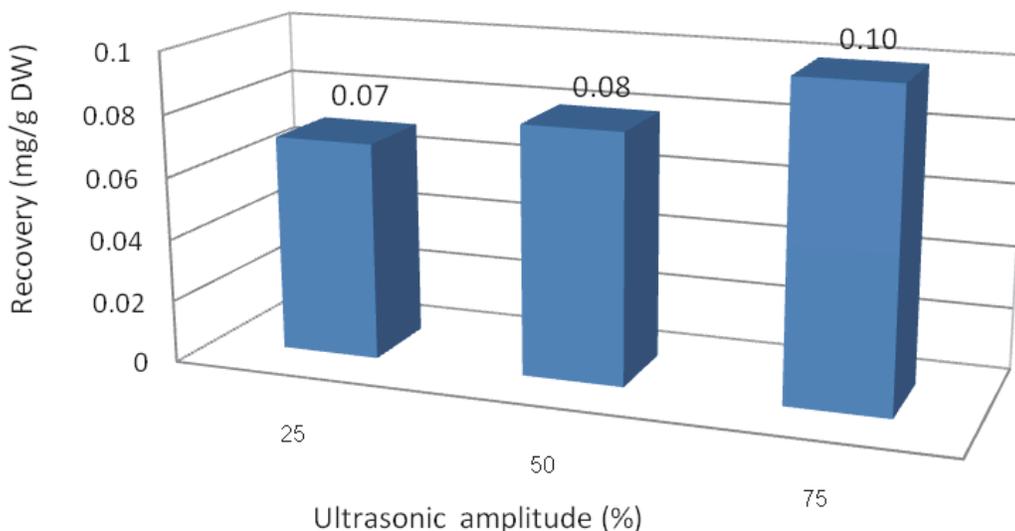


Figure 3. Effect of ultrasonic amplitude on the ultrasonic assisted extraction of xanthenes at 33°C ultrasonic temperature in 95% (v/v) ethanol.

was also increased because of the increase in surface tension and the decrease in viscosity.

The results of xanthone extraction using UAE at process conditions according to Box-Behnken design are shown in Figures 5a to c. The effects of ultrasonic amplitude and solvent on the recovery of xanthone as well as their interactions are shown in Figure 5a. The xanthone recovery decreased with increasing ultrasonic amplitude when water was applied as solvent. However, a reverse interaction between xanthone recovery and ultrasonic amplitude was observed when the solvent was close to 95% ethanol. Similar results were observed in the case of the effect of temperature and solvent compo-

sition on the xanthone extraction during ultrasonic extraction (Figure 5b). For 50% ethanol as solvent, increasing the process temperature resulted to decreasing the xanthone extractability. This was at higher ultrasound power obviously (Figure 5c).

Model fitting

The statistical model, representing xanthone recovery as a function of the independent variables under investigation can be expressed by the following quadratic Equation 1:

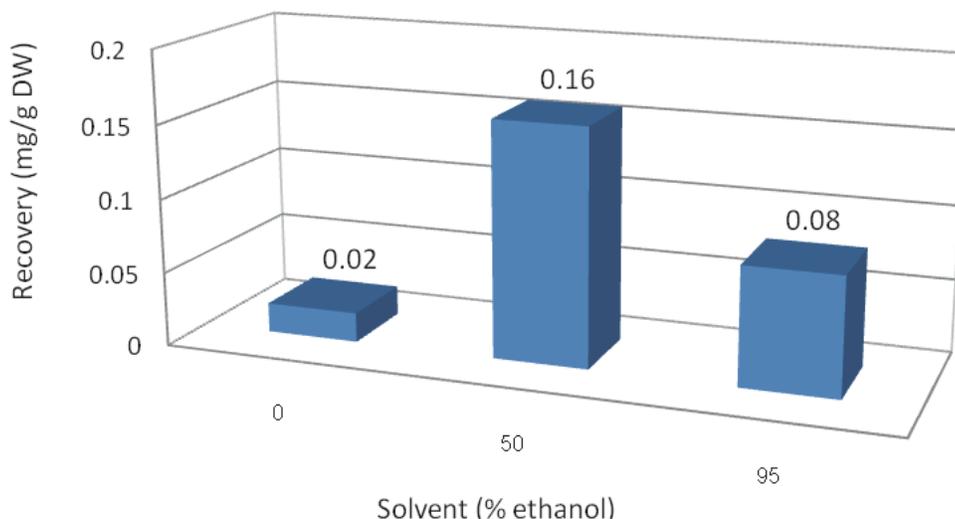


Figure 4. Effect of solvent (% ethanol) on the ultrasonic assisted extraction of xanthenes at 33°C ultrasonic temperature and 50% ultrasonic amplitude.

