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Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Journal of Medicinal Plant Research

Full Length Research Paper

Influence of oil content and yield of *Foeniculum vulgare* Mill. cv. Soroksary seeds by adapting different plant densities

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In this experiment, the effect of different plant densities on the oil content and yield of *Foeniculum vulgare* Mill. Cv. Soroksary seeds was studied at the Faculty of Agricultural Sciences and Engineering, Karaj, Iran (Latitude 35° 47´ N and Longitude 50° 59´ E) in 2008. Different spacing studied were 10, 15, 20, 25, and 30 cm and the distance between rows in all treatments was 40 cm using a complete randomized block design with three replicates. According to the results, the effect of plant density on oil content and yield was significant (P<0.01). The highest oil content (3.33%) and yield per hectare (116.73 L) was obtained with the lowest plant density.

Key words: *Foeniculum vulgare* cv. Soroksary, plant density, oil content, oil yield.

INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.) is one of the most important medicinal plants, native of Mediterranean regions and belongs to the Apiaceae family (Omidbaigi, 2007). The plant has abundant applications in various industries; for instance, the essential oil obtained from seeds is added to perfumes, soaps, pharmaceuticals and cosmetics. Fennel oil, seeds or extracts are also used for flavoring food preparations including meats, ice cream, candy, baked goods and condiments. Recent studies have shown that essential oil of this plant can be used as a valuable antioxidant, antibacterial and antifungal agent (Lucinewton et al., 2005). One of the major restraints in crop production is improper crop spacing in the field (Dupriez and Deleener, 1989). The effect of spacing on growth and secondary metabolites is largely due to change in the interception of radiant energy (Yao and Shaw, 1964). When crops are over crowded, there will be competition for water, sunlight and nutrient uptake. In the wider spacing, there will be no competition for water, sunlight and nutrient uptake between the plants (Ozar, 2003). Plant density is one of the most important factors affecting yield, yield components, oil and essential oil in medicinal plants. Masood et al. (2004) investigated the effect of row spacing (40, 50, 60, and 70 cm) on morphological characters and seed yield of fennel and reported that the highest plant height, seed yield per bed, and seed yield per hectare were obtained with the lowest row spacing. Arabaci and Bayram (2004) reported that

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Table 1. Physico-chemical properties of soil.

Soil sample	Property
pH (in 2:1 water)	8.1
Sand (%)	30
Clay (%)	32
Silt (%)	38
Ca (g/kg)	293
Fe (mg/kg)	12.1
Organic matter (g/kg)	0.78
N (g/kg)	0.092
P (cmol/kg)	12.83
K (cmol/kg)	305

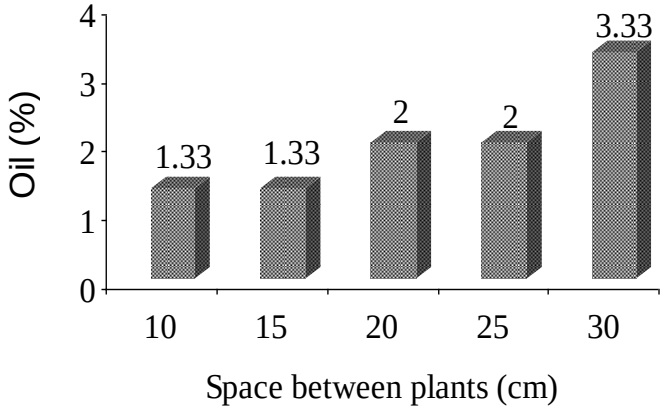

Figure 1. Relationship between plant density and oil percentage.

Table 2. Oil content and yield of seeds in different plant cultivation densities.

Space between plants (cm)	Oil content (%)	Oil yield (L/ha)
10	1.33[a]	86.19[c]
15	1.33[a]	65.27[a]
20	2[b]	85.89[c]
25	2[b]	72.94[b]
30	3.33[c]	116.73[d]

Different letters in each column indicating significant difference at P<0.01.

the highest yield in the Basil (*Ocimum basilicum* L.) was obtained in lower plant density. The maximum oil percentage and oil yield in Coriander (*Coriandrum sativum* L.) were obtained in density 30 plant per m^2 (Masood et al., 2004). Based on the aforementioned facts in view, the study was conducted, to evaluate the percentage variation and oil yield of *F. vulgare* cv. soroksary in different plant densities.

MATERIALS AND METHODS

A field study was conducted at the Faculty of Agricultural Sciences and Engineering, Karaj, Iran (Latitude 35° 47´ N and Longitude 50° 59´ E) to determine the effect of different plant densities on the oil content and yield of *F. vulgare* cv. Soroksary seeds, in 2008. A result of soil analysis is shown in Table 1. Experiment was conducted in a completely randomized block design with three replicates and different plant densities. The plot size was 2.5×1.5 m. The distance between blocks and plots were 1 m. Different plant spacing studied were 10, 15, 20, 25, and 30 cm. The distance between rows in all treatments was 40 cm. Each plot consisted of five rows. The bitter fennel seeds were sown on the 7th of March 2008. The following irrigation regime is as follows: (1) Irrigation interval of 2 to 3 days until germination stage, (2) Irrigation interval of 4 to 5 days from germination to appearance of first flowers stage, (3) Irrigation interval of 7 days from appearance of first flower to harvest stage. Thinning was done when plants had 4 to 5 leaves.

General agronomic practices were done for all the treatments. The seeds were harvested twice after ripening (20th August and 30th August) and dried in a shade for 72 h. After drying, 15 g of seeds were powdered and their oil content was extracted using a soxhlet apparatus with hexane solvent method (Harwood et al., 1999). After isolation, the oil was purified in a rotary vacuum evaporator apparatus (Buchi, Switzerland). Data collected were analyzed using Duncan Multiple Range Test (Duncan, 1955) and statistical software (SPSS).

RESULTS AND DISCUSSION

Different plant densities influenced the oil content and seed yield of bitter fennel. With increasing the spacing between plants, the oil content of seeds was increased but the pattern of changes in oil yield was irregular. Generally with increase in spaces between plants, the oil percentage increased significantly (P<0.01). The maximum oil percentage (3.33%) and yield (116.73 L/ha) were obtained in the lowest plant density. While the minimum oil percentage (1.33%) was obtained in the highest plant density and the minimum oil yield (65.27 L/ha) was obtained in 15 cm space between plants (Figures 1 and 2). Comparison of treatments indicated that there was no significantly difference in the oil percentage between 10 and 15 cm of plant densities. Similarly, there was no significant difference in the oil percentage between 20 and 30 cm of plant densities (Table 2). These results are in concomitant with the findings of Akbarinia et al. (2006) and Ozer (2003). The studies in most of the plants have shown that plant density is an important factor affecting yield. In the lower plant density, the competition between the plants for nutrients, sunlight, water and air is very less and therefore the plants grow better and finally produce the higher oil content and yield. But in the higher plant densities, they have restricted conditions for development and thus produce the lower yield. In conclusion, to reach the maximum oil content and yield of *F. vulgare* cv. Soroksary seeds, the minimum plant density is suggested.

In this study, increases observed in the yield of seeds

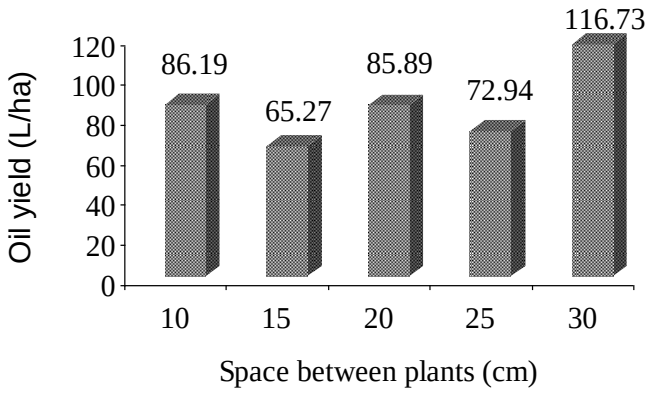

Figure 2. Relationship between plant density and oil yield.

can be attributed to the better growth of plants and subsequently the better canopy development which led ultimately to the better use of solar irradiance and higher photosynthesis. Considering the significant effect of different plant densities, it can be argued that seed yield increases in suitable plant densities are due mainly to production of more seeds in each umbel.

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Full Length Research Paper

Phytochemical analysis and biological activity of a precipitate from *Pavetta crassipes*

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Precipitate obtained from the aqueous extract of a *Pavetta crassipes* K. Schum (Rubiaceae) was analyzed for biological activity and phytochemical makeup. Phytochemical studies of the precipitate showed the presence of flavonoids. Antimicrobial studies showed that the precipitate inhibited the growths of *Corynebacterium ulcerans*, *Escherichia coli* and *Pseudomonas aeruginosa* at 6.25 mg/ml with corresponding minimum bactericidal concentration (MBC) of 12.5 mg/ml. It also inhibited the growths of *Streptococcus pyogenes, Klebsiella pneumoniae* and *Neiserria gonorrhea* at 12.5 mg/ml with a corresponding MBC at 25 mg/ml. It however showed no inhibitory effect against *Staphylococcus aureus, Bacillus subtilis, Salmonella typhii* and *Candida albicans*. The precipitate is undergoing purification and characterization and will be reported in due course.

Key words: *Pavetta crassipes*, Rubiaceae, flavonoid, antimicrobial screening.

INTRODUCTION

Plants have a long history of use all over the world for the treatment of different diseases and complaints. In certain African countries, up to 90% of the population still relies exclusively on plants as a source of medicines and many of these plants have been documented. The available knowledge on the use of plant preparations in traditional medicine is enormous but if this is not rapidly researched, indications as to the usefulness of this vegetable treasure-house will be lost with succeeding generations (Hostettman et al., 2000).

Africa is reputed for the extraordinary richness of its flora, totaling several tens of thousands of species. Environmental degradation provides a threat to biological diversity but the sub-Saharan region still hosts a wide variety of indigenous species. Based on careful observation and a judicious choice of plants, it is possible to discover very new natural products (Hostettman and Marston, 1990).

Pavetta crassipes K. Schum. (Rubiaceae) is a low glabrous shrub of the savannah with stout sub-quadrangular branches covered with pale corky bark which splits and falls off. In Nigeria, the leaves of this plant are used medicinally in the management of respiratory infections and abdominal disorders. The leaves are also used in Tanzania in the treatment of gonorrhea. In Central Africa, the acid infusion of the leaves is taken as a cough remedy (Watt and Breyer-Brandwijk, 1962). The leaves are eaten by some native tribes pounded up with other food, or boiled in slightly fermented water in which cereals have been left to steep, and mixed with pap. The sap is a coagulant of rubber latex (Dalziel, 1956).

Alkaloid extracts from the plants have been shown to have significant anti-malarial activity (Sanon et al., 2003). The ethanol extract has been shown to lower the blood

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pressures of cats and rats in a dose dependent manner (Amos et al, 1993). In view of this, *P. crassipes* is a good candidate for screening based on its therapeutic uses. It is imperative that a study of the plant be carried out with a view to justifying the claims by the traditional users and possibly isolating and characterizing the compound(s) responsible for the perceived activity.

MATERIALS AND METHODS

Plant extraction

The plant was collected in Zaria in the month of October, 2008 and identified at the Herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria as specimen with voucher number 3115. 1 kg of the fresh plant leaves was extracted using hot water and filtered. A yellow precipitate was observed. This was filtered using a Buchner funnel and trap under vacuum. It was dried and weighed and kept in a desiccator until needed.

Phytochemical studies

Phytochemical analysis was carried out on the precipitate using the method set out by Brain and Turner (1975).

Antimicrobial screening

The antimicrobial activity was determined using some pathogenic microorganisms. The microorganisms were obtained from the Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital, Zaria, Nigeria. All isolates were checked for purity and maintained in slants of blood agar. A solution of 0.5 g of the precipitate was made using 10 ml dimethyl sulphoxide (DMSO). This solution was used to check the antimicrobial activities of the precipitate. A control experiment was also set up using DMSO. Blood agar base (Oxoid, England) was prepared according to the manufacturer's instructions. This was then sterilized at 121°C for 15 min using an autoclave and was allowed to cool. The sterilized medium (20 ml) was pipetted into sterilized Petri dishes, covered and allowed to cool and solidify. The Petri dishes containing the medium were seeded with the test organisms by the spread plate technique and were left to dry for half an hour. Filter paper discs were cut and sterilized at 160°C for 30 min. The sterilized paper discs were then dropped into the solutions of the extracts and were dried at 45°C. The dried discs were then planted on the medium previously seeded with the test organisms. The plates were incubated at 37°C for 24 h after which they were inspected for the zones of inhibition of growth. The zones were measured and recorded in millimetres by the use of a pair of dividers and a ruler.

Minimum inhibition concentration (MIC)

Minimum inhibition concentration of the precipitate was carried out on the microorganisms that were susceptible to it and was carried out using the broth dilution method as described by Bauer et al. (1966). Nutrient broth (Oxoid, England) was prepared according to the manufacturer's instructions. 10 ml each was dispensed into 5 sets of screw cap test tubes and sterilized at 121°C for 15 min. The test tubes were allowed to cool. McFarland's turbidity standard scale number 0.5 was prepared. 10 ml normal saline was used to make a turbid suspension of the microorganisms. Dilution of the microorganisms was done continuously in the normal saline until the turbidity matched that of the McFarland's scale by visual comparison. At this point the microorganisms had a density of 3×10^8 cfu/ml. Serial dilution of the precipitate was made using the nutrient broth and the following concentrations were obtained; 50, 25, 12.5, 6.25 and 3.125 mg/ml. Having obtained the different concentrations, 1 ml of the microorganism in the normal saline was inoculated into the different concentrations of the precipitate in the broth and was incubated at 37°C for 24 h. The lowest concentration that showed no turbidity (clear solution) was recorded as the MIC.

Minimum bactericidal/fungicidal concentration (MBC/MFC)

This was carried out in order to determine whether the microorganisms could be completely killed or their growth could only be inhibited. Blood agar base (Oxoid, England) was prepared according to the manufacturer's instructions. The solution was sterilized at 121°C for 15 min using an autoclave and poured into sterilized Petri dishes. The contents of the MIC test tubes in the serial dilution were sub-cultured on the Petri dishes by dipping a sterile wire loop into each test tube and streaked on the surfaces of the Petri dishes. The Petri dishes were incubated at 37°C for 24 h after which they were observed for growth. The minimum bactericidal/fungicidal concentration (MBC/MFC) was the Petri dish with the lowest concentration of the precipitate that had no growth of the micro organisms.

RESULTS AND DISCUSSION

The phytochemical studies revealed the presence of flavonoids in the precipitate. Flavonoids are widely distributed in plants. They are known to be responsible for the yellow or red/blue pigmentations in flowers and also provide protection from attack by microorganisms and insects. The widespread distribution of flavonoids, their variety and their relatively low toxicity compared to other active plant precipitates (for instance alkaloids) had led to many animals, including humans, ingesting significant quantities in their diet without problems. Flavonoids have been referred to as "nature's biological response modifiers" because of the strong experimental evidence of their inherent ability to modify the body's reaction to allergens, viruses, and carcinogens. They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity (Cushnie and Lamb, 2005).

The results of the antimicrobials studies showed that the precipitate had remarkable activity at 50 mg/ml against six of the ten microorganisms tested with zones of inhibition between 15 to 22 mm. It could not inhibit the growth of *Staphylococcus aureus, Bacillus subtilis, Salmonella typhii* and *Candida albicans.* MIC and MBC studies showed that the precipitate inhibited the growths of *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Neiserria gonorrhea* at concentration of 12.5 mg/ml with anMBCat25mg/ml.*Corynebacteriumulcerans,Escherichia*

Table 1. Summary of MIC and MBC of precipitate (mg/ml).

Organism	MIC	MBC
Escherichia coli	6.25	12.5
Pseudomonas aeruginosa	6.25	12.5
Streptococcus pyogenes	12.5	25.0
Corynebacterium ulcerans	6.25	12.5
Klebsiella pneumoniae	12.5	25.0
Neiserria gonorrhea	12.5	25.0

coli and *Pseudomonas aeruginosa* were all inhibited at concentration of 6.25 mg/ml with corresponding MBC at 12.5 mg/ml (Table 1).

