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References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

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## ARTICLES

### Research Articles

**Effects of allopurinol and melatonin on warm renal ischemic- reperfusion injury in dogs**
NeshatGharamaleki Mehrdad, Shahbazi Mohammad Amin, Safarmashaei Saeid

**Justifying the use of the bluish liquid from the African giant snail (*Achatina marginata*) in traditional male circumcision surgery in Western Nigeria**
S. O. Olagbende-Dada, A. A. Adeniyi, F. O. Adefolaju, A. Adepoju-Bello, M. O. Ologunagba

**Effects of *Juniperus phoenicea* extract on uricemia and activity of antioxidant enzymes in liver, erythrocyte and testis of hyperuricemic (oxonate-treated) rats**
Nesrine GDOURA, Jean-Claude MURAT, Abdelwaheb ABDELMOULEH, Abdelfattah ELFEKI

**Lavender aromatherapy massages in reducing labor pain and duration of labor: A randomized controlled trial**
Abbaspoor Zahra, Mohammadkhani Shahri Leila

**Goldcrest honey and its solvent extracts: A natural product with anti-Helicobacter pylori activity**
Christy E. Manyi-Loh, Roland N. Ndip, Anna M. Clarke
ARTICLES

Research Articles

Influence of naringin on the myocardial ultrastructure and NF-κB expression in rats with diabetic cardiomyopathy
Keng Wu, Qiong You, Yan-Ming Tu 439

Function of human insulin-like growth factor-1 (hIGF-1) transgene towards the regeneration of peripheral nerves in vivo
Jia-Xiang Gu, Nai-Chen Zhang, Hong-Jun Liu, Jun-Bo Pan, Heng Tian, Wen-Zhong Zhang, Jing-Cheng Wang 444

Preparation and characterization of toltrazuril polyethyleneglycol 6000 solid dispersions with improved solubility
Haixia Lü, Suying Ma 450

Antioxidant, anti-cholinesterase and antibacterial activities of the bark extracts of Garcinia hombroniana
Jamila Nargis, Khairuddean Melati, Choon-Sheen Lai, Osman Hasnah, Keng-Chong Vikneswaran, Kooi-Yeong Khaw 454

Effects of leptin on colorectal cancer cell line HT-29
Ying Wang, Yan Liao, Hong-gang Yu, He-sheng Luo 460
ARTICLES

Research Articles

Toxicological evaluation of zerumbone on antitumor effects in mice  
Yeung Bae Jin, Woo-Duk Seo, Yoon-Jin Lee, Yun-Sil Lee, Hae-June Lee  
466

Antidiarrheal activity of methanol extract and major essential oil contents  
of *Saussurea lappa* Clarke  
J. S. Negi, V. K. Bisht, A. K. Bhandari, V. P. Bhatt, M. K. Sati, J. P. Mohanty,  
R. C. Sundriyal  
474
Effects of allopurinol and melatonin on warm renal ischemic- reperfusion injury in dogs

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INTRODUCTION

It has been previously observed that reperfusion of ischemic kidneys produces much more damage than that caused by the period of ischemia alone. However, the nature of the cellular insult that produces these changes is unknown. In this study, 30 male domestic native dogs were selected and randomly divided into five groups (n=6): G1:sham-operated(sham)(n=6), G2:ischemic-reperfusion induced group(I/R); about 45 min ischemia and 3 h reperfusion (n=6) , G3: I/R+melatonin(3 mg/kg) (n=6), G4: I/R + allopurinol (10 mg/kg)(n=6), G5: I/R +allopurinol + melatonin (n=6). Unilateral ureteral obstruction by ventral midline celiotomy in the left kidney was performed and after 14 days, renal samples were taken by ultrasound guided biopsy and samples were fixed in 10% buffered formalin and sent to pathology for grading. Data were analyzed by Mann-Whitney U test. P<0.05 was considered statistically significant. The degree of protection against renal dysfunction was less in comparison to animals treated with melatonin; we would suggest that melatonin-evoked changes in glutathione content and redox state results from increased activities of the enzymes of glutathione metabolism. There are a number of possible explanations for this finding, the most likely being that factors other than oxygen free radicals (for example, irreversible loss of mitochondrial function and ATP depletion and/or activation of proteolytic enzymes) contribute to renal dysfunction as the period of ischemia is extended.

Key words: Allopurinol, melatonin, renal ischemic reperfusion, dog.
has been tested under a remarkably large number of experimental situations in which free radical damage causes the accumulation of molecular debris and ultimately, cellular loss in the brain (Russel and Pappolla, 2004). In the present study, we studied the effect of allopurinol, a xanthine oxidase inhibitor, and melatonin co-administration to compare the safety of these two combinations on ischemia and reperfusion-induced renal injury in dog in order to investigate the importance of xanthine oxidase-linked free radical and melatonin in renal injury.