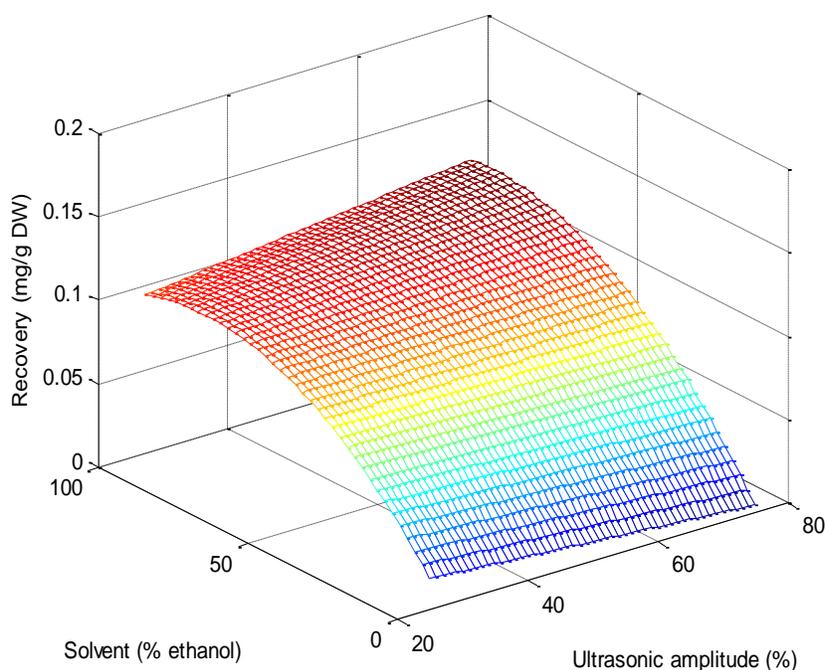


Figure 5a. Response surface plot showing the effect of ultrasonic amplitude and solvent on xanthere recovery. The process temperature was constant at 45°C.

$$Y = 0.078894 - 0.00116X_1 + 0.00137X_3 - 1.58 \times 10^{-5}X_3^2 - 6.52 \times 10^{-6}X_1X_2 + 1.79 \times 10^5X_1X_3 + 7.85 \times 10^{-6}X_2X_3 \quad (1)$$

Where Y is xanthere recovery (mg/g dried sample), X₁, X₂ and X₃ are the coded variables for ultrasonic temperature, ultrasonic amplitude and solvent (% ethanol), respectively.

In this research, the value of R² (0.9330) indicated a good agreement between the experimental and predicted values of xanthere recovery. The value of adjusted R² (0.8828) suggests that the total variation of 88% for xanthere recovery was attributed to the independent variables, and about 12% of the total variation could not be explained by the model. However, the value of the model was not significant (P > 0.05) that indicated the

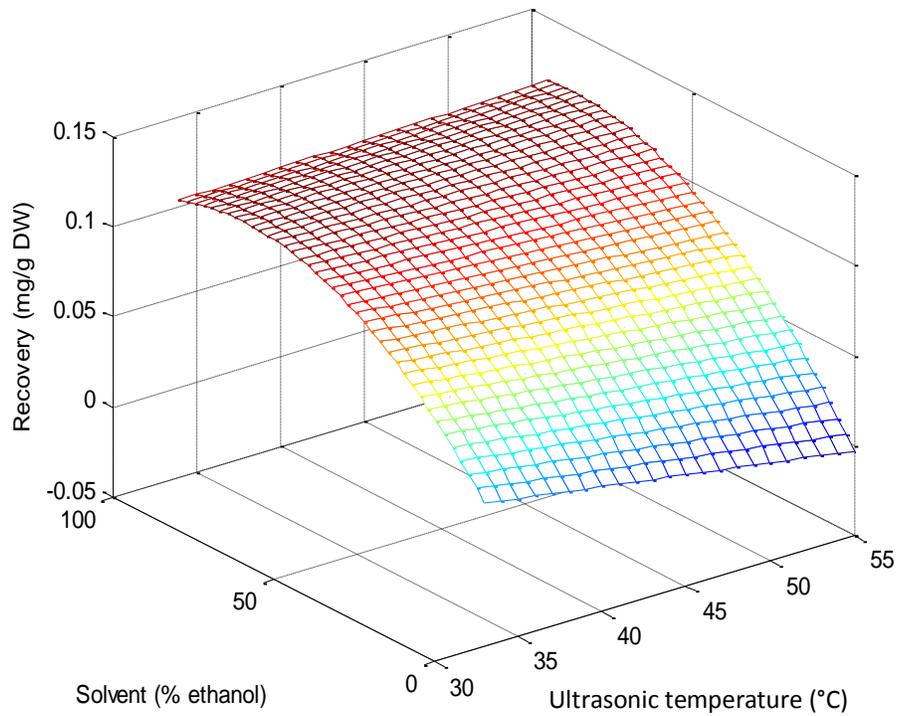


Figure 5b. Response surface plot showing the effect of ultrasonic temperature and solvent on xanthone recovery. The ultrasonic amplitude was constant at 50%.