Conclusion

The results from this research have supported the ethno-medicinal uses of this plant in the treatment of respiratory infections, abdominal disorders, gonorrhea and as a cough remedy. These diseases can be caused by the respective microorganisms tested. The precipitate is being purified and characterized and *in vitro* activity-related studies are on-going to establish the toxicity if any of the precipitate.

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Full Length Research Paper

Recovery effect of *Zingiber officinale* on testis tissue after treatment with gentamicin in rats

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Ginger rhizome (*Zingiber officinale* R., family: Zingiberaceae) is used medicinally and as a culinary spice. Gentamicin is synthetic antibacterial agent antibiotic with a very broad spectrum against microbial pathogens, especially the gram-negative. The aim of this study was to investigate the recovery effect of ginger on testis after treatment with gentamicin in rats. The forty male Wistar rats were selected and randomly divided into control (n = 10) and experimental (n = 30) groups. The experimental groups were split into three groups. First group, second experimental group which received 50 mg/kg (IP) gentamicin and one group treated with 100 mg/kg/rat/day of ginger rhizome via gavages daily for 30 days, respectively. However, the control group just received normal saline (IP). On the thirteeth day, after taking biopsy from testis of each group, tissue preparation was performed and analyzed for apoptosis. There was a significant increase in apoptosis in gentamicin groups when compared with other groups (P < 0.05). Gentamicin antibiotic have negative effect on sperm parameters and testis histology in rats. However, these side effects are less seen in the gentamicin group that received 100 mg/kg/rat of ginger. Therefore, it is recommended that usage of ginger with gentamicin has fewer side effects on male fertility.

Key words: Apoptosis, gentamicin, *Zingiber officinale*, testis tissue.

INTRODUCTION

Ginger rhizome (*zingiber officnale* R.,family: zingiberaceae) is used worldwide as a spice. Both antioxidative (Khaki et al., 2009) and androgenic activity (Kirtikar et al., 1991) of *Z. officnale* were reported in animal models. All major active ingredients of Z. *officnale* such as zingerone, gingerdiol, zingibrene, gingerol and shogaols have antioxidant activity (Nassiri et al., 2009). Besides, other researchers showed that ginger oil has dominative protective effect on DNA damage induced by H_2O_2 and might act as a scavenger of oxygen radical and might be used as an antioxidant (Khaki et al., 2009). Antibiotics are commonly prescribed for a multitude of everyday condition. Not surprisingly, a proportion of male patients attending fertility clinics may have been prescribed antibiotics by their general practitioner to treat these unrelated infections. In addition, some patients requiring assisted conception occasionally show evidence of infection of the male reproductive tract. The antibiotic aminoglycoside (gentamicin, neomycin, streptomycin and ofloxacin) are routinely used by urologists and fertility specialists to treat such bacterial infections occurring prior to *in vitro* fertilization treatment or when

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Table 1. Apoptotic cells percent of male rats exposed to ginger rhizome.

Group	Control	Gentamicin+Ginger	Ginger	Gentamicin	P-level
Apoptotic cell (%) (spermatogonia and spermatocytes)	6±2.11	15.05±1.11	0.15±0.14	22.11±1.11	p<0.05

high concentration of leukocytes are present in the semen of these patients, irrespective of microbial evidence of infection (Khaki et al., 2009, 2008; Mosher and Pratt, 1991).

Therefore, the present study was designed to investigate the protective effects of ginger rhizome on toxicity of gentamicin on testis of rats.

MATERIALS AND METHODS

Forty adult male Wistar rats weighting 200 ± 10 g (Tabriz university of medical sciences, Iran) were used in this study. They were fed with standard diet pellets and allowed food and water for an acclimation period of two weeks. The animals were maintained in a strictly controlled temperature (18 ± 1°C). Humidity was kept at 50% and the lighting cycle was 7.00 to 19.00 h light and 19.00 to 7.00 h dark, with adequate ventilation. Animals were handled with human care in accordance with the National Institutes of Health guidelines. The rats were randomly divided into 4 groups each consisting of ten animals. The experimental groups were split into three groups. The first group, second experimental group which received 50 mg/kg (IP) gentamicin and the other group treated with 100 mg/kg/rat/day of ginger rhizome via gavages daily for 30 days, respectively. However, the control group just received normal saline (IP). At the end of 4 weeks of treatment, testis was dissected from each rat under anesthesia exactly 24 h after the last administration then tissue preparation was performed to investigate apoptosis by TUNEL.

Tunel analysis of apoptosis

The *in situ* DNA fragmentation was visualized by TUNEL method. Briefly, dewaxed tissue sections were predigested with 20 mg/ml proteinase K for 20 min and incubated in phosphate buffered saline solution (PBS) containing 3% H_2O_2 for 10 min to block the endogenous peroxidase activity. The section were incubated with the TUNEL reaction mixture, fluorescein-dUTP (*in situ* cell death detection, POD kit, Roche, Germany), for 60 min at 37°C. The slides were then rinsed three times with PBS and incubated with secondary antifluorescein-POD-conjugate for 30 min. After washing three times in PBS, diaminobenzidine-H_2O^2 (DAB, Roche, Germany) chromogenic reaction was added on sections and was counterstained with hematoxylin.

As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections and nucleotide mixture in reaction buffer was used instead. Apoptotic germ cells were quantified by counting the number of TUNEL stained nuclei per seminiferous tubular cross section. Cross section of 100 tubules per specimen were assessed and the mean number of TUNEL positive germ cells per tubule cross section was calculated. In this method, the apoptotic cells can be identified by their darkly stained nuclei (Khaki et al., 2008).

RESULTS

Compared to the control group, number of apoptotic germ cells (spermatogonia and spermatocytes) per tubule cross section decreased following administration of 100 mg/kg/rat/day of ginger rhizome. Administration of 50 mg/kg/day gentamicin caused a significant increase in the apoptotic germ cells percent. When this dose of gentamicin was administrated together with 100 mg/kg/rat/day of ginger rhizome, apoptotic cells percent was significantly decreased from 22.11 ± 1.11 to 15.05 ± 1.11 indicating the protective effect of ginger rhizome against gentamicin-induced apoptosis (Table 1).

DISCUSSION

Infertility is one of the major problems in match's life, about 25 and 35% of infertility is regard to man and woman receptivity (Carlsen et al., 1992; Cummings and Bingham, 1998). The importance of many of these factors is not yet clearly understood. A better understanding of underlying mechanisms in fertility and better study results clarifying the effectiveness of nutritional and biochemical factors are important to improve diagnosis and treatment. Smart choices for better foods might prevent body from many diseases (Reddy et al., 2006; Suryavathi et al., 2005). As all spermatogenesis stages occur in seminiferous tubule of testis, it is possible to evaluate the extent of spermatogenesis by determination of number of spermatozoa produced per one gram of testicular parenchyma (Acharya et al., 2008; Hew et al., 1993). The sperm count is considered as important parameter assesses the effects of chemical on spermatogenesis (Yousef, 2005).

It has also been reported that there is a direct correlation between the epididymal sperm count and motility with fertility in animals (Dawson et al., 1992; Timmermans, 1989; Yu et al., 2005). The oxidative damage, elevated lipid peroxidation and the alteration of membrane properties can lead to germ cell death at different stages of development and the sperm count decrease (Bestas et al., 2006). Accordingly, it is expected that antioxidant therapy acts as a protective defense against oxidative stress and improve fertility parameters. The ability of antioxidants such as ascorbic acid in semen to protect spermatozoa from oxidative damage has been shown by some authors (Timmermans, 1989).

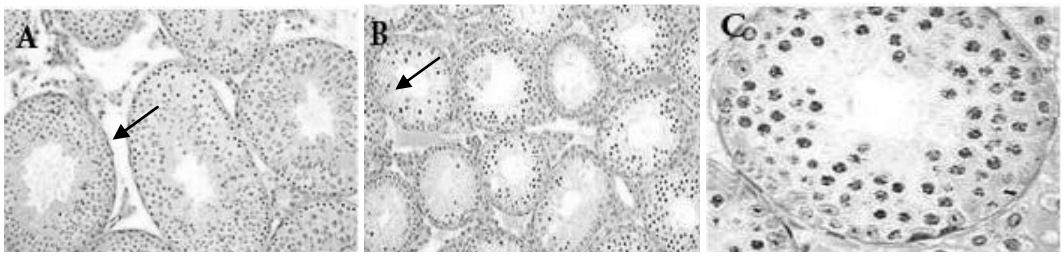

Figure 1. (A) Photomicrograph of testis in Ginger group, there are no apoptotic germ cells(tunel method) × 320. (B) Photomicrograph of testis in Gentamicin group that received Ginger, there are apoptotic germ cells, arrow (tunel method) × 320. (C) Photomicrograph of testis in Gentamicin group, there are apoptotic germ cells, arrow (tunnel method) ×320.

The main pharmacological actions of ginger and compounds isolated there include immuno-modulatory, anti-tumorigenic, anti-inflammatory, anti-apoptotic, anti-hyperglycemic, anti-lipidemic and anti-emetic action. Ginger is a strong antioxidant substance and may either mitigate or prevent generation of free radicals. It is considered a safe herbal medicine with only few and insignificant adverse/side effects (Dawson et al., 1992). Oxidants and antioxidants have attracted widespread interest in nutrition research, biology and medicine. It has become clear that constant generation of pro-oxidants, including oxygen free radical, is an essential attribute of aerobic life (Acharya et al., 2008). A disturbance in the pro-oxidant/antioxidant system has been defined oxidative stress. Reactive oxygen species (ROS) are very reactive molecules ranked as free radicals owing to the presence of one unpaired electron such as a superoxide ion, nitrogen oxide and hydroxyl radical, administration of this extract with gentamicin was also able to counterbalance the negative effect of gentamicin on sperm count.

Gentamicin can reduce the sperm count as it was demonstrated in this study and others (Khaki et al., 2009). Gentamicin is able to generate destructive reactive oxygen species including superoxide, hydrogen peroxide and hydroxyl radical and frequently used to produce oxidative and necrotic damages (Khaki et al., 2008). The role of gentamicin in the induction of apoptosis and oxidative damage has also been reported. Ciprofloxacin, gentamicin, neomycin, streptomycin and ofloxacin induce apoptosis in testis (Hong et al., 2006). Accordingly, the administration of carrot seed extract with gentamicin showing the effectiveness of this extract in the prevention of cell necrosis and apoptosis. This could be indicative of free radical scavenging properties of carrot seeds which has been reported previously (Polat et al., 2006).

The results of other study showed the ability of ginger in the enhancement of caudal epididymal sperm reserves of rats resulting from increased testicular resulting decrease of apoptosis in testis. Our finding showed that ginger can cause a decrease in rat male germ cell apoptosis rate which is in agreement with other studies (Morakmyo et al., 2008; Khaki et al., 2008). Morakmyo et al. (2008) showed that ginger caused a significant increase ($P < 0.05$) in the weight of the testis, epididymis and serum testosterone level. Khaki et al. (2008) showed that ginger caused increase of spermatogenesis. This study demonstrated that the administration of ginger can overcome reproductive toxicity of gentamicin. This natural extract was also able to reduce apoptosis in testis (Figure 1).

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Full Length Research Paper

Effects of Huanglian Jiedu Decoction (HLJDD) on fever induced by lipopolysaccharide and inflammation induced by carrageenan

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This study aimed to investigate the antifebrile and anti-inflammatory effect of Huanglian Jiedu Decoction (HLJDD). The rabbits received intravenous injection of lipopolysaccharide (LPS) after orally administered with HLJDD and the rectal temperatures of rabbits were monitored. The concentrations of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) in serum were assayed using rabbit IL-1β and TNF-α enzyme-linked immunosorbent assay (ELISA) kits. Carrageenan-induced paw edema in rats and the anti-inflammatory effects of HLJDD were also explored. HLJDD treatment group resulted in a significant fall in body temperature in biphasic fever peak ($p < 0.05$), and there were no significant differences between HLJDD treatment group and Ibuprofen (IBR) treatment group in biphasic fever peak at time 1 and 3 h ($p > 0.05$). HLJDD treatment group reduced the concentrations of IL-1β in serum at time 1 and 3 h to control febrile responses. Moreover, paw edema of carrageenan-treated rats were significantly attenuated in rats pretreated with HLJDD. Mechanistic studies showed that HLJDD effectively decreased expressions of COX-2 and inducible nitric oxide synthase (iNOS) proteins. These results suggested that HLJDD would be a valuable candidate for further investigation as a new anti-arthritic drug.

Key words: Lipopolysaccharide, Huanglian Jiedu Decoction (HLJDD), anti-inflammatory, interleukin-1 beta, tumor necrosis factor alpha.

INTRODUCTION

Infection and inflammation result in a number of metabolic changes that are often characterized by negative energy balance, increased thermogenesis and anorexia (Johnson et al., 1998). In experimental animals, these changes can be induced by exposure to the bacterial cell wall product lipopolysaccharide (LPS), which suppresses appetite and triggers a number of other behavioral responses including sleepy, general malaise and fever as part of the brain-coordinated host defensive mechanisms. These responses are mediated by cytokines, such as interleukin-1 (IL-1) (Dinarello et al., 1987) and tumor necrosis factor (TNF), and are the most likely candidates for endogenous pyrogen. Nonsteroidal anti-inflammatory drugs (NSAIDs) and immunosuppressants are commonly prescribed in clinical medication.

However, some of them can cause serious adverse effects such as gastric mucosal damage, water and salt retention and carcinomas. Thus, alternative agents with less severe side effects are required, and botanical products are important candidates.

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Huanglian Jiedu Decoction (HLJDD), a traditional Chinese medicine (TCM), had been described in “Waitai Miyao” a classical piece of TCM literature of the Tang dynasty (about 752 a.d.), and consists of four herbs, namely, *Coptidis rhizoma*, *Scutellariae radix*, *Phellodendri cortex* and *Gardeniae fructus*. HLJDD has been used as a therapy for various clinical symptoms associated with gastrointestinal disorders, inflammation and cardio-vascular diseases for one thousand years (Ohta et al., 1999; Wang and Mineshita, 1996). It was found to exert a preventive effect on the development of stress-induced acute gastric lesions in rats due to an inhibitory action on tissue neutrophil infiltration. HLJDD had protective effects against the impairment of learning and memory induced by transient cerebral ischemia in rat (Xu et al., 2000).

In the present study, we investigated the effect of HLJDD on fever induced by the LPS model in rabbit and the role of HLJDD upon inflammation.

MATERIALS AND METHODS

Animals

Female New Zealand white rabbits weighing 2.0 to 3.0 kg and Ihara's cataract rat (ICR) male rat weighing 20 ± 2 g were obtained from the Laboratory Animal Services Center, Liaoning University of TCM, and maintained in plastic cages at 21 ± 2°C with free access to pellet food and water. They were kept on a 12 h light: 12 h dark cycle. All animals used in this experiment were cared according to the ethical regulations on animal research of our university.

Regents and preparation of herbal extract

LPS from *Escherichia coli* 0127:B8 was purchased from Sigma Aldrich (China Mainland). Rabbit IL-1β and TNF-α ELISA kits were purchased from Shanghai Lang-dun Biologic Technology Company. Preparation of herbal extract was defined according to ancient records. The ingredients of the HLJDD (1000 g) were *C. rhizoma* (300 g), *S. radix* (200 g), *P. cortex* (200 g) and *Gardeniae fructus* (300 g). The herbs and ibuprofen were purchased from the Tong Ren Tang Medicinal Materials Company of Shenyang, China. The herbs were immersed in distilled water (1,500 ml), and boiled for 20 min up to the volume of the material until 200 ml was left. The extract obtained was filtered, and then stored at -20°C until use. Ibuprofen was made in the third plant of Harbin Pharmaceutical Industry (Harbin, China), and the lot number is, 080135. Other drugs and reagents, carrageenan and Tween 80, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The HLJDD groups were orally administered with HLJDD 5.25 g/kg for rabbits after intravenous injection of LPS and in the carrageenan-induced paw edema rats were orally administered with HLJDD 18.75 g/kg for rats.