**METHODS**

In this study, 30 male domestic native dogs were selected and randomly divided into 5 groups (n=6): G1: sham operated (sham)(n=6), G2: ischemic reperfusion induced group (I/R); about 45 min ischemia and 3 h reperfusion(n=6), G3: I/R + melatonin (3 mg/kg)(n=6), G4: I/R + allopurinol (10 mg/kg)(n=6), G5: I/R + allopurinol + melatonin (n=6), for anesthesia of acepromazine, ketamine and maintenance by propofol; cephalosporin for prevention of infection were also used. Unilateral ureteral occlusion of ventral midline celiotomy in the left kidney was performed and after 14 days, renal samples were taken by ultrasound guided biopsy and samples were fixed in 10% buffered formalin, and embedded in paraffin. Paraffin sections (5 µ thick) were stained with hematoxylin and eosin (H-E) and Masson trichrome (Satoh et al., 2001; Shaffer et al., 1987). The observers scored with a semiquantitative scale designed to evaluate the degree of tubule-interstitial injuries including tubular atrophy and dilation with cast formation, degeneration and necrosis of the tubular epithelium, and interstitial inflammation and fibrosis. The tubule-interstitial injury score ranging from 0 to 4+ was determined as follows: 0, normal kidney; 1+, mild change; 2+, moderate change; 3+, severe change; and 4+, injury to the whole tissue (Sunami et al., 2004). The level of glomerular injury was assessed using the 0 to 4+ scale, where 1+ injury involved less than 25% damage to the glomerulus, 2+ = 25 to 50% injury, 3+ = 51 to 75% damage and 4+ = 76 to 100% damage. Data were represented as percentage of damaged glomeruli (N=100) showing any level of injury (scale 1+ to 4+). The total numbers of damaged glomeruli including all levels of injury were also measured and represented as total percentage of damaged glomeruli (N=100) (Sunami et al., 2004). The scores were determined in each section selected at random, and 20 fields were examined under 100 magnification. All measurements and scoring were performed on blinded slides.

**Statistical analysis**

Results are presented as mean ± SE. Histologic (non-parametric) data were analyzed by Mann-Whitney U test. P<0.05 was considered statistically significant.

**RESULTS**

Light microscopic studies revealed that renal I/R produced severe tubular damage characterized by flattened epithelial cells and lumen dilatation as well as presence of hyaline cast, tubular epithelial cells degeneration or necrosis and detachment from tubular basement membranes observed in both renal cortex and outer medulla. In the interstitium of renal tissues exposed to I/R, there was severe inflammatory cells infiltrations, hemorrhage and fibrosis. These lesions were significantly (P<0.05) alleviated in I/R rats treated with melatonin while, treatment by allopurinol or allopurinol in combination with melatonin had no significant protective activity against renal tissue injury induced by ischemia/reperfusion in the rats (Figures 1 to 5). Figure 6 depicts comparison of the effects of administered drugs on kidney tissue injury induced by ischemia/reperfusion among the experimental groups. The total number of damaged glomeruli (all levels of injury) was 4±1% in shams, which is normal for this strain and age (Erdely et al., 2004; Szabo et al., 2003), and greater in all I/R groups (I/R: 40±3%, p<0.01; I/R + A: 35±2 %, p<0.01; I/R + A+M: 29±2%, p<0.05; I/R + M: 20±1%, p<0.05). The total injury was also greater in the I/R + A group vs. the I/R + A+M (p=0.05) and I/R + M (p=0.01). As shown in Table 1, there were more damaged glomeruli at 2+, 3+, and 4+ levels of severity in the I/R group vs. sham, while only the 2+ injury severity was greater in I/R + M group vs. sham. In general, the severity of injury was intermediate in the I/R + M between shams and other I/R treated groups. Comparison of the percentage and severity of glomerular injury on the 1+ to 4+ scales among the experimental groups is presented in Table 1.

**DISCUSSION**

Free radicals affect all cells in the organism and lead to lipid peroxidation. This can be controlled, and its damage can be reduced by antioxidants in the plasma and tissues (Shaffer et al., 1987; Sunami et al., 2004; Stein et al.,
Figure 2. Microscopic appearance of renal tissue from a dog belonged to IR group shows considerable thickening of parietal layer of the Bowman’s capsule as well as periglomerular sclerosis. Acute cell swelling as ballooning degeneration narrowing the lumens and characteristic features of coagulative necrosis of tubular epithelial cells are prominent (Masson trichrome, ×250).

Figure 3. Microscopic view from the kidney of a dog belonged to IR + allopurinol treated group shows thickening of the glomerular basement membrane, shrinkage of glomerular capillary and hypertrophy of squamous epithelial cells, which cover the visceral and parietal layers of the Bowman’s capsule (Masson trichrome, ×250).

1990 Stein et al., 1990). In this study, we used allopurinol (which is a specific inhibitor of xanthine oxidase), melatonin and, whether separately or in a combination, against renal damage caused by ischemic reperfusion stress. We observed that the application of melatonin produced more protective effect when compared with administration of allopurinol. Indeed, the effect of allopurinol in comparison to ischemic group was significant in controlling oxidative stress. Allopurinol and its metabolite oxipurinol have a protective role against free radical-induced damage by inhibiting xanthine oxidase. Allopurinol decreases the formation of xanthine oxidase products, thereby, reduces both the cell necrosis and increased microvascular permeability (Satoh et al., 2001;

Figure 4. Photomicrograph of the renal tissue from a dog belonged to IR + melatonin treated group. Glomerular structure is near to normal and tubular cells show only a mild degenerative changes. Balloning degeneration of tubular epithelial cells narrowing of the lumen is prominent (Masson trichrome, ×250).