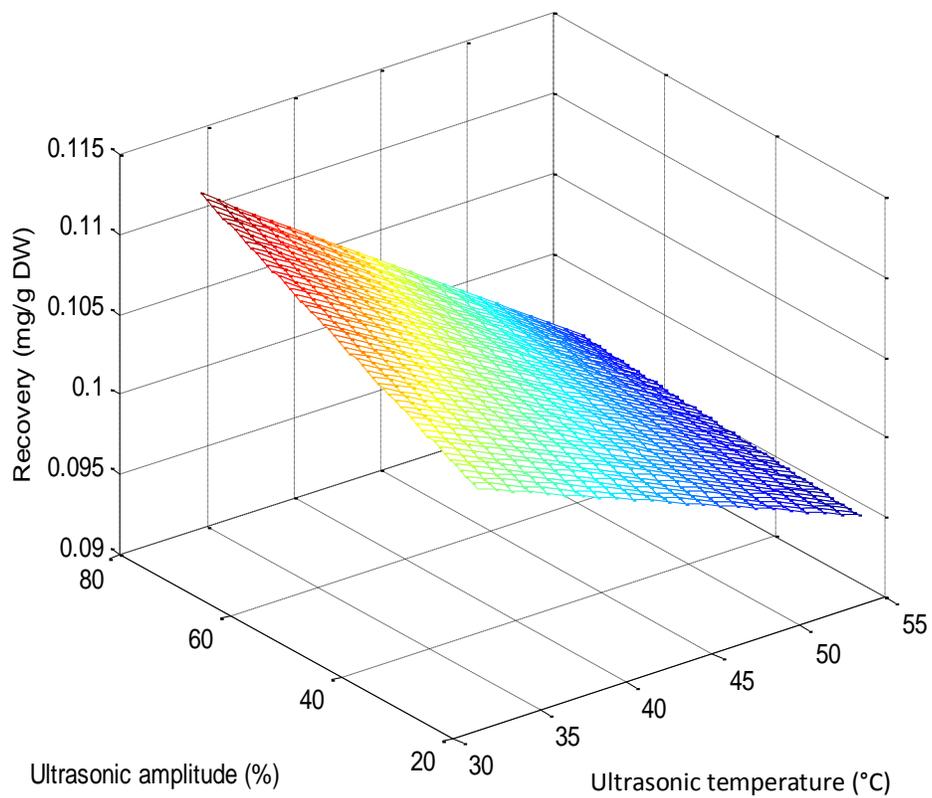


Figure 5c. Response surface plot showing the effect of process temperature and ultrasonic amplitude on xanthone recovery. The solvent was constant at 50% ethanol.

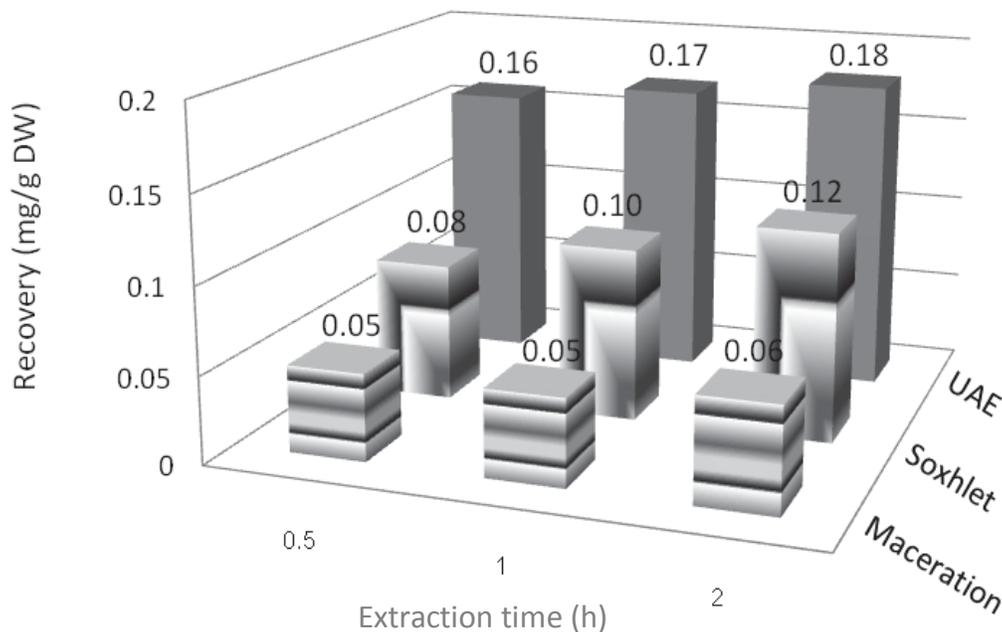


Figure 6. The xanthone recovery by maceration, soxhlet extraction and UAE applied with Box-Behnken design.

model did not exhibited a good fitness to the true behavior.

Validation of the model

In order to validate the adequacy accuracy of the model equation (Equation 1), a verification experiment was carried out under the optimum condition (temperature 33°C, 75% amplitude and 80% ethanol) within the experimental range. Under the optimum condition, the model predicted a maximum response of 0.1273 mg/g dried sample. The experimental result showed xanthone extraction of 0.1681 mg/g dried sample. It is not significantly different from the predicted value within the 95% confidence interval.

Comparison of ultrasonic assisted extraction and conventional technique

The xanthone recovery by ultrasonic assisted extraction was compared to maceration and Soxhlet extraction by applied extraction time 0.5, 1 and 2 h as shown in Figure 6. UAE was extracted by optimum condition from Box-Behnken design (33°C, 75% amplitude and 80% ethanol). This recovery by UAE applied with Box-Behnken design, Soxhlet extraction and maceration increased with increasing extraction time. The optimum conditions of UAE applied with Box-Behnken, Soxhlet extraction and maceration presented that extracted xanthenes in 2 h were 0.1760, 0.1221 and 0.0565 mg/g

of dried sample respectively. The UAE applied with Box-Behnken design showed the highest xanthone recovery (Figure 6). The response surface methodology was proved to be useful for investigating the optimum conditions of xanthone extraction. The statistical analysis showed that the optimum conditions for ultrasonic assisted extraction were ultrasonic temperature 33°C, ultrasonic amplitude 75 and 80% ethanol as solvent.