Chromatographic system and conditions

High performance liquid chromatography (HPLC) analysis was carried out on an Agilent 1100 series HPLC (Palo Alto, CA, USA) incorporating an ultra violet (UV) detector. The analytes were determined at room temperature on an analytical column (Diamonsil C18, 150 × 4.6 mm, i.d., 5 µm) (Dikma Technologies, Beijing, China). The mobile phase consisted of a mixture of acetonitrile-1% aqueous phosphoric acid (28:72, v/v). The mobile phase was passed under vacuum through a 0.45 µm membrane filter before use. The analysis was carried out at a flow rate of 1 ml/min with the detection wavelength set at 280 nm.

Measurement of changes in body temperature

The rectal temperatures of rabbits were monitored by a copper-constantan thermocouple for 6 h after an intravenous injection of LPS, and the rectal temperature change (ΔT) was calculated by subtracting the temperature before the injection from the temperature at each time point. Before the formal experiment, the body temperatures of rabbits were monitored for 2 days and the basic temperatures were noted. On the day of the body temperature experiment, animals were minimally restrained in conventional rabbit stocks, at an ambient temperature of 21 ± 1°C between 09:00 and 16:00. Throughout the experiment, the rectal temperature was measured every 20 min with a copper-constantan thermocouple. The rectal temperature in each animal was allowed to stabilize for at least 90 min before any injections were made. The intravenous injection of LPS was made into the marginal ear vein.

Drug treatment and tissue preparation

All rabbits (n = 60) were randomly divided into three parts of experiments, and the rabbits (n = 20) of each part experiment were separated into four groups: Control (n = 5, orally administered with 0.9% saline solution only), LPS (n = 5, received intravenous injection of LPS only, 200 ng/kg), HLJDD + LPS (n = 5, received intravenous injection of LPS 30 min after orally administered HLJDD, 5.25 g/kg), Ibuprofen + LPS (n = 5, received intravenous injection of LPS 30 min after orally administered with ibuprofen 10 mg/kg). To determine the suitable dosage of HLJDD, the conversion ratio of dosage between person and animals were performed as described elsewhere (Xu, 2009), and pilot studies were performed where HLJDD dosage 5.25 g/kg for rabbits and 18.75 g/kg for rats were used as suitable middle dosage, respectively.

In the first series of experiments, the body temperatures were observed for 6 h only to prove the antifebrile effect of HLJDD. In the second and third series of experiments, we observed the body temperature for 1 and 3 h, respectively. Then the blood samples were collected in tubes from the ear center artery of rabbits and centrifuged after depositing for 2 h. The blood serum was stored at -80°C for IL-1β and TNF-α activity assays. In carrageenan-challenged paw edema of rats, paw soft tissues were removed from individual rat and homogenized in Radioimmunoprecipitation assay (RIPA) buffer and incubated at 37°C on a rotator for 2 h. After washing the plate, biotinylated antibodies were added (100 µl per well) and plates were incubated at 37°C on a rotator for 1 h. Then the plates were incubated for 0.5 h with streptavidin-horseradish peroxidase at a dilution of 1: 20000, and the antibodies were detected with 3,3′,5,5′-tetramethylbenzidine (TMB) dissolved in dimethyl sulfoxide to a concentration of 1% in a solution containing 0.1 M citric acid, 0.1 M sodium acetate (pH 6), and 0.016% H_2O_2 for 30 min. The reaction was stopped by the addition of 1.5 M H_2SO_4. Plates were read using a wavelength (450 nm) on a microplate reader (BIO-RAD iMark^{TM}, Made in Japan 2010, Serial No.12843), and total cytokine concentrations were calculated using the standard curve prepared from recombinant cytokines. The lower limit of detection for the cytokines based on the standard curves ranged from 10 to16 pg/ml. The ELISA for rabbit IL-1β detects both precursor and mature IL-1β but not TNF-α.

Similarly, the ELISA for rabbit TNF-α does not cross-react with rabbit IL-1β. The protocol of the ELISA for rabbit IL-1β is similar to that for rabbit TNF-α. The sensitivities of the assays were: IL-1β, 10 pg/ml and TNF-α, 16 pg/ml.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β and TNF-α in serum were assayed using a commercially available rabbit IL-1β and TNF-α ELISA kits (R&D Systems, Inc., Minneapolis, Minn. Catalog No. F2019; Catalog No. F2041), following the manufacturer's instructions. The plasma samples and working standards were added (100 µl per well) in duplicate and concentrations of cytokines at the site of inflammation compared to those in the circulation, some samples required large dilutions.

Induction and assessment of acute inflammation in rat hind paws by carrageenan

The assay was conducted as previously described by Winter et al. (1962). Oral administration was conducted with 18.75 g/kg doses of HLJDD, the reference drug (Ibuprofen, 10 mg/kg), or the control, at 1 h prior to inflammation induction. At induction of the paw edema, each rat was injected with 0.05 ml freshly prepared carrageenan (1% w/v) in physiological saline (0.9% w/v NaCl) into subplantar tissues of the right hind paw. The left hind paws without injection were used as controls. The volumes (ml) of both hind paws of each animal were measured using a plethysmometer (YLS-7A, Anhui, China) at 1 h before inflammation induction and the time intervals of 1, 2, 3, 4, 5 and 6 h after induction. The rates of increase in paw volume (paw edema) of the right hind paws of rats were calculated by the following equation:

The increase rate (%) = (A - B) / B × 100
where A represents the paw volumes at different time points after injection, and B represents the paw volume before injection. The mean values of the treated animals were compared with the mean values of the control animals, and results were analyzed using statistical methods.

Western blot analysis of iNOS, COX-1, and COX-2 protein expressions

Protein levels of iNOS, COX-2, and COX-1 were assessed by Western blot analysis as previously described (Li et al., 2008). Paw soft tissues were removed from individual rat and homogenized in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 20 µg/ml leupeptin, 20 µg/ml aprotinin, 0.1% Triton X-100, and 1% SDS for 1 h. After centrifugation at 15,000 × g at 4°C for 10 min, the supernatants were collected, and the protein amount in each sample was measured by a Bio-Rad DC kit (Bio-Rad, Hercules, CA). The equal amount of sample (50 µg of protein) was subjected to electrophoresis on either 10 or 12% SDS-polyacrylamide gel. Following the electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution, and incubated with the corresponding primary antibodies in the blocking solution at 4°C for 12 h. After washing three times with TBST solution (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20), the membrane was incubated, with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:1000) at room temperature for 1 h. The detected protein signals were visualized by the enhanced chemiluminescence reaction system according to manufacturer's recommendation (Amersham Biosciences, Indianapolis, IN).

Statistical analysis

All results were confirmed in at least three separate experiments. Data were expressed as mean ± standard error of mean (SEM). A one-way analysis of variance (ANOVA) was used for multiple comparisons. A value of $p < 0.05$ was considered significant.

RESULTS

Effects of HLJDD on body temperature during LPS induced fever in rabbits

In the first series of experiment, we injected LPS intravenous at time 1 h after orally administering with HLJDD or ibuprofen, and the changes of body temperature of rabbits were in the Figure 1. Figure 2 showed changes in rectal temperature in the four different groups. Injection of LPS (200 ng/kg) induced a biphasic fever, in which the first peak occurred at 1 h and the second peak occurred at 3 h after injection. In contrast, HLJDD treatment group resulted in a significant fall in body temperature in both peaks ($p < 0.05$), and treatment with ibuprofen also attenuated both peaks of the fever ($p < 0.05$). There were no significant differences between HLJDD treatment group and Ibuprofen treatment group in both peaks ($p > 0.05$). Control animals administered only saline failed to show any change in body temperature.

The concentrations of IL-1β and TNF-α in serum after intravenous injection of LPS for 1 h in the four different groups

In order to discuss the mechanism of HLJDD in reducing the first peak of the body temperature in LPS induced fever, the concentrations of IL-1β and TNF-α in serum were measured at time 1 h, and the results showed that the level of IL-1β had significant differences between Control and LPS groups ($p < 0.05$). Both HLJDD and Ibuprofen treatment groups resulted in a significant fall in the concentrations of IL-1β in serum comparing with that of only treated with LPS ($p < 0.05$). Besides, Ibuprofen treatment group resulted in a significant fall in the concentrations of TNF-α in serum comparing with that of only treated with LPS group ($p < 0.05$). HLJDD did not reduce the concentrations of TNF-α in serum comparing with the LPS group ($p > 0.05$). Ibuprofen could reduce both the concentrations of IL-1β and TNF-α in serum to lower the first peak of the body temperature in LPS induced fever, while HLJDD reduced the concentrations of IL-1β in serum to lower the first peak of the fever (Table 1).

The concentrations of IL-1β and TNF-α in serum after intravenous injection of LPS for 3 h in the four different groups

The blood samples were collected at time 3 h after injecting LPS, and the concentrations of IL-1β and TNF-α were assayed (Table 2). The concentrations of IL-1β in

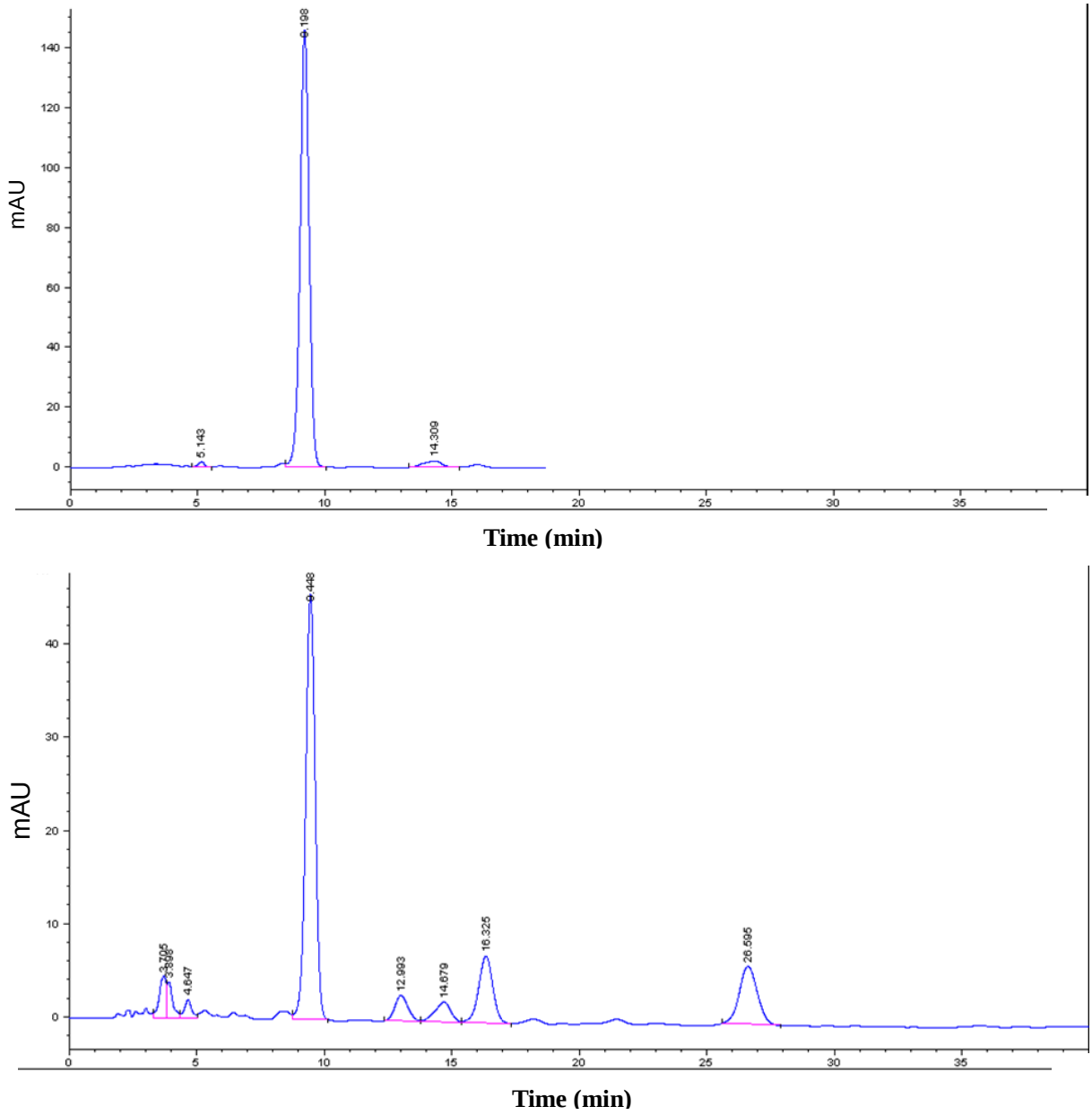

Figure 1. A. HPLC chromatogram of Baicalin. B. HPLC chromatogram of HLJDD.

serum of HLJDD and Ibuprofen treatment groups were significantly decreased comparing with the concentrations of LPS group ($p < 0.05$). There was no significant change on the concentrations of TNF-α in serum between LPS and Control groups ($p > 0.05$). HLJDD treatment group resulted in a significant decrease in the concentrations of IL-1β comparing with the group that only treated with LPS ($p < 0.05$), but there was no remarkable difference in the concentrations of TNF-α comparing with Ibuprofen treatment group ($p > 0.05$). From our experiment, we found that IL-1β in serum played the important role in the second peak of LPS induced fever.

Inhibition of the paw edema of rat by oral treatment of HLJDD

Figure 3 shows the effect of HLJDD on inhibition of the acute paw edema in rats evoked by carrageenan injection into the subplantar tissues of the right hind paws. It can be seen that the maximum phlogistic response of carrageenan was observed at 4 to 6 h after the injection