Figure 5. Microscopic appearance of the renal tissue from a dog belonged to IR+ allopurinol+melatonin treated group. Shrinkage of the glomerulus along with dilation of urinary space and thickening of parietal layer of the Bowman’s capsule is prominent. Acute cell swelling and necrosis of tubular epithelial cells is obvious (Masson trichrome, ×250).
Figure 6. Comparison of tubulointerstitial injury among the experimental groups. *Significantly different from the sham, P<0.05; #Significantly different from the I/R group. Error bars represent SE.

Table 1. Comparison of the % and severity of glomerular injury on the 1+4+ scale among the experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>% of damaged glomeruli</th>
<th>Total % of damaged glomeruli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+1</td>
<td>+2</td>
</tr>
<tr>
<td>Sham</td>
<td>4±1^a</td>
<td>3±1^a</td>
</tr>
<tr>
<td>I/R</td>
<td>4±0.3^a</td>
<td>12±0.5^b</td>
</tr>
<tr>
<td>I/R+A</td>
<td>5±0.2^a</td>
<td>10±0.6^b</td>
</tr>
<tr>
<td>I/R+M</td>
<td>4±0.3^a</td>
<td>10±0.4^b</td>
</tr>
<tr>
<td>I/R+A+M</td>
<td>5±0.3^a</td>
<td>9±0.6^b</td>
</tr>
</tbody>
</table>

Significantly different from the control: *P<0.05, **P<0.01 vs. sham. ^aDifferent superscripts shows significant difference in each row (P<0.05).

Shaffer et al., 1987; Sunami et al., 2004; Stein et al., 1990; Stein et al., 1990. Exogenous melatonin is able to preserve renal functional status following I/R induced injury by increasing glutathione and reducing lipid peroxidation in the early reperfusion phase, without any apparent effect on neutrophil infiltration in the late reperfusion phase (Sergio and Caridad, 2004). Oxygen free radicals have been implicated in the pathogenesis of cellular damage associated with oxygen toxicity and phagocyte-mediated inflammation. In addition, superoxide anion radicals have been implicated as mediators in the increased capillary permeability induced by temporary ischemia in the small intestine and the lung, brain damage resulting from cerebral ischemia, the ischemia-induced necrosis observed in island skin flaps, and finally, as mediators in the morphologic disruption of small bowel epithelium and intestine noted to occur following temporary ischemia and reperfusion. Superoxide anion radicals have previously been shown to play a role in the pathogenesis of ischemia-induced renal dysfunction. Importantly, superoxide radicals are generated immediately after the onset of reperfusion (Guarnieri et al., 1980). Superoxide radical-induced cytotoxicity appears to depend largely on the subsequent production of a highly reactive species, the hydroxyl radical (or its redox equivalent) catalyzed by iron, which is probably in the form of an organic iron complex.

Reactive oxygen species are implicated as the cause of increased permeability of cell membranes of endothelium and epithelium. Increased permeability in cell membranes of tubular epithelial cells or capillary endothelial cells of kidney could lead to disruption of vital transport functions. Membrane damage by reactive oxygen species could occur at the plasmalemna with secondary release of other mediators such as lipid endoperoxides, arachidonic acid, and hydroperoxides. Either these mediators or the...
initiating species could induce endothelial cell contraction, terminating in a macromolecular leak. Hydroxyl free radical has been shown to degrade hyaluronic acid, which is one of the principal constituents of the interstitial matrix and the capillary basement membrane. Oxygen free radical-induced damage to the capillary basement membrane could exacerbate the microvascular leak already established as a result of endothelial cell damage. Although superoxide radicals might act as primary mediators of ischemic tissue injury, with more severe or prolonged period of ischemia, other factors such as ATP depletion and proteolysis may play a more important role in the chemical and structural alteration of tissue. These other factors could then negate the protective effects of SOD following severely prolonged renal ischemia similar to that which occurs upon occlusion for 1 h of the superior mesentery artery. Allopurinol was employed in several earlier studies to prevent an irreversible loss of purine nucleotides from cells during ischemia. During ischemia, purine nucleotides are converted to adenosine and guanosine and subsequently degraded to uric acid. When purines have been catabolized from xanthine to uric acid enzymatically, they are irreversibly lost from the nucleotide pool. The degraded purines are thus no longer available to the cell for the resynthesis of ATP when oxygen delivery is restored. Allopurinol was previously thought to provide protection against ischemic damage by preventing the loss of purine nucleotides from the intracellular nucleotide pool during periods of hypoxic stress via inhibition of xanthine oxidase. Allopurinol provided protection against renal dysfunction following temporary ischemia. This protection was however not complete. The degree of protection against renal dysfunction was less in comparison to animals treated with melatonin; we would suggest that melatonin-evoked changes in glutathione content and redox state result from increased activities of the enzymes of glutathione metabolism. There are a number of possible explanations for this finding, the most likely being that factors other than oxygen free radicals (for example, irreversible loss of mitochondrial function and ATP depletion and/or activation of proteolytic enzymes) contribute to renal dysfunction as the period of ischemia is extended.