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Full Length Research Paper

Comparative mosquito repellent efficacy of alcoholic extracts and essential oils of different plants against *Anopheles Stephensi*

Mohammad Barat Shooshtari¹, Hamed Haddad Kashani², Siamak Heidari³ and Ruhollah Ghalandari^{4*}

¹Department of Plant Breeding, Shahrekord University, Shahrekord, Iran.

²Anatomical Sciences Research Center, Kashan University of Medical Sciences, Kashan,

³Department of Biology, Science faculty, Tarbiat Modares University, Tehran, Iran.

⁴Biotechnology Research Center, Science and Technology Institute, Tehran, Iran.

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Mosquitoes control and personal protection from mosquito's bites is one of the serious ways for preventing of contagious diseases distribution. Mosquitoes in addition to the local symptoms (itching, redness and irritation) can cause transmission of fatal and dangerous disease especially in tropical areas. In recent years, interest in plant-based products has been revived because of the development of resistance, cross-resistance and possible toxicity hazards associated with synthetic insecticides and their rising cost. Various plant-based products as herbal repellents are safe and biodegradable alternatives to synthetic chemicals for use against mosquitoes. In the present study, essential oils and extracts of six plants "*Melissa officinalis*, *Rosmarinus officinalis*, *Lavandula officinalis*, *Citrus limonum*, *Eucalyptus globulus* and *Ocimum basilicum*" were evaluated compared against mosquitoes of *Anopheles Stephensi*. Finally, repellent properties of essential oils and extracts as experimental groups and of *N, N-diethyl 3 - methylbenzamide* (DEET) as a positive control group were compared. We used Duncan's multiple range tests to determine the significant differences at 1% level between the experimental group and the control group. Results of statistical analysis showed significant differences between the extracts and essential oils. Essential oils indicated more effectiveness rather than extracts.

Key words: Malaria, *Anopheles stephensi*, insect repellent, essential oil, plant extract.

INTRODUCTION

Problems with chemical insecticides and possible effect of essential oils attracted the attention of researcher. In the recent years, long term application of chemical substances for controlling, repelling and killing of hazardous insects make serious anxieties for environment and human health (Nerioa, 2010; Yang, 2002).

Therefore, uses of environment friendly and biodegradable natural insecticides of plant origin have received renewed attention as agents for mosquitoes control (Nerioa, 2010). The years before 1940, volatile substances such as Citronella oil, Clove seed oil and

Camphor were the common insect repelling substances. In addition some plants such as *Marquise*, *eucalyptus*, *fennel*, *oregano*, *pepper*, *wormwood* plant and tea tree are known to show such properties (Wilkinson, 1996; Sima, 2012).

Natural Iranian flour consists of many herbs which are traditionally used as repelling insects especially against *malaria* vectors. According to WHO report, eastern Mediterranean countries like Iran are the source of contagious disease by insects specially mosquitoes species (WHO, 2010; Jinous and Fereshteh, 2012). So far, 64 species of mosquitoes have been reported from Iran, which included 28 species of *Anopheles*, 3 species of *Ades* and 19 species of *Culexus* and 14 species are from other genus (Azari, 2007). Among all the insect

*Corresponding author. E-mail: hamedir2010@gmail.com.

Table 1. Six tested plants.

No	Plant name	Family	Parts used for extracts and essential oils	Common name
1	<i>Lavandula officinalis</i>	Lamiaceae	Flower	Lavender
2	<i>Melissa officinalis</i>	Labiatae	Leaf	Melissa
3	<i>Rosmarinus officinalis</i>	Lamiaceae	Leaf	Rosemary
4	<i>Citrus limonum</i>	Rutaceae	Peel	Lemon
5	<i>Eucalyptus globolus</i>	Myrtaceae	Leaf	Eucalyptus
6	<i>Ocimum basilicum</i>	Lamiaceae	Herb	Basil

vectors of human disease, *Anopheles* is the most popular and worst species of mosquitoes (Collins, 1995; Curtis, 1994).

Among the species, *Anopheles stephensi* mosquito is the most important malaria vector especially in Iran. This mosquito in India and some of West Asian countries are annually the reason of 40 to 50% of malaria disease. Globally, malaria kills 3 million people each year, including 1 child every 30 s (Shell, 1997). The search for effective vaccines against malaria is still in progress. Individual protecting actions, comprising repellents are extensively applied to put off the transmission of arthropod – borne diseases by decreasing access between persons and vectors (Coleman, 1993; Walker, 1996). World War II and the needs of military groups in the tropical lands promoted the scientists to extensive research for discovery and preparation of insect repellent drugs (Wilkinson, 1996). Undoubtedly, Iranian military forces are face to such disease by the insects; thus, more than ten thousands chemical compounds have been tested for the insect repelling effects. Nevertheless, long term use of chemical insecticides has side effects on health. To avoid the adverse effect, researches on repellents that are derived from plant are promising in that they are effective, safe to users and also inexpensive (Abu-Qare, 2001; Fradin, 1998; Barnard, 2004).