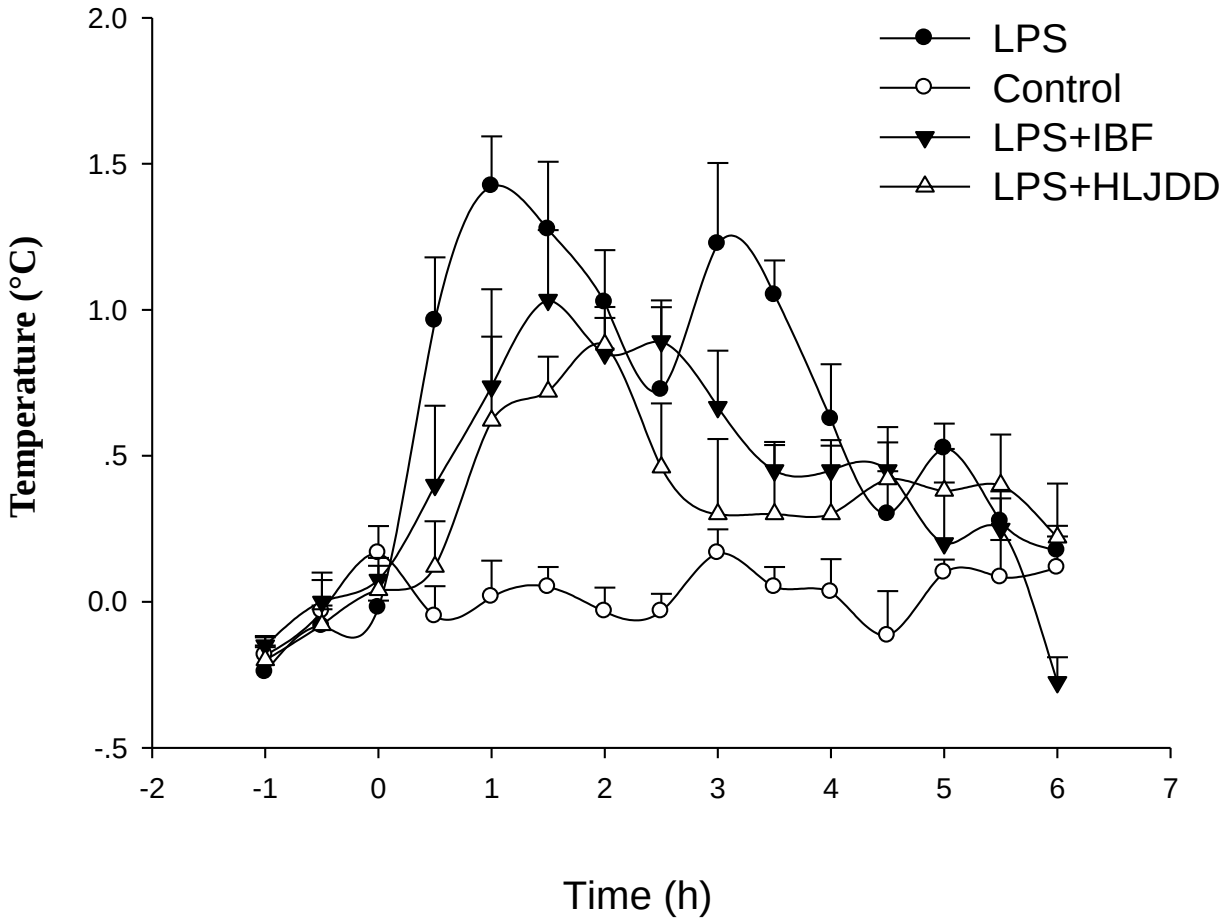

Figure 2. The changes of body temperature in different groups. Rabbits were injected of LPS intravenously 30 min after orally administered with HLJDD or Ibuprofen and the Control animals were orally administered with 0.9% saline solution only.
●.LPS (n = 5); ○.Control (n = 5); ▼.LPS + Ibuprofen (n = 5); △.LPS + HLJDD (n = 5). Each point represents mean ± S.E.M. At time 1 h LPS vs Control, $p < 0.05$; LPS vs LPS + HLJDD, $p < 0.05$; LPS vs LPS + Ibuprofen, $p < 0.05$; and at time 3 h LPS vs Control, $p < 0.05$; LPS vs LPS + HLJDD, $p < 0.05$; LPS vs LPS + Ibuprofen, $p < 0.05$.
There were no significant differences between HLJDD treatment group and Ibuprofen treatment group in biphasic fever peak at time 1 h and 3 h ($p > 0.05$).

in the control animals. The paw volumes from HLJDD-treated animals with dosages of 18.75 g/kg, at 1, 2, 3, 4, 5 and 6 h after induction of paw edema showed remarkable decrease in comparison with the data of non-treated animals at the same time points ($p < 0.05$), and there were no significant differences between the HLJDD-treated and the Ibuprofen-treated animals ($p > 0.05$). These results indicated that the anti-acute inflammatory effect of HLJDD in rats were effective.

Inhibition of iNOS, COX-1 and COX-2 protein expressions in the paw tissues of rats by treatment of HLJDD

It can be seen in Figure 4 that HLJDD dose-dependently attenuated the protein expression of COX-2 in carrageenan-injected paw tissues. Reduction was achieved by treatment with HLJDD at dosages of 18.75, 9.38, and 4.69 g/kg, respectively. Figure 4 also showed that the protein expression of iNOS in carrageenan-injected paw tissues was dose-dependently attenuated in HLJDD treated rats. HLJDD at 18.75/kg can significantly inhibit the iNOS protein expression. Ibuprofen at a dosage of 10 mg/kg also demonstrated significant inhibition in iNOS protein expression. Examination of COX-1 protein expression showed that the level of expression was not suppressed by administration of HLJDD at any dosage. These results suggested that HLJDD may have a selective inhibitory effect on COX-2 protein expression in the paw edema tissues of rat. However, Ibuprofen at a dosage of 10 mg/kg demonstrated significant inhibition on

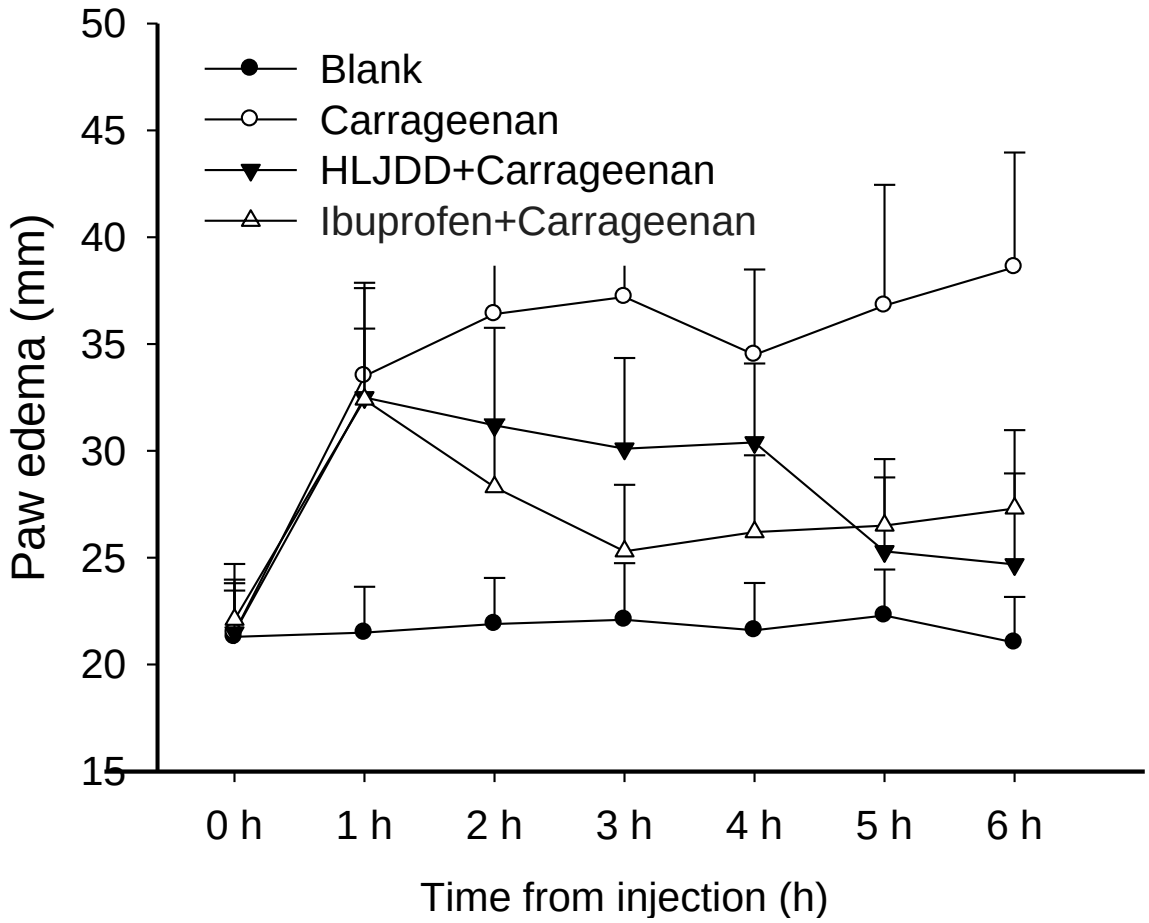

Figure 3. The inhibition of carrageenan-evoked paw acute inflammation of rat by HLJDD. The paw volumes from HLJDD-treated animals with dosages of 18.75 g/kg, at 1, 2, 3, 4, 5 and 6 h after induction of paw edema showed remarkably decrease in comparison with the data of non-treated animals at the same time points ($p < 0.05$).

both COX-2 and COX-1 protein expressions.

DISCUSSION

Fever is a regulated rise in body temperature and one of the most common responses to infection, injury or trauma. Administration of bacterial endotoxin LPS is widely used as a laboratory model of fever. IL-1 is thought to be an endogenous pyrogen during LPS-induced fever. The pro-inflammatory cytokine IL-1 is a pivotal mediator of local and systemic responses to infection and inflammation, of which fever is the most widely studied experimentally and clinically (Dinarello, 1996; Kluger, 1991). Rabbit TNF injection also elicited biphasic fever in rabbits, the second phase of which was found to be mediated by the similar endogenous pyrogen, and endogenous TNF played an important role in eliciting a febrile response to endotoxin (Kawasaki et al., 1989).

We made a biphasic fever model in rabbits using a high dosage of LPS (200 ng/kg) (Chen et al., 2008), that was conformed as experiments had been made by others, and the first phase of biphasic fever appeared at about time 1 h and the second phase of biphasic fever appeared at about time 3 h, that was similar as the investigation reported before (Nakamori et al., 1994). The main findings of this study were that treatments with the crude extract of HLJDD could remarkably reduce the biphasic fever induced by LPS. Ibuprofen, a specific cyclo-oxygenase inhibitor, not only reduced the first phase of biphasic fever clearly, but also affects the latter fever peak. This result of ibuprofen antifebrile action was consistent with the investigation reported before that it could result in a significant fall in body temperature in both fever peaks (Sobrado et al., 1983), and the reasons for this might be the different dosages of ibuprofen that the rabbits were administrated or the difference of animal species which are needed for further investigation.

The present results showed that IL-1β and TNF-α were increased in serum during biphasic fever induced by intravenous injection of LPS (200 ng/kg). In addition, at time 3 h, there were no differences in TNF-α level in the

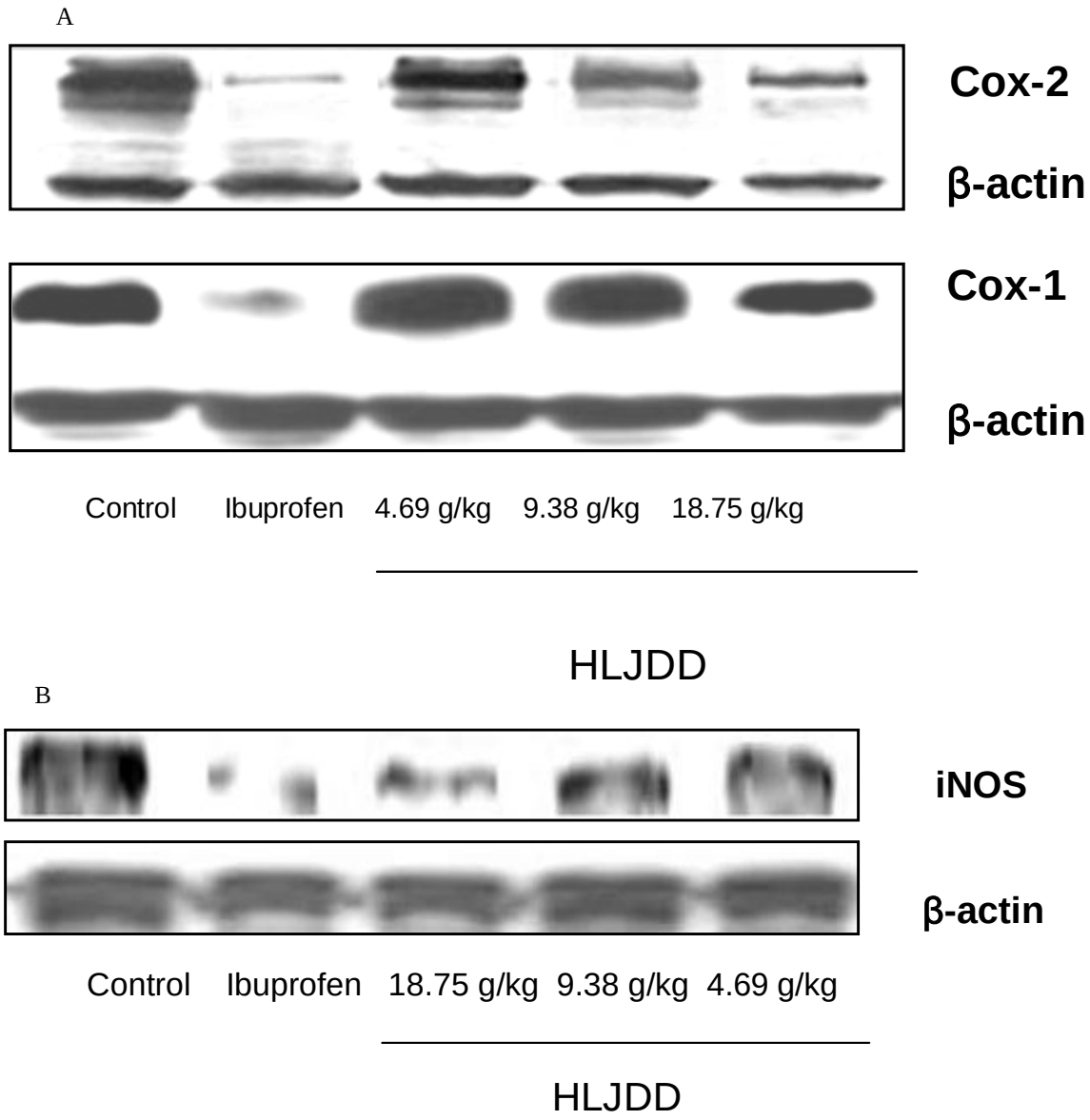

Figure 4. A. Effects of HLJDD on COX-1 and COX-2 protein expressions in carrageenan-injected paw tissues of rats. HLJDD at dosages of 4.69 g/kg, 9.38 g/kg, and 18.75 g/kg was orally administrated 1 h before the carrageenan injection. At 4 h after the injection, paws were removed. Then COX-1 and COX-2 protein expressions in the paw tissues were detected by Western blot analysis, using β-actin as the internal control. HLJDD dose-dependently attenuated the protein expression of COX-2, while the COX-1 protein expression was not suppressed by administration of HLJDD at any dosage. Ibuprofen at a dosage of 10mg/kg demonstrated significant inhibition on both COX-2 and COX-1 protein expressions. B. Effects of HLJDD on iNOS protein expressions in carrageenan-injected paw tissues of rats. HLJDD at dosages of 4.69 g/kg, 9.38 g/kg, and 18.75 g/kg was orally administrated 1 h before the carrageenan injection. At 4 h after the injection, paws were removed. Then iNOS protein expression in the paw tissues was detected by Western blot analysis, using β-actin as the internal control. HLJDD at 18.75/kg can significantly inhibit the iNOS protein expression. Ibuprofen at a dosage of 10 mg/kg also demonstrated significant inhibition in iNOS protein expression.

serum between LPS and Control groups. Therefore, it was possible that the early peak fever was induced by the indirect action of IL-1β and TNF-α produced in serum by intravenous injection of LPS, and the latter fever peak appeared at 3 h which was mediated by LPS induced endogenous IL-1β in serum. The previous study had shown that endogenous TNF activity was detected in 1 h blood in an endotoxin dose dependent manner coincident with the early peak fever but was not detected in 2.5 h blood (Kawasaki et al., 1989). HLJDD reduced the concentrations of IL-1β in serum to control the febrile responses at time 1 h; and the levels of IL-1β in serum

Table 1. Concentrations (ng/ml) at time 1 h (mean ± SEM).

Test group	IL-1β	TNF-α
LPS	11.00±4.24	95.15±8.05
Control	3.72±0.53*	92.47±16.27#
IBR+LPS	2.94±1.76*	83.74±5.41*
HLJDD+LPS	5.11±3.06*	106.39±7.49#

The concentrations of IL-1β, TNF-α in serum at time 1 hour after the injection of LPS in the different groups were detected. Data are expressed as mean ± S.E.M. (n = 5). Compared with LPS group, *p < 0.05; #p > 0.05.

Table 2. Concentrations (ng/ml) at time 3 h (mean ± SEM).

Test group	IL-1β	TNF-α
LPS	8.66±0.92	81.28±1.52
Control	6.79±0.61*	76.54±3.88#
IBR+LPS	4.91±0.46*	97.61±1.38#
HLJDT+LPS	5.66±2.17*	79.00±9.99#

The concentrations of IL-1β and TNF-α in serum at time 3 hour after the injection of LPS in the different groups were detected. Data are expressed as mean ± S.E.M. (n = 5). Compared with LPS group, *p < 0.05; # p > 0.05.

were remarkably decreased in HLJDD group than that of in LPS group at time 3 h. But ibuprofen failed to reduce the rising concentrations of TNF-α in serum in the latter fever peak, and the concentrations of TNF-α in serum between HLJDD and Ibuprofen groups had significant differences ($p < 0.05$). The reasons might be the dissimilar anti-inflammation mechanism between ibuprofen and HLJDD.