REFERENCES

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In contemporary African tradition, the male child is circumcised. One of the requirements for successfully carrying out this surgical feat by the traditional birth attendants in the western part of Nigeria is to immediately bath the exposed penis surface with the fresh bluish liquid from the African giant snail (*Achatina marginata*). This study screened the bluish liquid for anti-bacteria/anti-fungi activities and examined it for coagulatory effect. The coagulatory effect was assessed through the Prothrombin time (PT) of three categories of people, (a) normal persons, (b) patients on warfarin, (c) hemophilic patients, and compared with the PT of calcified tissue thromboplastin (reference) on the same people. The anti-bacterial/anti-fungal effects were studied using three bacteria and three fungi grown on nutrient agar; the inhibitory effect of the bluish liquid on their growth was compared with that of standard antibacterial (gentamicin) and antifungal (clotrimazole) drugs. The studies were carried out using the two known varieties of *A. marginata* (suturalis and ovum) in order to establish any variation in their effectiveness. Anti-bacterial or anti-fungal property was not exhibited by the bluish liquid, but a stronger (than the reference) coagulatory effect which was also effective on hemophilic blood was revealed in the bluish liquid from the two snail varieties. *A. marginata* ovum showed higher potency over the suturalis variety. Elemental analysis of the bluish liquid from the two snail varieties carried out showed three elements (calcium, magnesium and zinc) in relatively large amount when compared with other detected elements. The result on the prothrombin time justified the use of the snail bluish liquid as a strong blood coagulant while the elemental results supported the observed higher potency of the ovum snail variety over the suturalis variety. The result also suggests that the hemophiliacs can benefit from this liquid.

**Key words:** Male circumcision, Western Nigeria, blood clotting, *Achatina marginata* (African snail), prothrombin time, hemophiliacs.

**INTRODUCTION**

Male circumcision originally was notably and mostly known to be a religious ritual practiced among the Jews but has widely spread in conception and spanned over three millennia (Rubin, 2003). Circumcision as defined by the University of Michigan Health system in 2007 is the surgical removal of some or the entire foreskin (prepuce) from the penis. Religious male circumcision is considered a commandment from God in Judaism as recorded in the Holy Bible, and it is customary in many Christian churches in Africa; most Muslims also practice and see it as sunnah (Rizvi et al., 1999) hence it is of general...
acceptance, especially in the Middle East, North and West Africa (Weiss et al., 2007).

Medically, many reports have advocated it as a good preventive measure for a number of diseases like urinary-tract infection, sexually transmitted diseases, penile cancer, prostate cancer, cervical cancer and acquired immunodeficiency syndrome (AIDS) (Weiss et al., 2007; Nigerian Punch, 2011). Throughout the world, human immunodeficiency virus (HIV) prevalence is reported to be generally lower in populations that practice male circumcision than in populations where most men are uncircumcised. Many trials have been reported that brought out the conclusion that male circumcision is an effective risk-reduction measure for men, and should be used in addition to other known strategies for the prevention of heterosexually acquired HIV infection in men (Joint UN programme on HIV/AIDS, 2008).

Traditionally in Western Nigeria, circumcision is usually performed at a very tender age (usually 8th or 10th day after birth) and very early in the morning by the local traditional birth attendant (TBA) who is usually a female. The bluish liquid from the giant African snail Achatina marginata is one of the materials required for this feat. Immediately the foreskin is surgically removed, the shell of the life snail is washed, broken at the tip end and the clear blue liquid which exudes is allowed to flow directly on the cut surface. No further dressing or medication is applied on this day; but on the second day either methylene blue dye or a preparation of some herbs in palm oil is applied on the wound with the aid of a feather; this is done daily after the child’s bath. The sore heals within one to two weeks. The question to address is, what is the role being played by the snail bluish liquid in this surgery, is it coagulatory or anti-bacterial/antifungal?

Snails are the largest group of molluscs which constitutes the largest animal group after arthropods (Glick, 2005). Land snail’s habitats in Nigeria range from the dense tropical high forest in the north to the fringing riparian forests of the derived Guinea Savanna in the middle belt of the country (Yoloye, 1984). The snail meat have been established as a good source of protein (Ajayi et al., 1980) and they are relished delicacies among the Calabars, Isekeris, Yorubas and many other coastal tribes of West Africa (Ajayi et al., 1980; Brender, 1992) who now breed them commercially to meet demand (Imevbore and Ademosun, 1988; Odaibo, 1997).

The giant African snail A. marginata exists in two different varieties, ovum and suturalis (Ajayi, 1980); both varieties were used in these studies in order to determine which of the two gives the better result; though the ovum variety is more commonly encountered and used (Odaibo, 1997; Ademolu et al., 2004).

Coagulation is a complex process by which blood forms clots to stop bleeding and begin repair of any damaged vessel. It is an important part of hemostasis wherein a damaged blood vessel wall is covered by a platelet and fibrin-containing clot (Dahl, 2000). Coagulation involves a cellular (platelet) and a protein (coagulation factor) component. It begins almost instantly after an injury to the blood vessel has damaged the endothelium; exposure of the blood to proteins such as tissue factor initiates changes to blood platelets which immediately form a plug at the site of injury; this is called primary hemostasis. Secondary hemostasis occurs simultaneously.