For this reason and achieving better results, we select the plants that can be grown in Iran and are easily propagated. An enormous amount of plant products have been stated to have mosquito larvicidal and/or repellent action against mature mosquitoes.

In the previous studies, 10 medicinal herbs were used as repelling and killing of ova, larva and mature insects of *Anopheles stephensi*, *Aedes Aegypti* and *Culex quinquefasciatus*. Finally, ginger and rosemary essential oils were introduced respectively as a killer and repellent of three mentioned mosquitoes. Also, repellent effects of either essential oil and extract of lemon and Melissa against *A. stephensi* were examined and the results showed better effect of essential oil rather than extract, but differences were not significant (Veena, 2005; Vatandoost, 2004). In another study, the essential oil of five different plants leaves and their repellent effects was investigated against the *Anopheles* mosquito. Other scientists also were introduced essential oils as repelling of the malaria-carrying insect (Oshaghi, 2003; Rajkumar,

2007). Essential oils repellency of aromatic plants that grow in Argentina against *Aedes aegypti* have also evaluated: *Acantholippia seriphioides*, *Achyrocline satureioides*, *Aloysia citriodora*, *Anemia tomentosa*, *Baccharis spartioides*, *Chenopodium ambrosioides*, *Eucalyptus saligna*, *Hyptis mutabilis*, *Minthostachys mollis*, *Rosmarinus officinalis*, *Tagetes minuta* and *Tagetes pusilla*. Most essential oils were effective (Gillij, 2008; Muhammad, 2012).

In recent years, several plants extracts including neem (*Azadirachta indica*, *A. bJuss*), Citronella grass (*Cymbopogan nardus Rendle*), basil (*Ocimum basilicum L.*, *Ocimum gratissimum L.*, *Ocimum americanum L.*), clove (*Syzygium aromaticum L.*), prickly straggler (*Solanum trilobatum L.*), musk basil (*Moschosma polystachyum L.*) and thyme (*Thymus vulgaris L.*) have been studied as possible mosquito repellents (Gillij, 2007). Consequently, the aim of this study is to compare the repellent activity of plant essential oils and plant extract of six plants against the *A. stephensi*.

MATERIALS AND METHODS

Plant selection

In this study plants according to ancient data bases for insect repelling were selected. Also tried to choose plants that are easily propagated and using of them be safe for human and does not induce toxicity in person. Table 1 showed six plants that were tested. The plants from the farm of Karaj Agriculture Faculty (Tehran University) were prepared. Leaves and spire of the plants which contain the effective substances were selected and in a proper condition with suitable air circulation and far from direct sun light were dried. Dried herbal parts were milled. Then from powder by the maceration method and use of 70% ethanol, alcoholic extract were prepared. In this method 100 g powder in 1 L of ethanol as a solvent macerated by vacuum machine and in temperature of 40°C were concentrated. For essential oil preparation, for each 100 g of herbal powders, 1 L double distilled water was added and essential oil extraction was done in Clevenger machine (Tyler, 1988). Then repellent effects of both herbal extracts and essential oils separately on *Anopheles* mosquitoes were investigated (Buescher, 1985; Klun, 2000).

To prepare different concentrations, the products were further diluted using alcohol as diluents. Extract and essential oil solutions were formulated on a volume- volume basis at a concentration of 3 and 1%, respectively. The compounds were applied as 4-ml aliquots of ethanol solution and were spread evenly over the animal skin as explained previously (Buescher, 1985; Klun, 2000).

Table 2. Relative repellent effectiveness of 3% extracts and 1% oils of *Lavandula officinalis*, *Melissa officinalis*, *Rosmarinus officinalis*, *Citrus limonum*, *Eucalyptus globulus*, *Ocimum basilicum* laboratory mosquitoes of *Anopheles stephensi* on guinea-pigs in the laboratory. Efficacy of each experimental substance was repeated three times. Highest efficacy were supposed for *Lavender oil* and lowest efficacy for *Ethanol*.

Repellent	Replicate I*	Replicate II	Replicate III	Mean	Mean \pm SE (%)
Lavender oil	100	97	94.5	97.1667 ^a	97.16 \pm 2.75
Melissa oil	93.5	84.5	100	92.6667 ^a	92.66 \pm 7.78
Rosemary oil	84.4	93	97.5	91.6333 ^a	91.63 \pm 6.65
Lemon oil	95.9	90	92	92.6333 ^a	92.63 \pm 3.00
Eucalyptus oil	96	95.45	100	97.1500 ^a	97.15 \pm 2.48
Basil oil	96.4	93.4	94.2	94.6667 ^a	94.66 \pm 1.55
DEET(standard)	97	100	94	97.0000 ^a	97.00 \pm 3.00
Lavender extract	95	100	94.73	96.5767 ^a	96.57 \pm 2.96
Melissa extract	71.42	83.3	74.2	90.0667 ^a	76.30 \pm 6.21
Rosemary extract	79.16	71	66.8	76.3067 ^b	72.32 \pm 6.28
Lemon extract	29.41	58.8	62.15	72.3200 ^b	50.12 \pm 18.01
Eucalyptus extract	93.7	92.5	84	73.9333 ^b	90.06 \pm 5.2
Basil extract	73.3	82.5	66	50.1200 ^c	73.93 \pm 8.2
Ethanol (control)	0	0	0	0	0

Averages that have the same letters are not statistically significant at 5%. *Efficacy of each experimental substance was repeated three times and finally we calculate the mean.