To compare with ibuprofen, according to TCM selective treatment based on the differential diagnosis, the TCM prescription HLJDD has some advantages in reducing fever induced by various diseases. Salvemini et al. (1993) proposed that NO stimulates COX activity in macrophages through direct interaction with active sites of the COX enzyme. Inhibition of NO production by the suppression of the enzyme activity of iNOS is one of the pathways for anti-inflammatory effect (Cai et al., 2005; Salvemini et al., 1993). Furthermore, they reported that NO produced by iNOS is involved in maintenance of the carrageenan evoked inflammatory response, while peripheral or central administration of iNOS inhibitors could effectively inhibit carrageenan-induced hyperalgesia in rats (Salvemini et al., 1996).

In the present work, the anti-inflammatory and analgesic effects of HLJDD in carrageenan induced rat paw edema were also studied. The possible effective mechanisms were investigated with regard to the iNOS, COX-1, and COX-2 protein expressions in paw tissues. The anti-inflammatory effect of these drugs is believed to result from their ability to inhibit the formation of prostaglandins by cyclooxygenases (COXs). Two isoforms of cyclooxygenase, COX-1 and COX-2, have been identified. COX-1 constitutively expressed in normal tissues functions as necessarily physiological activities including protection of the gastric mucosal lining; while COX-2 is overproduced in the sites of inflammation (Smith et al., 1998). In clinical use, some NSAIDs have been proven to block both COX-1 and COX-2 activities resulting in induction of gastric ulcerization and kidney failure. Moreover, the marketing of new COX-2 inhibitors which emphasized the advantages of not blocking the necessary COX-1 pathway and gastrointestinal problems from the use of those drugs are still being reported (Silverstein et al., 2000).

Conclusion

The effects of TCM prescriptions were multi-target and complex, but at least in part. The present study showed that the prescription could evidently reduce the increased concentrations of IL-1β, and have selective inhibitory effect on COX-2 protein expression. All these results indicated that the HLJDD would be a valuable candidate for further investigation as a novel anti-arthritic botanical drug.

ACKNOWLEDGEMENT

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ABBREVIATIONS

HLJDD, Huanglian Jiedu Decoction; **IBR,** ibuprofen; **TCM,** Traditional Chinese Medicine; **LPS,** lipopolysaccharide; **IL-1β,** interleukin-1 beta; **TNF-α,** tumor necrosis factor alpha; **NSAIDs,** nonsteroidal anti-inflammatory drugs; **ELISA,** enzyme-linked immunosorbent assay; **COX,** cyclooxygenase; **iNOS,** inducible nitric oxide synthase.

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Full Length Research Paper

Comparative genetic and chemical profiling performed on *Alstonia scholaris* in China and its implications to standardization of Traditional Chinese Medicine

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Validity of traditional Chinese medicine (TCM) is oppugned very often because of the bugs of quality, effectiveness, and repeatability. Standardization of TCMs is one way to make a change. How to realize standardization of TCMs and with what measures to shape the quality control system, is provoking much debate in the TCM field. Genetic and chemical profiling were comparatively performed on *Alstonia scholaris* in China through amplified fragment length polymorphism (AFLP) analysis and alkaloid high performance liquid chromatography (HPLC) scanning, aiming to evaluate the attributes of chemical and DNA fingerprinting as applicable tools for quality control of TCMs and explore theoretic strategy of TCM standardization. Each individual of *A. schlolaris* displayed a unique AFLP or HPLC profile, indicating well individual-distinguishing ability of the two techniques. Patterns of variance structure disclosed by the HPLC and AFLP profiling were similar, and more than 50% of alkaloid loci were kept constant across any single population, suggesting a genetic basis of the alkaloid secondary metabolism in the plant and justifying DNA fingerprinting as a qualified identifier for quality control of the TCM. Compared to the alkaloid HPLC scanning, the AFLP analysis produced much more loci with lower polymorphic loci percentage, suggesting that AFLP can be more statistically informative and with moderate sensitivity. In passing, a strategy pursuing genetically identical TCMs by popularizing plantation of selected germplasm of medicinal plants was suggested for TCM standardization.

Key words: Traditional Chinese Medicine (TCM), standardization, *Alstonia scholaris*, alkaloid scanning, genetic profiling.

INTRODUCTION

Alstonia scholaris is an evergreen tree with white perfumed flowers, growing in mixed forests and village groves from 200 to 1000 m a.s.l. in the tropical and subtropical areas such as Southern Yunnan and South-western Guangxi, and cultivated in Fujian, Guangdong, Hainan, Hunan, Taiwan and as an ornamental in China (Li et al., 1995). It has been widely used in traditional Chinese medicine (TCM) to treat various diseases such as headache, influenza, malaria, bronchitis and pneumonia (Zhou et al., 2005). Various alkaloids from its bark and leaves were confirmed to be the active ingredients (Khyade and Vaikos, 2009; Shang et al., 2010).

Morphologically, the species is a ‘good’ species, very homogenous without much difference recognized between populations from different geography areas, and easily distinguished from other *Alstonia* species. It represents a useful simple model to test the difference between chemical and DNA fingerprinting as two marker

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systems to identify medicinal plants, since taxon with multiple morphological variation will make the comparison more complicated and elusive.

Traditional Chinese medicine (TCM) has been seeing an increasing business volume in China in recent years. According to PricewaterhouseCoopers (2009), sales revenue for TCM products in China reached approximately US$ 21 billion in 2007, accounting for around 40% of the total pharmaceutical market in China. In 2008, TCM represented, in terms of sales volume, around two-thirds of drug sales in China. The widespread use of TCM had already posed substantial competition to the conventional drug industry, and sales in TCM might see a further increase (PricewaterhouseCoopers, 2009). TCM is not only one of the important historical cultural heritages in the world, but also one of the important scientific heritages of humans. The long history of application of TCM by Chinese is in nature a history of clinic experiment of TCMs directly upon human bodies. The effectiveness and safety of TCM is rationally based. This is the very reason that TCM has been seeing an increasing business volume in China. However, TCM still faces many challenges, one of which is to address the inconsistencies found in its manufacturing processes (Business Monitor International, 2008). Raw materials for many TCMs are from wild resources in current practice. A stockpile of a specific TCM in a drugstore might come from different geographical area and might differ in heredity and chemical constituents. This is one of the reasons that the patients often complain about the quality, effectiveness and repeatability of TCMs and that some countries refuse TCM usage. Standardization of TCMs is the most important way to make the quality of TCMs appropriately controlled, qualifying TCM to serve people in modern society, and relieving TCM of the present embarrassed situation. How to realize standardization of TCMs and with what measures to shape the quality control system is provoking much debate in the TCM field. Chemical and DNA fingerprinting, as two applicable tools to identify medicinal plants, are often the focus of this kind of controversies. What are the attributes of these two kinds of marker? How effective are the two measures when used as identifier? How about their distinguishing ability? Are they perfect identifiers throughout the whole industrial production process of a specific TCM? Such questions have remained to be resolved since phytochemical and genetic studies are often separately performed on different medicinal plants by different investigators. Systematic comparative studies of phytochemical and genetic diversity of medicinal plants are still limited.

Therefore, in this paper, we used genetic and chemical profiling as a framework to look at the genetic and alkaloid variation within *A. scholaris* in China, evaluated the attributes of chemical and DNA fingerprinting as applicable tools for quality control of TCMs and explored theoretic strategy to realize TCM standardization.

MATERIALS AND METHODS

Taxa and sampling

Four wild geographical populations of *A. scholaris* were selected, with each population being at least 200 km away from its closest neighbor (Table 1). The sampling strategy was to include most variations of *A. scholaris* in China and allowing detection of chemical and genetic variation within and among populations. During the early fall of 2005, 15 flowering individuals were sampled randomly for each population at intervals of at least 10 m. All studied materials are listed in Table 1, and all vouchers were deposited in the herbarium of Kunming Institute of Botany, CAS (KUN).

DNA extraction and amplified fragment length polymorphism (AFLP) experiment

Total genomic DNA was extracted from dried leaf material according to the CTAB protocol of Doyle and Doyle (1987), treated with RNAase (30 min at 37°C), and adjusted to a concentration of ca. 25 ng/µl. The AFLP analysis followed the protocol described by Vos et al. (1995) with minor modifications. 250 ng/10 µl of genomic DNA were restricted with the enzyme combination *Mse*I and *Eco*RI and ligated to *Mse*I and *Eco*RI adapters. Pre-amplifications were performed in an ICYCLER thermal cycler [BioRad] using primer pairs with a single selective nucleotide (*Eco*RI-A/*Mse*I-A). Sixteen different primer combinations with three selective nucleotides were tested for the selective amplification. On the basis of consistency of results and the number of scoreable bands, two primer combinations were chosen to carry out selective amplification for all samples, namely *Eco*RI-AGA/*Mse*I-AAG and *Eco*RI-AAC/*Mse*I-ACC. The products of final amplification were denatured and loaded on 6% polyacrylamide denaturing gels with 100 bp DNA Ladder [Promega] as a standard. The bands were sequentially visualized by silver staining (Figure 1) (Bassam et al., 1991). The gels were scanned on an A3 sized scanner and bands were visually scored as present or absent with the aid of LabWorks Analysis Software version 4.0 (UVP). Only bands that could be scored confidently were recorded.

Total alkaloid extraction and HPLC analysis

The dried and powdered leaves of *A. scholaris* (5.0 g) were extracted with MeOH (each 25 ml) five times at room temperature each, for 24 h, and the solvent was evaporated *in vacuo*. The residue was dissolved in 1% HCl, and the acidic solution was adjusted from pH 9 to 10 with ammonia. The basic solution was partitioned with EtOAc, and then EtOAc layer was evaporated *in vacuo* to afford total alkaloids which were subjected to further analysis. HPLC analysis was carried out on a Waters 2695 separation module equipped with a Waters 2996 photodiode array detector and Millenium32 software using a Xterra RP18 column (4.6 × 250 mm, 5 um) and a solvent system of 20:80 MeCN/0.5% CF_3COOH-H_2O isocratic, with a flow rate of 1 ml/min. The column temperature was 35°C and 40 min running time for each sample. Peaks in the HPLC profiles were visually scored as present or absent.

Statistical analysis

Based on the constructed present/absent data matrix, the molecular diversity indices, total number of loci, percentage of polymorphic loci, mean pairwise differences and average gene/alkaloid diversity over loci were generated by Arlequin 2.01. An Analysis of Molecular

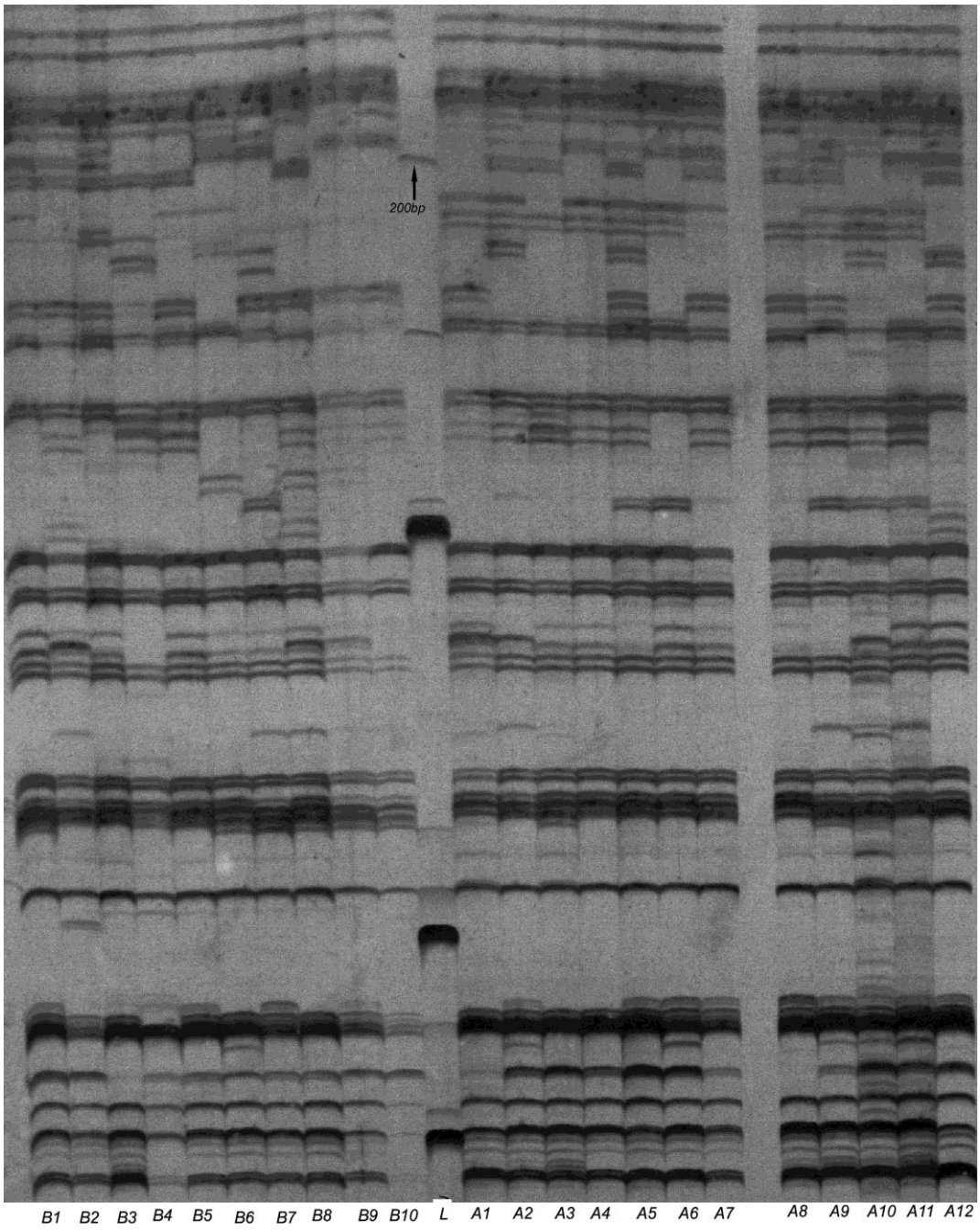

Figure 1. Example of AFLP profile obtained for *Alstonia scholaris* with the primer combination *Eco*RI-AGA/*Mse*I-AAG. B1-A12, codes for individuals. B1= the first individual of population B. L= 100 bp DNA ladder [Promega].

Table 1. Material resources, vouchers, and population codes.

Taxon	Population code	Location	Voucher	Sample size
Alstonia scholaris	A	Kengma	ZZY2005002	15
	B	Malipo	LS2005001	15
	C	Chiangcheng	ZZY2005001	15
	D	Jinping	LS2005002	15

Table 2. Biodiversity within the *Alstonia scholaris*, which was evaluated by number and percentage of polymorphic loci, mean pairwise difference within population, and average diversity over loci (with 5% allowance of missing data; standard deviation in parentheses).

population	Number of polymorphic loci		Percentage of polymorphic loci		Mean number of pairwise differences		Average diversity over loci	
	Gen.	Alk.	Gen.	Alk.	Gen.	Alk.	Gen.	Alk.
A	41	10	31.80	31.3	15.90 (7.48)	3.87 (2.10)	0.12 (0.07)	0.12 (0.07)
B	41	8	31.80	25	14.55 (6.87)	3.51 (1.94)	0.11 (0.06)	0.11 (0.07)
C	46	15	35.70	46.90	16.44 (7.72)	5.31 (2.78)	0.13 (0.07)	0.17 (0.10)
D	43	14	33.30	43.80	15.79 (7.43)	5.40 (2.82)	0.12 (0.06)	0.17 (0.10)
Total	54	21	41.80	65.60	18.19 (8.17)	5.93 (2.89)	0.14 (0.07)	0.19 (0.10)

Total loci are 129 for AFLP and 32 for alkaloid profile. Percentage of polymorphic loci = Number of polymorphic loci/total loci; Gen. = genetic statistics based on AFLP profiling; Alk. = statistics base on total alkaloid profiling.