Proteins in the blood plasma, called coagulation factors or clotting factors, respond in a complex cascade to form fibrin strands which strengthen the platelet plug. One of the tests commonly employed in the determination of blood clot is the prothrombin time (Dacie and Lewis, 2001).

Prothrombin time (PT) is a blood test that measures how long it takes a blood clot to form and it is an important coagulatory test because it measures the presence and activity of five different blood clotting factors (Factors I, II, V, VII and X). It is a test of extrinsic and common pathway, and the normal PT is about 12 seconds (Fritsma, 2002).

These studies were designed to determine the effect of the snail liquid on some blood plasma samples clotting time and compare with that of a standard coagulator (thromboplastin). Likewise, the bacterial and fungal inhibitory effect of the blue liquid was studied using three bacteria and three fungi and compared with the inhibitory effect of standard antibacterial (gentamicin) and antifungal ( clotrimazole) drugs. The studies further investigated the elemental composition of the blue liquid from the two snail varieties in order to explain variation in the observed clotting time, as some elements, calcium in particular, are essential for the clotting process. The atomic absorption spectrometer which is commonly used for elemental analysis of metals in solution (Ademolu et al., 2004; Fagburo et al., 2006; Adepoju-Bello et al., 2009; Momodu and Anyakora, 2010) was used for the analysis of the metal ions after proper digestion with aqua regia to get rid of organic impurities. A similar work carried out on snail reported solely on the elemental composition of the meat of four species of snail which included the two under study (Ademolu et al., 2004; Fagburo et al., 2006) but report on this bluish liquid was lacking.

MATERIALS AND METHODS

Materials used in the studies include ten snails, A. marginata (five of ovum and five of suturalis varieties); spectrophotometer (Perkin Elmer Analyst 200) acetylene gas, calcified tissue thromboplastin (Fisher Diagnostic Ltd, U.K), calcium chloride (Mayer & Baker PLC), saboraund dextrose agar (SDA), Mueller Hilton agar (MHA) (Biotech, U.K), gentamicin, clotrimazole (Drugfield), Aqua regia (concentrated nitric and hydrochloric acids 3:1), sulphate salts of the elements under evaluation (Zn, Cu, Fe, Ca, Pb) (May & Baker PLC). Plasma from appropriate patients (three patients on warfarin and one hemophilic) were obtained from Hematology Outpatient Clinic of Lagos University Teaching Hospital. Normal (control) plasma was obtained from a healthy laboratory staff.
EXPERIMENTALS

Antibacterial/antifungal screening

Three universal bottles each containing 20 ml Mueller Hilton agar were autoclaved at 121°C for 15 min and inoculated with 1 ml standardized suspension of Escherichia coli, Staphylococcus aureus and Bacillus subtilis, respectively after cooling to 40°C. Three bottles of Saboraund dextrose agar were similarly prepared and inoculated with 1 ml standard suspension of Aspergillus niger, Candida albican and Trichophyton rubrum, respectively. Each of the inoculated agar was poured into three petri dishes, (two for test liquid that is, the bluish liquid from A. marginata ovum and A. marginata suturalis and the other for the control that is, the standard gentamicin or clotrimazole), allowed to set and four equal sized wells were bored with a Durham tube in each. Four different concentrations of the snail bluish liquid (100, 50, 25 and 12.5%) from the two snail varieties were filled into the wells of the two petri-dishes, respectively while four different concentrations (180, 80, 40 and 20) of the control (gentamicin) were filled into the wells of the other petri-dish of each bacterium containing agar with corresponding labels for identification as shown in the table of results. Incubation was done at 37°C for 24 h, after which the plates were inspected for zones of inhibition. The same procedures were carried out for the anti-fungal screening using 800, 400, 2000 and 100 µg of clotrimazole as control and incubating at room temperature.

Preparation of blood plasma

Nine milliliters (9 ml) of blood obtained from the patient’s antecubital fossa of the arm through a clean puncture at the vein was delivered into a 15 ml tube containing one ml of 0.1 M trisodium citrate. The content of the tube was properly mixed and then centrifuged at 40,000 rpm for 15 min. The supernatant plasma was gently removed using a pasteur pipette and used immediately.

One-stage prothrombin time

A one stage prothrombin time test was carried out using calcified tissue thromboplastin on both test and control plasma. Another series of the test were carried out where the snail bluish liquid was substituted for the calcified tissue thromboplastin. The stop watch was immediately set after the expiration of 10 seconds in the presence of calcium ion, this splits the phospholipids released from the platelets to form the phospholipid complex which contains an importaant proteolytic enzyme.

Elemental composition

Each sample (5 ml) of the two snails’ water was digested separately in equal volume of aqua regia in order to get rid of all organic impurities and was then prepared into standard solutions using distilled de-ionized water. Standard solutions of each elemental salt (in three different concentrations (2, 4 and 6 ppm)) were prepared and their various absorbances were determined using the atomic absorption spectrometer (Perkin Elmer Analyst 200) in order to plot a calibration curve for each element to be quantified. The absorbance of the prepared standard snail waters was also taken for each element, absorbance readings were taken in triplicates in order to get an average value. The concentration of each element was then obtained from the absorbance regression on each calibration curve.