Insect selection

The mosquitoes of *A. stephensi* was obtained from a well-established laboratory colony from school of Public Health and Institute of Health Research, Tehran University of Medical Sciences. Mosquitoes were reared and maintained at 27 \pm 3°C and 80 \pm 10 relative humidity (RH) under a 12:12 (L:D) photoperiod. Larvae were reared on a diet of floating catfish food. Female mosquitoes are only the *malaria* vectors, so they were selected. The adults were maintained in screen cages on 10% sucrose solution but 24 h before experiments the sucrose solution was removed from cages. Repellency was tested against 3 to 5-day-old, blood-starved mosquitoes, and for each test 25 mosquitoes were used (Barat, 2012; Norashiqin, 2008).

Animal testing

The extracts and essential oils were tested on animal guinea pigs. The animals were laboratory reared albino males with average 400 to 450 g weight. A 4 x 6 cm of animal abdomen hairs was removed then washed and cleaned by ethanol. Treatments were 4 ml of either the extracts containing 0.12 and 0.04 g of active ingredients of extracts and oils, respectively, and 0.4 ml DEET (N, N-diethyl-3-methylbenzamide) in the same way were used as a positive control. After treatment, the animal was bound on top of the cage in which the treated position was exposed to mosquitoes for 30 min. Each test was repeated three times replacing new mosquitoes and new animal, and number of bites through the fabrics was recorded. Animal experimentation was approved by the Animal Research Committee of Tehran University.

Statistical analysis

To compare the repellent efficacy of the compounds, we used Duncan's multiple range tests to determine the significant differences at 1% level between the experimental group and the control group.

RESULTS

Results of laboratory study on animals comparing extracts and oils of *Melissa officinalis*, *R. officinalis*, *Lavandula officinalis*, *Citrus limonum*, *Eucalyptus globulus*, *Ocimum basilicum* and DEET against *A. Stephensi* are presented in Table 2. Results showed that oils were significantly more effective than extracts. *L. officinalis* and *E. globulus* oils have most effectiveness. In this study, there was no significant differences between oil and extract of *Lavandula* (97.16 and 96.57%, respectively) and *Eucalyptus* (97.15 and 90.06%, respectively) that could indicate the better effectiveness of these two herbs in repelling of *A. Stephensi*. There are significant differences in the effectiveness rate of oils and extracts of *M. officinalis*, *R. officinalis*, *O. basilicum* and especially *C. limonum*, which repelling effect of the extracts were less than oils (Table 3). In this study, comparing repellent effect of oils rather than chemical control (DEET) was acceptable but comparing of *DEET* with extracts was not significant.

DISCUSSION

Present study shows herbal essential oils have better repellent efficacy rather than herbal extracts. Hence, essential oils could be also used as a better and safer substitution of chemical repellent substances such as *DEET* (Barat, 2012).

Previous studies regarding the extracts and essential oils of *Melissa*, *Rosemary*, *Lavender*, *lime* and *ginger* that have been done by others support our study and they

Table 3. Influence of variance analysis of extracts and essential oils of six herbs on the repellent rate of *Anopheles stephensi* in guinea pigs.

Source of variant	Mean squares	Sum of square	df	F ₅	F ₁	F _c
Treatment (between groups)	601.6	7219.23	12	2.15	2.96	12.09**
Error (within groups)	49.75	1293.567	26			

**Significant at 1% probability.

have also proposed the essential oils as a suitable substitution of chemical repellent (Oshaghi, 2003; Rajkumar, 2007; Barat, 2012; Kweka, 2009). In the animal experiments, *Lavender* and *Eucalyptus* oils rather than other oils had a better repellent effectiveness, 97.16 and 97.15% respectively, against *anopheles* (Gillij, 2007). Therefore, they could be recommended as a safe and suitable substitution of chemical repellent. In this work, we have tested the repellents against only one species and do not know if these compounds are protective against other mosquito species or medically important insects. By more clinical trial we may introduce the essential oils in the insect's repellent herbal cream formulation. Different factors may interfere in insect's repellent efficacy that the main factor could be effective substances of essential oils and extracts. Therefore, analysis of different fractions of herbal oils and extracts and its effect on the insects is recommended.

Most of these essential oils are highly volatile and this contributes to their poor longevity as mosquito repellents. However, this problem can be addressed by using fixatives or careful formulation to improve their longevity. For example, oils from turmeric and hairy basil with addition of 5% vanillin repelled 3 species of mosquitoes under cage conditions for a period of 6 to 8 h depending on the mosquito species (Tawatsin, 2001). The exception to this is para-menthane 3, 8 diol which has a lower vapour pressure than volatile monoterpenes found in most plant oils (Barasa, 2002) and provides very high protection from a broad range of insect vectors over several hours (Carroll, 2006).