Table 3. AMOVA-designed variance among and within populations of *Alstonia scholaris*.

Source of variation	D. F.	Sum of squares		Variance components		Percentage of variation	
		Gen.	Alk.	Gen.	Alk.	Gen.	Alk.
Among populations	3	97.78	34.25	1.65	0.92	17.40	28.82
Within populations	56	483.80	81.40	7.83	2.26	82.60	71.18
Total	59	536.58	115.65	9.48	3.18	-	-

Gen. = Genetic statistics based on AFLP profiling; Alk. = statistics base on total alkaloid profiling.

Variance (AMOVA; Excoffier et al. 1992) was also performed using Arlequin 2.01 with 1000 permutations and the same general settings as for the calculation of molecular diversity dices. The structure was defined to partition the total variance into components among populations and among individuals within populations. The variance components of AFLP were also used to calculate the fixation index Fst.

RESULTS

Overall genetic/alkaloid variation for *A. scholaris* obtained by high performance liquid chromatography (HPLC) and AFLP analysis was evaluated by total number of loci, number and percentage of polymorphic loci, mean pairwise differences, and average gene/alkaloid diversity over loci (Table 2). Each individual displayed a unique AFLP and HPLC profile in the analysis, indicating extensive genetic/alkaloid variation within *A. scholaris*. For genetic variation, a total of 129 reliable loci were recorded with two primer combinations, of which 54 loci (42%) were polymorphic at species level and 75 bands were shared by all individuals. The percentages of polymorphic loci at population level were lower than that at species level, ranging from 32 to 36%. While for alkaloid variation, a total of only 32 peaks were recorded, of which 21 peaks were being polymorphic at species level and 11 peaks shared by all individuals. The mean value of pairwise differences at species level was 18 loci in the AFLP analysis and 6 peaks in the HPLC analysis. The average diversity over loci at species level was 0.14 for the amplified DNA fragments and 0.19 for the alkaloids. With respect to variance components within and among populations, *A. scholaris* displayed similar patterns in the AFLP and the HPLC profiling. AMOVA analysis attributed 17% of total genetic variance (29% of alkaloid variation) among the populations and 83% (71% of alkaloid variation) within the populations (Table 3). Moreover, the average genetic fixation index between populations was estimated to be 0.17.

DISCUSSION

Diversity pattern displayed by *A. scholaris*

A. scholaris is a ‘good’ species, very homogenous without much difference recognized between populations from different geography areas, and easily distinguished from other *Alstonia* species. Nevertheless, the AFLP and HPLC analysis disclosed considerable genetic and alkaloid variation within the species. Each individual presents a unique AFLP and HPLC profile. With respect to structure of variance, the *A. scholaris* displayed limited divergence and strong gene flow among populations. Only little portion of total variance resided among populations whether in AFLP or HPLC analysis, indicating a strong out-crossing tendency of the species. This meets the expectation of the species. *A. scholaris* is a perennial

tree with height of 20 to 40 m, which can facilitate the long-distance dissemination of pollen and seeds. Its strongly perfumed flowers are very attractive to insects, which can promote out crossing. Its pendulous fruits can stay on the branches for a long time and keep releasing the numerous small seeds. The seeds, bearing a tuft of hairs 7 to 13 mm long at each end, can easily float in the air (Li et al., 1995). These characteristics together make long-distance dispersal of the seeds possible, promoting populations in different geographical areas to be homogenized. Therefore, it is not surprising if strong gene flow is detected among populations of the species.

Attributes of DNA and chemical profiling

Chemical fingerprinting is based on chemical constituents which are the physical foundation of medicinal function of a TCM, while DNA fingerprinting is an in-direct reflection of the chemical constituents. Therefore, chemical fingerprinting apparently seems in authority on quality control of TCMs, which is the main argument of phyto-chemists supporting chemical fingerprinting as the only legitimate tool. As expected, more than 50% of alkaloid loci were kept constant across any single population (Table 2), and the patterns of variance structure disclosed by the alkaloid scanning was similar to that by AFLP profiling (Table 3), suggesting that the alkaloid secondary metabolism in the plant can be genetically based on nature. The genetic basis of the alkaloid secondary metabolism justifies DNA fingerprinting as a qualified tool for quality control of the TCM.

Each individual of *A. schlolaris* displayed a unique AFLP or HPLC profile. This implies that both AFLP and alkaloid scanning method possess well individual-distinguishing ability, powerful enough to be identifier of TCMs. Nevertheless, there are still some subtle differences between the two kinds of marker system that should be kept in mind. First, with two primer combinations, the AFLP analysis produced 129 reliable loci, whereas the alkaloid scanning produced only 32 information loci which could not be increased further by adjusting experimental parameters. This suggests that AFLP method can produce much more loci for succedent statistical analysis, which will be helpful for eliminating influence of experimental error and other random factors and therefore improve the confidence coefficient. Secondly, polymorphic loci percentage for the limited alkaloid loci in the HPLC profiling is higher than that in the AFLP profiling. This can be explained as the result of environmental plasticity of secondary metabolism and indicates that chemical scanning is more sensitive. Thirdly, DNA fingerprinting like AFLP cannot play role on TCMs in the form of chemical extract, while chemical scanning can play role on TCMs in various dosage forms, having a wider application range. Therefore, with respect to information capacity and moderate sensitivity, genetic fingerprinting such as AFLP is a preferred identifier especially in the case of identifying plant materials.

Strategy to standardize TCM

Chemical compounds are the physical foundation of function of TCM. Therefore, the most ambitious intention of TCM standardization is to make a specific TCM chemically completely identical so that TCM can meet the standard of modern medicine. Frustratingly, a ‘good’ species like *A. scholaris* bears considerable genetic and chemical variation all the same, not to say other species with multiple varieties or ecological races. Pursuing chemically identical TCM is practically doomed to fail, because no two identical biological individuals exist in the world owing to environmental plasticity and individual history, even for twin brothers or clones which are genetically identical. Does this mean that standardization of TCM is impossible or we should give up TCM thoroughly? Of course the answer is no. The key point is that we should adopt an appropriate standard with appropriate flexibility. In fact, it is one of the logic foundations of TCM pharmaceutics that different individuals of a species bear similar chemical constituents and function similarly as medicine. What should be done is to control the strictness when to define or delimit a specific TCM.

In view of the alkaloid diversity displayed by *A. scholaris* and other complex species like *Spiraea japonica* (Zhang et al., 2006), it can be confirmed that much chemical variation exist within traditional defined TCMs. However, the identity control of many TCMs is routinely based on traditional morphological classification, which might ignore the much variance residing within a species. In addition, it has often happened that several closely related and morphologically undistinguishable species or varieties are un-discerned and used as substituted agents of a trueborn TCM, which makes things worse. This practice is one of the root reasons hindering further healthy development of TCM. Homonym of TCMs will make it impossible for doctors or pharmacologists to accurately evaluate the aimed TCM by recording and comparing the curative effect or bio-activity of the TCM among different cases, different periods and different recipe. Also, it will be difficult for doctors or pharmacologists to exchange their experiences or views on a specific TCM or on a recipe. Therefore, identity control of TCMs ought to be strengthened in TCM practice. On another hand, ambition to make a TCM chemically completely identical is unpractical and also unnecessary in practice. From perspective of long-term development of TCM, pursuing genetically identical TCMs is a less ambitious and achievable goal and may be adequate. Advocating plantation and popularization of germplasm selected is the way to gradually achieve the goal.

Conclusion

Conclusively, it was confirmed that much genetic and chemical variation exist within traditional defined TCMs. Identity control of traditionally defined TCMs ought to be strengthened and standardization is necessary for further development of TCM. In practice, pursuing genetically identical TCM is a less ambitious and achievable goal. Plantation and popularization of germplasm selected is the way to achieve the goal gradually. For identity control of TCMs, genetic and chemical fingerprinting are two powerful tools with several advantageous and disadvantageous aspects, respectively. With respect to information capacity and moderate sensitivity, genetic fingerprinting like AFLP is a preferred identifier especially in the case of identifying plant materials.

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In vitro antiviral activity of fisetin, rutin and naringenin against dengue virus type-2

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In vitro **antiviral activities of three flavonoids: fisetin, naringenin and rutin against dengue virus type-2 (DENV-2; NGC strain) were evaluated. Inhibitory effects of each compound at the different stages of DENV-2 infection were examined using foci forming unit reduction assay (FFURA) and quantitative real-time polymerase chain amplification (qRT-PCR). Fisetin, rutin and naringenin showed cytotoxic effects against Vero cells with 50% cytotoxicity (CC_{50}) values of 247, >1000, and 87 µg/ml, respectively. Fisetin when added to Vero cells after virus adsorption inhibited DENV replication with a half maximal inhibition concentration (IC_{50}) value of 55 µg/ml and selectivity index (SI) of 4.49. The IC_{50} value of fisetin was 43.12 µg/ml with SI=5.72 when Vero cells were treated for 5 h before virus infection and continuously up to 4 days post-infection. There was no direct virucidal activity or prophylactic activity of fisetin against DENV-2. Rutin and naringenin did not inhibit DENV-2 replication in Vero cells. Naringenin however, exhibited direct virucidal activity against DENV-2 with IC_{50} = 52.64 µg/ml, but the SI was <1. The present study suggests that among the flavonoids examined, only fisetin showed significant *in vitro* anti dengue virus replication activity.**

Key words: Infectious disease, virology, dengue, antivirals, flavonoid.

INTRODUCTION

Dengue virus (DENV) belongs to the Flaviviridae family which consisted of several important human pathogens. There are four different DENV genotypes: DENV-1, DENV-2, DENV-3 and DENV-4. All four endemic DENV genotypes are transmitted by the mosquitoes, *Aedes agypti* or *Aedes Albopictus*. Most primary DENV infections are asymptomatic or manifests as dengue fever (DF), a mild febrile illness with some vascular leakage tendencies. The severe forms of dengue usually occur in secondary infections which could manifest as dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) with severe vascular leakage. Currently, there is no effective antiviral drug or licensed vaccine against dengue. Finding effective antiviral compound(s) against the virus is crucial as the disease is rapidly spreading worldwide and causing many deaths (Che et al., 2009).

Among the different types of compounds and chemical derivatives studied for antiviral activities against DENV, include those from natural resources such as plant extracts and their purified compounds (Jain et al., 2008). Plant-derived compounds are desired due to the potential

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of low side effects and their easy accessibility from the nature. Among these plant-derived compounds, flavonoids have received considerable attentions as possible sources of new therapeutics for viral infections (Asres et al., 2005; Wang et al., 2006). Flavonoids are phenolic compounds found in many plants (Tapas et al., 2008). There have been many studies reporting the beneficial effects of flavonoids on general health such as their anti-oxidant activities, anti-tumor activities and also their anti-microbial activities (Chukwujekwu et al., 2011; Kale et al., 2008; Middleton, 1996; Tim Cushnie et al., 2005). Antiviral activities for a number of flavonoids have also been described against many different viruses (Lyu et al., 2005; Arena et al., 2008; Xu et al., 2010). The flavonoids have been shown to possess antiviral activities against HSV-1, HSV-2, Sindbis virus, parainfluenzavirus-3 and HCMV (Hayashi et al., 2007; Lyu et al., 2005; Orhan et al., 2010; Paredes et al., 2003).

A recent study reported the potential of flavonoids as antivirals against DENV (Muhamad et al., 2010). The present study examined the *in vitro* antiviral activity of the flavonoids, fisetin, naringenin and rutin against DENV-2.

MATERIALS AND METHODS

Chemicals

Fisetin and naringenin were purchased from Sigma Chemical Company (Sigma, St, Louis, USA) and rutin was purchased from Indofine Chemical Company (Indofine, NJ, USA). All compounds were initially dissolved in dimethyl sulfoxide (DMSO) (Sigma, St, Louis, USA) to prepare the stock solution (20 mg/ml) and stored at -20°C until needed. To prepare the different concentrations of each compound, the stock solution was serially diluted in serum free cell culture medium and sterile-filtered using syringe filter with 0.2 µm pore size. Fetal bovine serum (FBS) was added to the treatment medium to a final concentration of 2% when needed for the treatment of the infected cells.

Cells and virus

DENV-2 New Guinea C strain (NGC) was used for the study. Virus was propagated in *A. albopictus* C6/36 monolayer cells at 28°C in the presence of 3% CO_2 and harvested on day 7 post-infection (PI). Virus stock was prepared and titrated as previously described (Wong et al., 2007) and stored at -70°C until needed.

African green monkey kidney cells (Vero) was used for the foci forming unit reduction assay (FFURA). Cells were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS; Gibco, NY, USA) at 37°C in the presence of 5% CO_2. For maintenance medium, serum concentration was reduced to 2%.

Cytotoxicity assay

In vitro cytotoxicity of compounds used in the study was determined using 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) method performed strictly following the manufacturer's protocol and as previously described (Mosmann, 1983). Briefly, Vero cells were cultured in 96-well cell culture microplate. Confluent cells were treated by increasing concentration of each compound in triplicate wells. The final volume of cell culture medium and compound in each well was 90 µl. Treated cells were incubated for 4 days at 37°C. After 4 days, 15 µl of MTT solution (Promega, WI, USA) was added to each well. The microplate was kept at 37°C for an additional 4 h in a humidified 5% atmosphere. After the incubation period, 100 µl of the solublization/stop solution was added to each well. The absorbance value of each well was measured using a 96-well plate reader (TECAN, Mannendorf, Switzerland) at 570 nm.

In vitro antiviral assays

The *in vitro* antiviral assay was performed using three different treatment conditions. In the first instance, Vero cells were plated into 24 wells cell culture microplate. After attaining 80% confluence, virus inoculum consisting of 200 FFU DENV-2 was added to each well and virus was allowed to absorb to the cells for 1 h at 37°C. Unabsorbed viruses were removed by rinsing cells with sterile PBS twice. Different concentrations of each compound were mixed with 1.5% carboxymethylcellulose (CMC) containing cell-growth medium supplemented with 2% FBS and the plates were incubated at 37°C for 4 days. After 4 days, cells were fixed with 4% paraformaldehyde for 20 min and subsequently permeabilized with 1% NP40 solution (Sigma, St, Louis, USA) for 10 min at room temperature. Cells were washed 3 times with PBS and blocked with 3% skim milk solution prepared in PBS, for 2 h at room temperature. Dengue hyper-immune serum prepared in rabbit, diluted at 1:500 using 1% skim milk solution was added to each well. The cells were incubated at 37°C for 1 h. After incubation, cells were washed 3 times with PBS and similarly incubated with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) with final concentration of 1:250 in 1% skim milk solution (Sigma, St. Louis USA). Finally, 3' di-amino-benzidine (DAB) peroxidase substrate (Pierce, Illinois USA) was added to each well to stain the virus foci (Okuno et al., 1979). Foci were counted under a stereomicroscope and expressed as Foci-Forming-Unit (FFU). Antiviral activities of the compounds were determined by calculating the percentage of foci reduction (%RF) compared against the controls maintained in parallel using the following formula: RF (%) = (C-T) × 100/C, where C is the mean of the number of foci from triplicates treatment without compound added and T is the mean of the number of foci from triplicates of each treatment measures with the respective compound (Laille et al., 1998).

In a separate study, the effects of prophylactic treatment of cells with the compounds prior to DENV infection were examined. Vero cells were treated with the different concentrations of each compound for 5 h before viral infection. The treatment medium was aspirated after 5 h and cells were rinsed twice with sterile PBS and then infected with 200 FFU of DENV-2 in the presence of the various concentrations of the compounds. The microplate was kept at 37°C for 1 h to allow virus adsorption. After virus adsorption, the infected cell monolayer was rinsed twice with sterile PBS in order to remove the unabsorbed viruses and incubated in 2% FBS containing EMEM with 1.5% CMC and the different concentration of the compounds. The plates were incubated at 37°C for 4 days in the presence of 5% CO_2. Viral foci were stained and counted as described earlier.