RESULTS AND DISCUSSION

The bluish water from the two snail varieties lacks any anti-microbial/anti-fungi activity as no inhibition was observed even in the growth of media containing the highest concentration (100%) of the snail’s liquid (Table 1). Prothrombin result shows prominent clotting ability which is more potent than that of calcified thromboplastin that was used as reference. The time taken by the snail’s water to clot serum from all the tested patients was shorter than the time taken by the calcified thromboplastin, with the liquid of the ovum variety showing higher potency. While the control could not clot hemophilic blood after 10 min, the bluish water from both varieties of the snail was able to do this before the expiration of 10 min, with ovum and suturalis taking 6.4 and 7.9 minutes, respectively.

Apart from the fact that the ovum variety is more commonly distributed in Nigeria, this result supports its choice as the preferred variety at the circumcision table. The elemental analysis result also corroborate this; for all the elements analyzed with the exception of manganese, the liquid from the ovum variety recorded higher concentrations which in some cases (Magnesium and Calcium) actually doubled the value obtained for the suturalis variety. This finding differs from the report of Fagbura et al. (2006) on the meat in which only small variations existed in the mineral content of the two varieties. The extrinsic mechanism that initiates the formation of prothrombin activator begins with the traumatized vascular wall or extra vascular tissues and progresses stepwise in the presence of calcium as shown (Dacie and Lewis, 2001).

Firstly, the traumatized tissue releases a complex of several factors called tissue factor or tissue thromboplastin which is composed of phospholipids from the tissue membranes and a lipoprotein complex which contain an important proteolytic enzyme.

Secondly, the lipoprotein complex of the tissue factor complexes with blood coagulation Factor VII and in the presence of calcium ions, acts enzymatically on Factor X to form activated Factor X designated as Factor Xa. Factor Xa combines immediately with Factor V as well as the phospholipids released from the platelets to form the complex called prothrombin activator and within few seconds in the presence of calcium ion, this splits prothrombin to form thrombin. Thrombin is a proteolytic enzyme which acts on fibrinogen to form the fibrin monomers which polymerize each other to form the long fibrin fibers that form the reticulum of the clot.

The essential role of calcium in the processes of blood clotting has long been established (Dacie and Lewis,
Table 1. Anti-microbial/anti-fungi: Zone of Inhibition (mm) at stated concentrations.

<table>
<thead>
<tr>
<th>Drug (µg)</th>
<th>Test organisms</th>
<th>Drug (µg)</th>
<th>Test organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gentamicin</td>
<td>Clotrimazole</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. subtilis</td>
<td>A. niger</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>19.0</td>
<td>100</td>
<td>14.0</td>
</tr>
<tr>
<td>40</td>
<td>20.0</td>
<td>200</td>
<td>15.0</td>
</tr>
<tr>
<td>80</td>
<td>24.0</td>
<td>400</td>
<td>17.0</td>
</tr>
<tr>
<td>160</td>
<td>26.0</td>
<td>800</td>
<td>20.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drug (µg)</th>
<th>Test organisms</th>
<th>Drug (µg)</th>
<th>Test organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>C. albicans</td>
<td>T. rubrum</td>
</tr>
<tr>
<td>20</td>
<td>20.0</td>
<td>16.0</td>
<td>24.0</td>
</tr>
<tr>
<td>40</td>
<td>21.5</td>
<td>20.0</td>
<td>25.0</td>
</tr>
<tr>
<td>80</td>
<td>22.5</td>
<td>24.0</td>
<td>27.0</td>
</tr>
<tr>
<td>160</td>
<td>25.5</td>
<td>27.0</td>
<td>30.0</td>
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A. marginata (suturalis) %

<table>
<thead>
<tr>
<th>%</th>
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<tbody>
<tr>
<td>12.5</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>No activity</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
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</tr>
</tbody>
</table>

A. marginata (ovum) %

<table>
<thead>
<tr>
<th>%</th>
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</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>12.5</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>No activity</td>
<td>25</td>
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<td>50</td>
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<tr>
<td>100</td>
<td>100</td>
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</table>

2001; Heneghan et al., 2006), and that the bluish liquid of the African giant snail contains it in high amount can explain why its application immediately on the exposed penis after the foreskin is removed during circumcision is imperative. It facilitates clotting and prevents excessive bleeding. The application of methylene blue or/and other oil dispersed herbs from the second day of the operation on the other hand, suggests awareness that the bluish liquid from the snail cannot prevent microbial infestation; hence these were probably used to act as anti-bacterial agents. However, already studied plants with antibacteria activity like Cymbopogon flexuosus (lemongrass) and C. nardus (citronella) (Innsan et al., 2011) could be used in place of the methylene blue at least to avoid the mess of its stain.

Apart from calcium, magnesium and zinc elements were also found in high amount (Table 3) in the bluish liquid from the snail which clotted the hemophilic blood that the calcified thromboplastin (control) could not do (Table 2). Magnesium and zinc are cofactors in the synthesis of some proteins (Rutin, 1975; Narayan et al., 1997), and bearing in mind that the various clotting factors are proteineous, one may then reason that these other two elements may have an additional role to play alongside calcium in the clotting of blood, particularly in the hemophiliacs. In countries such as East and South Africa where circumcision is being advocated for AIDS control and performed at puberty, report of excessive bleeding has been given as a major setback (Nigerian Punch, 2011); use of this bluish liquid from the African giant snail (the ovum variety) may provide a succor.