The plants can be used alone or combined for effective protection against mosquitoes. They can also be used for control of mosquito breeding (Barnard, 2004; Trongtokit, 2005). They also offer safer alternative to synthetic chemicals and can be obtained by individuals and communities easily at a very low cost. However, toxicity tests of the active plants need to be done to ascertain their safety in administration (Robert, 1991; Rutledge, 1978).

Conclusion

The identification of these potential repellent plants from the local flora will generate local employment and stimulate local efforts to enhance public health. However, pilot studies are indicated to evaluate the epidemiological

impact and cost-effectiveness of the natural oils which are reported to be effective in mosquito control or provide protection against mosquito bites. Further investigations are needed to elucidate the six essential oils against a wide range of mosquito species and also to identify active compounds responsible for repellent activity and to be utilized if possible, in preparing a commercial product/formulation to be used as insecticidal.

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Short Communication

Effect of finasteride on lipid profile in individuals with androgenetic alopecia

Seied Reza Seied Mohammad Doulabi¹, Hossein Kavoussi^{1*}, Danial Isapour¹,
Amirhossein hashemian² and Ali Taheriniya³

¹School of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.

²Department of Biostatistics and Epidemiology, School of Health, Kermanshah University of Medical Sciences, Kermanshah, Iran.

³Department of Emergency Medicine, Alborz University of Medical Sciences, Karaj, Iran.

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Atherosclerosis constitutes one of the most frequent diseases and one of the important predisposing factors for atherosclerosis is lipid profile change. Androgen changes lipid profile, mainly high density lipoprotein (HDL), and oral finasteride are used for treating androgenetic hair loss as a risk factor for atherosclerosis. This study was conducted in order to determine the lipid profile changes by 1 mg finasteride tablets daily in patients with androgenetic hair loss. Twenty-five patients with androgenetic hair loss were prescribed one 1 mg finasteride tablet daily. Fasting plasma levels of low density lipoprotein (LDL), HDL, triglyceride, total cholesterol and HDL to LDL ratio of patients before therapy and after 3 and 6 months of therapy were measured. The study was conducted in the form of a before-after clinical trial. Data were analyzed using SPSS software version 16. A statistically significant rise in triglyceride plasma level ($p=0.014$) and significant decreases in HDL plasma level ($p<0.001$) was observed after 3 and 6 months of therapy, respectively. Plasma levels of LDL, total cholesterol and HDL to LDL ratio were not changed significantly but changes in ratio between third and sixth month were significant. Therefore finasteride may decrease dihydrotestosterone and increase testosterone that could lead to complications of the lipid profile by reducing HDL and increasing total cholesterol.

Key words: Atherosclerosis, finasteride, testosterone, lipid profile.

INTRODUCTION

Atherosclerosis constitutes one of the most common diseases, with an increasing frequency. It is predicted that atherosclerosis will be the top cause of death in 2020. Atherosclerosis may involve vessels of different regions of the body and thus induce stroke and myocardial infarction (Fauci et al., 2008). Lowering the low density lipoprotein (LDL) level through administration of statins may prevent atherosclerosis. Increasing high density lipoprotein (HDL) and lowering triglyceride are two other major factors for preventing atherosclerosis (McRobb et al 2009) indicated that androgens may cause vascular calcification.

Smoking is considered as a risk factor associated with elevated serum level of triglyceride, cholesterol and LDL but HDL was almost similar in both groups of smoker and non-smoker (Al-Ajlan, 2012). *Tulbaghia violacea* is a valuable medicinal plant used in South Africa for management of heart diseases and many human disorders and methanolic extract of it in rat reduces plasma level of triglyceride, cholesterol, VLDL and LDL (Olorunnisola et al., 2012).

Finasteride is used for treating benign prostatic hyperplasia and hormonal hair loss, exerting its effect through inhibition of 5- α reductase (Moorjani et al., 1987). Long-term therapy with 1 mg finasteride tablets may prevent androgenetic hair loss (Barud et al., 1999). It also suppresses the conversion of testosterone to dihydrotestosterone, the most potent metabolite of testosterone (Asscheman et al., 1994; Hämäläinen et al.,

*Corresponding author. E-mail: s8371916@kums.ac.ir. Tel: +98 9127016682. Fax: 00988318368013.

Table 1. Lipid profile changes.

Lipid	Before therapy	After 3 months		After 6 months	
	Mean \pm SD	Mean \pm SD	Percent change	Mean \pm SD	Percent change
HDL	43.6 \pm 9 ^a	44.91 \pm 10.7	3.6 \pm 17.1	38.5 \pm 8.1	-13.2 \pm 12 ^a
LDL	103.8 \pm 27.8	99.8 \pm 26.2	-1.7 \pm 22.1	103.2 \pm 27.2	-2.6 \pm 12.2
HDL/ LDL	0.43 \pm 0.1	0.48 \pm 0.16	8.9 \pm 22.6 ^b	0.39 \pm 0.11	-9.2 \pm 19.2 ^b
Triglyceride	93 \pm 32.9 ^c	117.2 \pm 48.2 ^c	32.8 \pm 51	94.8 \pm 28.3	16.5 \pm 48.2
Total cholesterol	162.1 \pm 36.6	166.1 \pm 32.9	5.3 \pm 24.6	160 \pm 38.3	-0.9 \pm 19.5

Values with different superscripts differ significantly ($p < 0.05$).