The potential direct virucidal effects of the compounds on DENV were examined by treating virus suspension containing 200 FFU of DENV-2 with increasing concentration of each of the compound for 2 h at 37°C. Vero cell monolayers in 24 wells cell culture microplate were infected with the compound-treated virus suspensions. After 1 h adsorption at 37°C, cells were rinsed twice with PBS to remove

unabsorbed viruses. Then, 1.5% CMC containing growth medium was added to each well and the microplate was incubated for 4 days in a humidified 37°C incubator in the presence of 5% CO_2. Viral foci staining were performed as described earlier.

Quantitative real-time polymerase chain amplification (RT-PCR)

DENV genome replication was estimated using the quantitative RT-PCR method described previously with some modifications (Wong et al., 2007). Briefly, DENV-2 RNA was extracted from the DENV-2 infected cells and cell culture supernatant using RNA extraction kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed using SensiMix SYBR green mixture (Quantace, Watford, United Kingdom) in a total reaction volume of 20 µl consisting of ddH_2O (7.4 µl), 2× SensiMix One-Step (10 µl), 50× SYBR Green solution (0.4 µl), RNase Inhibitor (10 units), 50 pmol each of forward (DNF) and reverse (D2R) primers, and the extracted DENV RNA (1 µl) (Seah et al., 1995). All amplifications were performed in triplicates using the DNA Engine Opticon system (MJ Research/Bio-Rad, Hercules, CA) and the following amplification cycles: reverse transcription at 50°C for 30 min, initial denaturation at 95°C (10 min), followed by 45 cycles of 95°C for 15 s, 59°C for 30 s and 72°C for 30 s. Melting curve analysis was performed at temperature from 60 to 98°C to verify the assay specificity. For absolute quantitation of the viral RNA, a standard curve was established with a serially diluted RNA extracted from virus inoculum with known infectious virus titer.

Statistical analysis

Statistical analysis performed using Graph Pad Prism for Windows, version 5 (Graph Pad Software Inc., San Diego, CA, 2005). Selectivity index value (SI) was determined as the ratio of CC_{50}/IC_{50} of each compound.

RESULTS AND DISCUSSION

Cytotoxicity of bioflavonoids

Potential cytotoxic effects of the compounds were determined using the MTT assays (Figure 1). Results from the assays suggested that the 50% cytotoxicity (CC_{50}) values for fisetin, rutin and naringenin were 247, >1000 and 87 µg/ml, respectively (Table 1). Naringenin was the most cytotoxic compound to Vero cells among the three examined compounds. Rutin showed no significant cytotoxicity against Vero cells. There were no measurable cytotoxic effects of 0.5% DMSO against Vero cells examined at the highest possible concentration as solvent for the compounds used in the study (data not shown).

Antiviral assays with different compounds against DENV-2

Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the flavonoids examined in the study. All the compounds were examined for the potential antiviral effects, (i) for their prophylactic activity, (ii) added subsequent to virus infection of cells, (iii) added to cells at 5 h prior to infection and then all throughout the assay period and (iv) directly to virus suspension to examine their direct virucidal effects.

Fisetin showed a dose-dependent inhibition of DENV-2 replication in the FFURA with a half maximal inhibition concentration (IC_{50}) value of 55 µg/ml when added subsequent to infection of the Vero cells (Figure 2a). Comparable IC_{50} inhibition value (IC_{50} = 50 µg/ml) was obtained for inhibition of 49% ± 1 DENV-2 genome copy number relative to the untreated Vero cell cultures, determined by qRT-PCR (Figure 2b). There was no significant direct virucidal activity of fisetin against DENV-2 (Figure 4). Fisetin when continuously present exhibited anti-dengue activity with IC_{50} = 43.12 µg/ml (Figure 3a). This finding was supported by the qRT-PCR results which showed that the DENV-2 RNA level was decreased by more than 65% ± 1.3 when Vero cells were similarly treated with 50 µg/ml fisetin (Figure 3b).

Table 1. *In vitro* cytotoxicity effects of flavonoids against Vero cells.

Flavonoid	CC_{50} (µg/ml)
Fisetin	247
Rutin	>1000
Naringenin	83

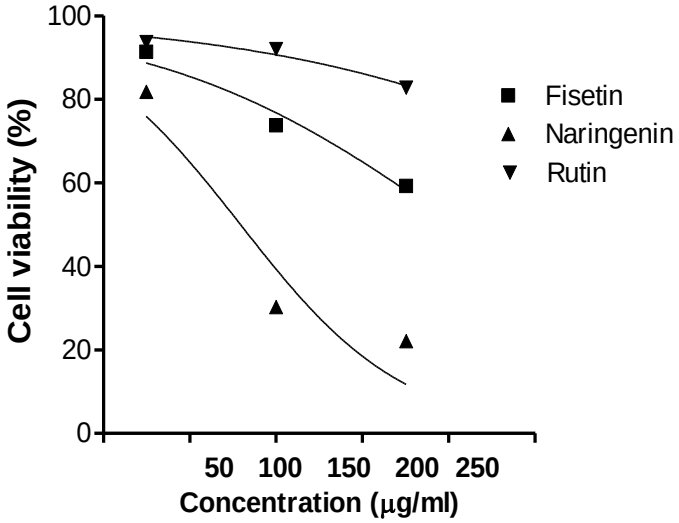

Figure 1. Cytotoxicity of flavonoids against Vero cells. MTT assay was used to evaluate the cytotoxicity of the flavonoids. All experiments were conducted in triplicates.

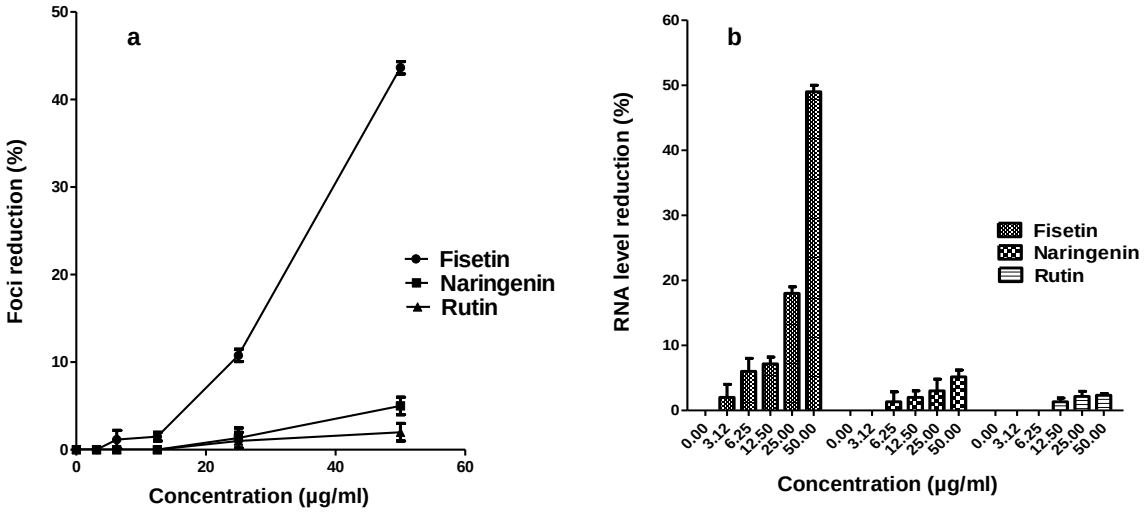

Figure 2. Assay of flavonoids against DENV-2 intracellular replication. Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the tested flavonoids after viral adsorption (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel.
Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

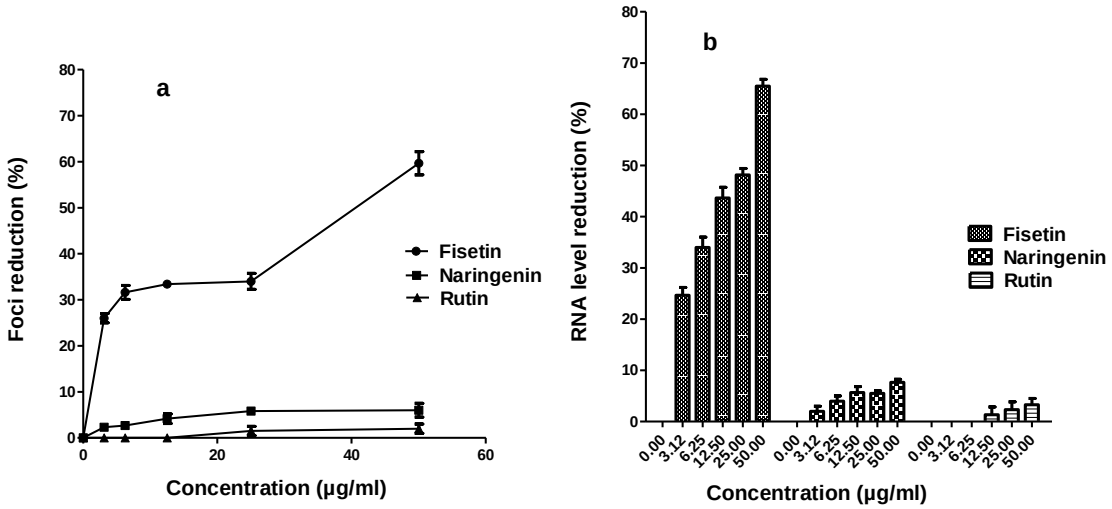

Figure 3. Evaluation of flavonoids continous treatment against DENV-2 replication. Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the tested flavonoids 5 h before infection to 4 days post infection (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel.
Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

Naringenin and rutin when added to cells subsequent to DENV-2 infection did not exhibit any anti-DENV-2 activities (Figure 2). No antiviral activity was also recorded when naringenin and rutin was added to cells continuously from 5 h before virus infection up to 4 days post infection (Figure 3). These findings were corroborated by results obtained from qRT-PCR which indicated no significant reduction in the amount of total DENV-2 genomic RNA levels (Figures 2b and 3b).

Naringenin, however, exhibited direct virucidal activity

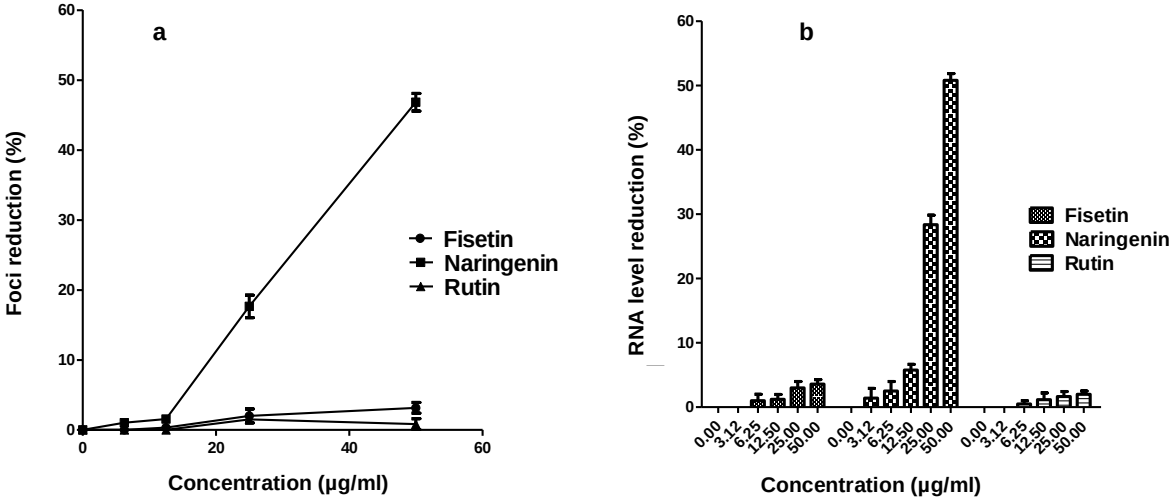

Figure 4. Direct virucidal activity of flavonoids against DENV-2. Foci forming unit reduction assay (FFURA) was used to evaluate the direct anti-dengue virus activities of the tested flavonoids by treatment of viral suspension with flavonoids prior to infection (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel.
Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA).

against DENV-2 with IC_{50} = 52.64 µg/ml when added directly to the virus suspension (Figure 4a). Naringenin at 50 µg/ml decreased the DENV-2 RNA level by 50% ± 1.3 when compared with the non treated virus inoculum (Figure 4b).

In an earlier study, it was described that the flavonoids, glabranine and 7-O-Methyl-Glabranine exhibited significant anti-dengue activity in cell culture (Sanchez et al., 2000). It was also described in an *in vitro* study that pinostrobin could inhibit DENV-2 NS2B/NS3 protease (Kiat et al., 2006).

Results from the present study suggest that fisetin ($C_{15}H_{10}O_6$), a flavonol commonly found in *Acacia greggii*, *Acacia berlandieri*, *Gleditschia triacanthos*, *Quebracho colorado* and also in mangoes, parrot tree and Euroasian smoketree, possesses anti-DENV activities. The anti-DENV activity is notable when cells were treated continuously for 5 h before infection until 4 days post-infection as well as when added subsequent to virus infection. Naringenin ($C_{15}H_{12}O_5$), a plant flavonone found in grapefruit, orange and tomato skin, on the other hand, showed no inhibition of DENV-2 replication. Naringenin however, exerted direct virucidal effects on DENV even though it is highly cytotoxic with selectivity index (SI) of <1. Rutin ($C_{27}H_{30}O_{16}$), one of the flavonoid glycoside found in buckwheat, asparagus, citrus fruits and some berries showed no inhibition of virus adsorption and DENV-2 replication as well as no prophylactic effect against virus infection.

The mechanism of how fisetin affects DENV virus replication is unclear. Fisetin is not likely to act directly on the virus, as there was no inhibition of the virus when fisetin was added directly the virus inoculum. These results also suggest that fisetin does not affect DENV-2 binding to cells. Results from the qRT-PCR assay suggest that fisetin could affect DENV genome copy number. One possibility is that fisetin could interfere with DENV-2 replication by binding directly to virus RNA, forming a flavonoid-RNA complex (Nafisi et al., 2009) or affecting the RNA polymerases (Ono et al., 1990) resulting in inhibition of virus replication. Prophylactic treatment of cells with fisetin could result in accumulation of intracellular fisetin, which in-turn increases inhibition of dengue virus replication. Further study to improve the efficacy of the anti-DENV properties of fisetin could be undertaken utilizing fisetin as a base molecule for further modifications.

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New antioxidant and cholinesterase inhibitory constituents from *Lonicera quinquelocularis*

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A phytochemical investigation on the ethyl acetate soluble fraction of *Lonicera quinquelocularis* (whole plant) led to the isolation of three new and two known compounds. The crude extract and the various soluble fractions were screened for cytotoxic activities. The ethyl acetate soluble fraction was found to be more cytotoxic. The chloroform soluble fraction showed moderate activity while the other fractions and crude extract were weakly cytotoxic. The isolated compounds were tested for antioxidant, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities. Almost all the compounds showed profound antioxidant activities in DPPH free radical scavenging assay. The 50% inhibitory effect (IC_{50}) values of compounds 3 and 5 against AChE were determined to be 1.65 and 3.43 µM, while the values obtained against BChE were 5.98 and 9.84 µM, respectively. Compounds 2 and 4 showed weak inhibition profile.

Key words: *Lonicera quinquelocularis,* phthalates and benzoates, cholinesterase inhibition, antioxidant activity.