Conclusion

The result on the prothrombin time justified the use of the snail bluish liquid as a strong blood coagulant, useful in preventing excessive blood loss during and after surgery while the elemental results supported the observed higher potency of the ovum snail variety over the suturalis variety. It further suggests that the hemophiliacs can benefit from this liquid. Further studies on this snail liquid...
Table 2. Prothrombin time.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thromboplastin (reference)</th>
<th>Bluish liquid of <em>A. marginata</em> (suturalis)</th>
<th>Bluish liquid of <em>A. marginata</em> (ovum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood plasma sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (control)</td>
<td>11.6±0.3</td>
<td>8.5±2.1</td>
<td>8.0±1.4</td>
</tr>
<tr>
<td>Patient 1 (on warfarin)</td>
<td>34.4±0.2</td>
<td>16.0±1.4</td>
<td>13.5±2.1</td>
</tr>
<tr>
<td>Patient 2 (on warfarin)</td>
<td>37.5±0.3</td>
<td>17.2±1.4</td>
<td>15.5±2.1</td>
</tr>
<tr>
<td>Patient 3 (on warfarin)</td>
<td>23.0±0.1</td>
<td>15.5±0.7</td>
<td>13.0±0.2</td>
</tr>
<tr>
<td>Hemophiliac patient</td>
<td>No clotting after 10 min</td>
<td>415.0±4.7 (7.9 min)</td>
<td>385.0±2.8 (6.4 min)</td>
</tr>
</tbody>
</table>

Table 3. Elemental composition.

<table>
<thead>
<tr>
<th>Element</th>
<th><em>A. marginata</em> (suturalis)</th>
<th><em>A. marginata</em> (ovum)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean concentration (mg/L)</td>
<td>Mean concentration (mg/L)</td>
</tr>
<tr>
<td></td>
<td>± standard deviation</td>
<td>± standard deviation</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>1.1176±0.0021</td>
<td>1.5294±0.0032</td>
</tr>
<tr>
<td>Iron (Fe)</td>
<td>2.3989±0.0054</td>
<td>2.5500±0.0108</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>0.5870±0.0117</td>
<td>0.6114±0.0024</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
<td>25.736±0.0328</td>
<td>54.9717±3.3190</td>
</tr>
<tr>
<td>Manganese (Mn)</td>
<td>4.2541±0.0361</td>
<td>2.7403±0.0052</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
<td>22.450±0.073</td>
<td>47.2487±2.6510</td>
</tr>
<tr>
<td>Potassium (K)</td>
<td>4.3214±0.0106</td>
<td>7.7381±0.0946</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>26.326±0.463</td>
<td>35.0233±1.7940</td>
</tr>
</tbody>
</table>

are recommended in order to establish the factor(s) inherent in it that support the clotting of hemophiliac blood.

ACKNOWLEDGEMENT

The assistance of Prof. Egbonmwan R.I. of Zoology Department, University of Lagos is acknowledged in the identification of the snail variety; Dr. Adeniran A. of Haematology Department, College of Medicine, University of Lagos, through whom the patient’s blood were obtained and Mr. Usman AR of Pharmaceutics Department, University of Lagos for technical assistance.

REFERENCES


Full Length Research Paper

Effects of *Juniperus phoenicea* extract on uricemia and activity of antioxidant enzymes in liver, erythrocyte and testis of hyperuricemic (oxonate-treated) rats

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The aim of the present study was to determine whether administration of *Juniperus phoenicea* extract would have any advantage over allopurinol therapy on (1) lipids peroxidation level and (2) antioxidant activity in liver, erythrocyte and testis of hyperuricemic rats by oxonate administration. In hyperuricemic rats, levels of lipids peroxidation in liver, erythrocyte and testis were found to be significantly increased as compared to control rats (\(p < 0.05\)). Activities of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT) were also significantly increased as compared to the controls (\(p < 0.05\)). An ameliorative effect was obtained in hyperuricemic rats by oral treatment with either allopurinol (10 mg/kg body weight (bw)) or *J. phoenicea* extract (100 or 200 mg/kg bw). The 100 mg/kg dose revealed to be efficient in reducing uric acid level in blood and the 200 mg/kg dose strongly reduced the activities of SOD, CAT and GPX in liver and erythrocyte. Our results support that the consumption of *J. phoenicea* extract should be recommended in ethnomedicinal practice to reduce the risk of gout by decreasing the uric acid level in blood and to confer some protection against oxidative stresses at organs level.