1987). Androgenetic hair loss is considered a risk factor for atherosclerosis (Dogramaci et al., 2009). The increase in HDL level following total testectomy and the subsequent decrease in testosterone have been demonstrated in patients with prostatic cancer (Moorjani et al., 1987). Furthermore, the impact of testosterone on lowering HDL and increasing cholesterol and triglyceride has been established (Asscheman et al., 1994). Also, an inverse relationship exists between the plasma level of free testosterone and serum triglyceride (Hämäläinen et al., 1987). HDL to LDL ratio has been confirmed to be not only an important factor for development of cardiovascular disease (Reaven, 1988; Onyesom et al., 2012) but also used to efficacy of anti-lipid drug therapy (Kannel, 2005).

There are a few studies that evaluated the effect of using finasteride tablets on lipid profile with variable results (Moorjani et al., 1987; Denti et al., 2000; Amory et al., 2008; Duskova et al., 2010). According to predictor factor of androgenetic alopecia for atherosclerosis and the effect of using oral finasteride tablets on lipid profile, we undertook the present study to determine the impact of using 1 mg finasteride tablets on lipid profile of patients with androgenetic hair loss.

MATERIALS AND METHODS

This study took place in Hajdaie Clinic of Kermanshah University of Medical Sciences in the city of Kermanshah, west of Iran. This is a before-after clinical trial.

Subjects

Twenty-five patients with androgenetic hair loss as confirmed by a dermatologist were selected. Prior to entry, the patients were inquired about using other drugs which may influence the lipid profile, such as statins and patients who used these drugs were excluded from the study. All patients were referred to one single laboratory for tests and they were fasting before the tests. During the study, patients were evaluated for nutritional status. Patients had different degrees of androgenetic hair loss and this difference did not affect our study. All patients expressed their informed consent in writing prior to the study. The proposal of the study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and registered in IRCT database.

Experimental design

Blood samples were obtained from patients at three stages (before therapy, after three months and six months of treatment with 1 mg finasteride tablets) and their fasting plasma levels of LDL, HDL, triglyceride and total cholesterol were measured. Patients who did not take finasteride regularly or had discontinued therapy were eliminated from the study.

Sample collection

Twenty-two patients underwent the second phase of tests and finally 16 patients with all three tests and regular consumption of 1 mg finasteride tablets during 6 months remained in the study.

Statistical analysis

Analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, U.S.A.). The statistical analysis was performed using the dependent t-test with an assumption that p -value ≤ 0.05 is significant.

RESULTS

Changes in parameters were compared. Analysis of data indicated a significant decrease in fasting plasma level of HDL after 6 months of therapy ($p < 0.001$) as well as a significant increase in plasma level of triglyceride after 3 months of therapy ($p = 0.006$). No significant change was observed in the fasting plasma levels of LDL and total cholesterol. Although HDL to LDL ratio did not show significant change after 3 ($p = 0.073$) and 6 months ($p = 0.056$), however, changes in ratio between third and sixth month were significant ($p = 0.006$) (Table 1).

DISCUSSION

Our study is the first to indicate that 1 mg finasteride tablets used for treatment of androgenetic hair loss causes a significant decrease in HDL after 6 months of therapy and a significant increase in triglyceride after 3 months of therapy also changes ratio of HDL/LDL between third and sixth months significantly (p -value = 0.006), all of which may increase the risk for

atherosclerosis. Moreover, a study by Moorjani et al. (1987) indicated that anti-androgenic medication such as finasteride may increase HDL in patients with prostatic cancer while leaving LDL intact. An Italian study on patients with benign prostatic hyperplasia indicated that using 5 mg finasteride tablets may increase HDL and decrease LDL after 6 months of therapy, which may be due to the direct impact of the drug on hepatic metabolism or through dihydrotestosterone (DHT) suppression (Denti et al., 2000), (Movérare-Skrtic et al 2006) reported that treatment with DHT may increase HDL and decrease TG. Amory et al. (2008) indicated that suppressing DHT with 5- α reductase inhibitors, such as finasteride tablet, does not lead to adverse modifications of the lipid profile.

In addition, Duskova et al. (2010) conducted a study on 12 patients to observe that finasteride an initial rise in LDL, HDL and total cholesterol which became constant with progression of the study. We assume that the different results from previous studies may be related to dosage or duration consumption of finasteride, age and nutritional status of patients and different genetic ability of drug hepatic metabolism. While HDL to LDL ratio has been proved to be a predictor factor of cardiovascular disease (Reaven., 1988; Onyesom et al., 2012), our study did not show significant change after 3 and 6 months but changes ratio between third and sixth month were significant.

The risk of stroke and myocardial infarction is higher in young men compared to young women. This change in lipid profile may be due to testosterone elevation by using finasteride. As earlier mentioned, it is recommendable to identify the risk factors for atherosclerosis in patients with androgenetic hair loss so that 1 mg finasteride tablets may be waived for high-risk patients. Moreover, the adverse effect of the drug may be countered through recommendations made to the patients regarding lifestyle modification and abstaining from high-fat food. Further studies aimed at the effects of finasteride exerted on vessels through lowered DHT may corroborate our findings.

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