INTRODUCTION

The genus *Lonicera* belongs to the family Caprifoliaceae. It contains about 12 genera and 450 species (Mabberley, 1997), and occurs mainly in temperate region of Northern Hemisphere. In Pakistan, it is represented by 4 genera and 27 species (Akhter, 1986). Various species of this genus are used for the treatment of acute fever, headache, respiratory infections (Houghton et al., 1993), bacterial infections (Puupponen-Pimia et al., 2001) and also used as an antioxidant (Kahkonen et al., 2001; Ali et al., 2013), cytoprotective (Chang and Hsu, 1992), heapto-protective (Liu et al., 1992; Shi et al., 1999), antiviral (Chang et al., 1995), antitumor (Wang et al., 2009; Yip et al., 2006) and anti-inflammatory agent (Yoo et al., 2008). Previous studies on this genus reported the isolation of a variety of constituents including iridoids, bisiridoids, sulfur containing monoterpenoids, alkaloids, glycosides, triterpenoids, saponins, coumarin glycosides and flavone glycosides (Machida et al., 1995; Bailleul et al., 1981; Souzu and Mitsuhashi, 1969, 1970). *Lonicera quinquelocularis,* a member of this genus is widely distributed in dry sunny places between 750 and 3000 m in many countries of Asia. In Pakistan, it is found in Baluchistan, Kurram, Chitral, Swat, Astor, Hazara, Murree hills, Poonch and Kashmir (Abdullah, 1972). Previous phytochemical study on this plant resulted in the isolation of triterpenoids, benzoates, lonicerin, loganin, coumarin and iridoid glycosides (Ali et al., 2013; Kumar et al., 2000). It is used as a sedative, hypotensive, anti-pyretic and antioxidant agent (Ali et al., 2013; Calis et al., 1984; Basaran et al., 1988). The diverse medicinal uses of the genus *Lonicera* has prompted the investigate of the chemical constituents of *L. quinquelocularis.*

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MATERIALS AND METHODS

General experimental procedure

Aluminium TLC plates (20 × 20, 0.5 mm thick) pre-coated with silica gel 60 F_{254} (20×20 cm, 0.2 mm thick; E. Merck, Darmstadt, Germany) were used for TLC to check the purity of the compounds. Column chromatography (CC) was carried out using silica gel of 230-400 mesh (E. Merck, Darmstadt, Germany). Ceric sulphate and potassium permanganate solutions were used as visualization reagents. The UV spectra (λ_{max} nm) were recorded on Shimadzu UV-2700 spectrophotometer (Shimadzu, Japan) in EtOH. Mass spectra was recorded on Bruker TOF Mass spectrometers (Billerica, USA) using electrospray ionisation (ESI). The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 NMR spectrometer (Billerica, USA) (400 MHz for ^{1}H and 100 MHz for ^{13}C-NMR), using $CDCl_3$ as solvents.

Plant

The whole plant of *L. quinquelocularis* was collected from Bara Galli, Hazara Division, District Mansehra, in June 2009. It was identified by Professor Dr. Manzoor Ahmad, Plant Taxonomist, Department of Botany, Government Degree College Abbotabad, Pakistan, where a voucher specimen has been deposited in herbarium (Accession No. C-0013).

Extraction and isolation

The shade dried whole plants (13 kg) was ground and extracted with ethanol at room temperature (3 × 25 L). The combined ethanol extract was evaporated under reduced pressure to obtain a thick greenish gummy material (crude), which was fractionated with *n*-hexane (151 g), chloroform (147 g), ethyl acetate (109 g), and n-butanol (53 g) soluble fractions, respectively.

The ethyl acetate soluble fraction was subjected to column chromatography over silica gel (70-230 mesh) eluted with *n*-hexane (100%), *n*-hexane: EtOAc (1:19–19:1), EtOAc (100%), EtOAc:MeOH (1:19–19:1), MeOH (100%) in increasing order of polarity to obtain 13 fractions (A-M).

Fraction C (4 g) was again subjected to a series of silica gel column chromatography eluted with *n*-hexane, *n*-hexane-EtOAc and EtOAc in increasing order of polarity to afford compound 1 and to a preparative TLC using *n*-hexane:EtOAc (4:1) as solvent system to afford compounds 2 and 3, respectively.

Fraction D (4 g) was re-chromatographed over silica gel eluted with *n*-hexane, *n*-hexane-EtOAc and EtOAc in increasing order of polarity followed by preparative TLC eluted with *n*-hexane:CH_2Cl_2 (2:3), and afforded compounds 4 and 5, respectively.

Identification of compounds

Compounds 1 and 2 were isolated as colourless oils and were identified as bis (2-ethylhexyl) phthalate (Nair et al., 2012) and dioctyl phthalate (Sultan et al., 2010). Identification of compounds 3, 4 and 5 were based on spectroscopic data analysis.

Bis (7-acetoxy-2-ethyl-5-methylheptyl) phthalate (3)

Colourless oil: UV (MeOH) λ_{max} nm (log ε): 296 (4.26) and 232 (5.18); IR (dry film) ν_{max} cm^{-1}: 1725 (Ester -C=O), 1610 (aromatic C=C) and 1130-1200 (C-O); HREIMS-TOF m/z 562.7642 $[M+H]^+$ (calcd. for $C_{32}H_{50}O_8$, 562.3603); ^{1}H NMR (400 MHz, $CDCl_3$). δ 7.73 (dd, *J* = 5.8, 3.3, H-3/H-6), δ 7.56 (dd, *J* = 5.8, 3.3, H-4/H-5), 4.15 (dd, *J* = 11.8, 6.1 Hz, H-1'a), 4.25 (dd, *J* = 12.1, 6.2 Hz, H-1'b), δ 4.09 (t, *J* = 6.8 Hz, H-7'), δ 2.07 (s, H-10'), 1.70 (m, H-2'), 1.64 (m, H-5'), 1.48 (1H, m, H-6'b), 1.32 (1H, m, H-6'a), 1.45 (m, H-11'), 1.35 (1H, m, H-4'b), 1.24 (1H, m, H-4'a), 1.29 (m, H-3'), 0.96 (d, *J* = 6.4, H-13'), 0.89 (t, *J* = 6.5, H-12'). ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.22 (C-1/C-8), 132.49 (C-2), 128.80 (C-3/C-6), 130.86 (C-4/C-5), 132.49 (C-7), 68.15 (C-1'), 38.76 (C-2'), 31.60 (C-3'), 28.94 (C-4'), 34.67 (C-5'), 30.39 (C-6'), 64.35 (C-7'), 167.75 (C-9'), 22.65 (C-10'), 23.76 (C-11'), 10.95 (C-12'), 14.09 (C-13').

Neopentyl-4-ethoxy-3, 5-bis [3-methyl-2-butenyl] benzoate (4)

Amorphous powder: UV (EtOH) λ_{max} (log ε): 210 (4.3), 260 (4.5) nm; IR (KBr) ν_{max}: 2940, 1705, 1600, 1440, 1300, 1230, 1010, 780 cm^{-1}; HREIMS-TOF *m/z* $[M]^+$ 372.3742 (calcd for $C_{24}H_{36}O_3$, 372.2156); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.39 (1H, d, *J* = 1.8 Hz, H-2/H-6), 5.69 (2H, t, *J* = 6.8 Hz, H-2'), 4.38 (2H, q, *J* = 7.8 Hz, OCH_2), 4.15 (s, 2H, H-3''), 3.27 (4H, d, *J* = 6.8 Hz, H-1'), 1.83 (6H, d, *J* = 1.1 Hz, H-4'), 1.74 (6H d, *J* = 1.1 Hz, H-5'), 1.36 (3H, t, *J* = 7.8 Hz, OCH_2CH_3), 0.96 (9H, s, H-5''); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 137. 2 (C-1), 129.3 (C-2), 124.8 (C-3), 154.6 (C-4), 124.8 (C-5), 129.3 (C-6), 27.6 (C-1'), 121.5 (C-2'), 133.5 (C-3'), 19.4 (C-4'), 24.7 (C-5'), 163.0 (C-1''), 83.2 (C-3''), 29.4 (C-4''), 23.1 (C-5''), 68.3 ($O-CH_2$), 15.5 (O-C-CH3).

Neopentyl-4-hydroxy-3, 5-bis [3-methyl-2-butenyl] benzoate (5)

Amorphous white solid: UV (EtOH) λ_{max} (log ε): 217 (4.2), 265 (4.5), 295 (4.3) nm; IR (KBr) ν_{max}: 2940, 1705, 1600, 1440, 1300, 1230, 1010, 780 cm^{-1}; HREIMS-TOF *m/z* $[M]^+$ 344.6403 (calcd. for $C_{22}H_{32}O_3$, 344.2549); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 7.48 (1H, d, *J* = 1.6 Hz, H-2/H-6), 5.72 (2H, t, *J* = 7.1 Hz, H-2'), 5.31 (1H, bs, OH), 4.19 (2H, s), 3.33 (4H, d, *J* = 7.1 Hz, H-1'), 1.85 (6H, d, *J* = 1.2 Hz , H-4'), 1.78 (6H, d, *J* = 1.2 Hz, H-5'), 0.98 (9H, s, H-5''); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 135.0 (C-1), 129.3 (C-2), 124.2 (C-3), 152.5 (C-4), 124.2 (C-5), 129.3 (C-6), 27.3 (C-1'), 123.1 (C-2'), 133.5 (C-3'), 19.1 (C-4'), 25.4 (C-5'), 165.4 (C-1''), 81.9 (C-3''), 29.7 (C-4''), 21.8 (C-5'').

Cytotoxic activity

Cytotoxic activities of the extract and fractions were determined using brine-shrimp (*Artemia salina*) lethality bioassay (Meyer et al., 1982). Artificial sea water was prepared by dissolving 3.7 g of sea salt per liter of double distilled water and was filtered. “Sea” water was placed in small tank, and brine shrimp eggs (1 mg) was added and was darkened by covering with aluminium coil. Twenty milligrams of concentrated sample was dissolved in 2 ml of $CHCl_3$ (20 mg/2 ml (10 mg/ml)) and transferred to 500, 50 and 5 µl vials corresponding to 1000, 100 and 10 µg/ml, respectively. Three replicates were prepared for each concentration making a total of nine vials. The vials containing the sample were concentrated, dissolved in DMSO (50 µl) and 5 ml “sea water” was added to each. Then, ten shrimps were added per vial. All the vials were incubated at 37°C for 24 h and the brine shrimps that survived were counted. The activities of these extract and fractions were compared with standard drugs Ampicillin (Mc Laughlin and Anderson, 1988; Rashid et al., 2002). The data was analyzed with a Finney computer program to determine LD_{50} values with 95% confidence interval.

Cholinesterase inhibition assay and determination of IC_{50} values

Acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (horse-

Table 1. Cytotoxic activities of crude extract and fractions.

Concentration (µg/ml)	Fractions and crude extract (% deaths at doses)					
	n-hexane (F1)	$CHCl_3$ (F2)	EtOAc (F3)	*n*-BuOH (F4)	Ethanol crude	Standard drug (Ampiciline)
10	15	40	50	30	20	40
100	25	50	60	40	30	80
1000	30	60	80	60	40	120
ED_{50}	650.55	320.60	120.25	450.40	540.65	70.50

serum E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, galanthamine and 5, 5´-dithiobis [2-nitrobenzoic-acid] (DTNB) were purchased from Sigma. All other chemicals were of analytical grade. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were measured according to a modified spectrophotometric method used by Ellman (Ellman et al., 1961). Protocol and assay conditions were the same as described by Rocha (Rocha et al., 1993).

Acetylthiocholine iodide and butyrylthiocholine chloride were used as substrates to assay acetylcholinesterase and butyrylcholinesterase, respectively. DTNB was used for the measurement of cholinesterase activity. 0.2 mM DTNB in 62 mM sodium phosphate buffer (pH 8.0, 880 µl), test compound solution (40 µl) and acetylcholinesterase or butyrylcholinesterase solution (40 µl) were mixed and incubated for 15 min (25°C). The reaction was then initiated by the addition of acetylthiocholine or butyrylthiocholine (40 µl), respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were monitored by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine and butyrylthiocholine, respectively, at a wavelength of 412 nm (15 min). All the reactions were performed in triplicate in a BMS spectrophotometer (USA). The concentrations of test compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) were determined by monitoring the effect of increasing concentrations of these compounds in the assays on the inhibition values. The final DMSO concentration in the reaction mixture was 6%.

DPPH radical scavenging activity

The DPPH assay was performed according to the standard procedure with little modifications (Gymfi et al., 1999). The fresh stock solution was prepared by dissolving 3 mg DPPH with 100 ml of methanol and then stored at 20°C. The working solution was obtained by diluting DPPH solution with methanol to obtain an absorbance of about 0.980 (± 0.02) at 517 nm using the spectrophotometer. A 900 µl aliquot of this solution was mixed with 100 µl of the compounds (10 µg/ml). The same was repeated with positive reference, that is, ascorbic acid (10 µg/ml). The solution in the test tubes were shaken well and incubated in dark for 30 min at room temperature. Then, the absorbance was taken at 517 nm. The scavenging activity was calculated from the percentage of DPPH radical scavenged as the following equation:

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

RESULTS AND DISCUSSION

The crude ethanol extract (430 g) was successively extracted with *n*-hexane (F_1), chloroform (F_2), ethyl acetate (F_3) and *n*-butanol (F_4). All these fractions and the crude extract were tested for their cytotoxic activities (Brine shrimp lethality assay). The F3 was found to be more effective and showed high activities, F2 and showed optimum while F1 and F4 showed low lethality in brine shrimp assay (Table 1).

In the present investigation, phytochemical study on the ethyl acetate extract of whole plant of *L. quiquelocularis* afforded bis (2-ethylhexyl phthalate (1), dioctyl phthalate (2), bis (7-acetoxy-2-ethyl-5-methylheptyl phthalate (3), neopentyl-4-ethoxy-3, 5-bis (3-methyl-2-butenyl benzoate (4) and neopentyl-4-hydroxy-3, 5-bis (3-methyl-2-butenyl benzoate (5) (Figure 1).

Compounds 1 to 5 from *L. quinquelocularis* were tested against AChE and BChE, which represent the most attractive target for drug design and discovery of mechanism-based inhibitors for the treatment of neurone degenerative disorders such as Alzheimer's disease (Zhang, 2004). The percentage of inhibition was first determined at 0.1 mM. Compounds for which enzyme inhibition was greater than 50% were subsequently assayed for IC_{50} (50% inhibitory effect) determination. Among the isolated compounds, 3 and 5 showed most effective inhibitory activity against AChE and BChE as compared to standard drugs; allanzanthane and galanthamine in a dose dependent manner. The IC_{50} values of compounds 3 and 5 against AChE were determined to be 1.65 and 3.43 µM, while against BChE, were measured as 5.98 and 9.84 µM, respectively. Compounds 2 and 4 showed weak inhibition profile against AChE and BChE (Table 2).

The DPPH radical scavenging activity assay was performed to evaluate the antioxidant property of the isolated compounds with positive reference ascorbic acid (10 µg/ml) according to the standard procedure with some modifications (Gymfi et al., 1999). All isolated compounds in (Figure 2) showed higher antioxidant property than ascorbic acid. Furthermore, the compound 5 was found more active than others. It may be due to different structure of the compounds.

Conclusion

Several antioxidant and anticholinesterase active compounds were isolated from *L. quinquelocularis*, which

Table 2. AChE and BChE inhibitory activities of compound **1**-**5** from *L. quinquelocularis* (IC_{50}, µM).

Compound	AChE ± SEM[a]	BChE ± SEM[a]
1	Nil	Nil
2	8.74 ± 0.07	20.12 ± 0.079
3	1.65 ± 0.03	5.98 ± 0.079
4	5.27 ± 0.04	14.76 ± 0.087
5	3.43 ± 0.02	9.84 ± 0.037
Allanzanthaneb	2.94± 0.45	12.96 ± 0.053
Galanthamine[b]	1.79 ±0.061	7.98 ± 0.01

[a]Standard error of mean of five assays. [b]Positive control used in the assays. Data shown are values from triplicate experiments.

which showed good activity. The ethyl acetate soluble fraction was found to be more cytotoxic. In the present work, several new compounds from ethyl acetate soluble fraction which showed good activities in cholinesterase inhibition and DPPH radical scavenging assays were isolated. This species is as one of the ingredient of the traditional medicine in some part of the world.

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