**Key words:** Oxidative stress, oxonate, uric acid, allopurinol, *Juniperus phoenicea*, rat.

**INTRODUCTION**

Hyperuricemia, that is, high level of uric acid in blood, is present in 5 to 30% of the general population and seems to be increasing worldwide. Besides being considered the major risk factor for gout (Bieber and Terkeltaub, 2004), it is associated with the development of other disorders such as cardiovascular diseases, hyperglycemia/diabetes mellitus, inveeterate alcoholism, renal failure, obesity, dyslipidemia and increased mortality (Vazquez-Mellado et al., 2004). Therefore, there is an obvious need for novel agents or therapeutic strategies that could act on the physiological regulation of uric acid levels and prevention of uric acid-related diseases. *In vitro* and *in vivo* studies show that natural products have good anti-inflammatory effects (Sy et al., 2009; Bedi et al., 2010; Mwale and Masica, 2010). *Juniperus L.* (Cupressaceae) species have been used to cure various inflammatory and infectious diseases in Turkish folk medicine (Akkol et al., 2009). Inflammation is a physiopathological response of living tissues to injuries that lead to the local accumulation of plasmatic fluid and white blood cells. Although it is a defence mechanism, the complex events and mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases. Therefore, the use of anti-inflammatory agents is helpful in the therapeutic treatment of these pathologies.

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In the traditional medicines worldwide, several Juniperus species are used as remedies against common cold, urinary infections, nettle-rash (urticaria), dysentery, hemorrhages, leucorrhea and rheumatic arthritis, to regulate menstruation and to relieve menstrual pain (Seca and Silva, 2007). For instance, Juniperus drupacea fruits are used to treat helmithes infections, stomach aches and hemorrhoids (Yesilada et al., 1993), decoction of fresh shoots is used for urinary inflammations, gout and to treat abdominal pain and the tar of this species is used against diarrhea (Yesilada et al., 1993). Fruits of another species, Juniperus communis, are swallowed like a pill against cough, to alleviate pain and to cure hemorrhoids, whereas tar of this species is used externally against scabies and heat rash (Fujita et al., 1995). The boiled fruit of Juniperus oxycedrus is widely used in the treatment of gastrointestinal disorders, common colds, as expectorant in cough, to treat calcinosis in joints, as diuretic to pass kidney stones, against urinary inflammations, hemorrhoids, and as antidiabetic (Yesilada et al., 1993; Abdou et al., 2011), while the resin was used for wound healing (Yesilada et al., 1993).

On the other hand, Juniperus phoenicea L. leaves were found to contain active components due to the anti-proliferative activity they show against a broad range of human tumors (Rizk et al., 2007) and antioxidant properties due to its content of flavonoid and phenolic compounds (Ibrahim and Risk, 2005). In spite of such a wide use of Juniperus species against pain and inflammatory conditions of various origins, it was discovered that only few studies have evaluated their anti-inflammatory and antinociceptive potentials in a reference survey (Moreno et al., 1998). Quite often, the real efficacy and/or the relevant active principles of many plants used in folk medicine remain unknown. Therefore, the aim of the present study was to find a scientific basis that could support the use of J. phoenicea in medicine. In this study, effects of J. phoenicea extract were compared to those of allopurinol, a standard non-steroidal anti-inflammatory drug, administered by oxonate in rats induced to be hyperuricemic.

**MATERIALS AND METHODS**

Leaves of J. phoenicea L. (Cupressaceae) were used in this study. Leaves were collected in Mars, 2009, in the region of Sfax, Tunisia. The plant was identified by (Chaiheb and Bokhri, 1998) botanists in the University of Science (Sfax, Tunisia). The vouchers specimen were deposited at the herbarium of the department of botany in the cited institute.

**Preparation of extracts**

Plant material, either 1 or 2 g in order to test two concentrations (see below), was boiled for 10 min in 200 ml of distilled water in an Erlenmeyer flask. This procedure was repeated twice and aqueous extracts were mixed and kept frozen, after which they were orally administered in the drinking water, and were given with J. phoenicea extract (equivalent to 100 or 200 mg fresh leaves/kg body weight (bw), respectively).

**Animals and experimental design**

Male Wistar rats (250 to 300 g) were used in this study. The animals were purchased from the Central Pharmacy of Tunisia (SIPHAT, Tunisia) and were housed in plastic cages and fed on standard chow pellets. They were given water ad libitum. All animals were maintained on a 12 h light/12 h dark cycle, at a constant temperature of 25°C. All procedures were in strict accordance with the sciences faculty legislation on the use and care of laboratory animals and with the guidelines established by Institute for Experimental Animals of Sfax University, being approved by the university committee for animal experiments. Experimental hyperuricemia was induced in rats by intraperitoneal (i.p) injections of the uricase inhibitor potassium oxonate (300 mg/kg bw) as proposed by Liu et al. (2008) (oxonic acid = 1,4,5,6-tetrahydro-4,6-dioxo-1,3,5-triazine-2-carboxylic acid). Animals were divided in five groups. Group one, the controls, were sham-injected with saline solution. Group 2 (OxoT) and the remaining 3 groups were i.p. injected with potassium oxonate (300 mg/kg bw) one hour before administering either the J. phoenicea extract or allopurinol. Allopurinol, an analog of hypoxanthine, is a common remedy to treat hyperuricemia (Liu et al., 2008) which was used in this study for comparison with the effect of Juniperus extract. This procedure was carried out each day for 7 consecutive days. Orally administered in the drinking water, group 3 (OxoT + allopurinol) was given allopurinol (10 mg/kg/bw) and groups 4 and 5 (OxoT + J1 and OxoT + J2, respectively) were given J. phoenicea extract (equivalent to 100 or 200 mg fresh leaves/kg bw, respectively).

**Sample preparation**

**Serum preparation**

At the end of experiments, animals were rapidly sacrificed by decapitation and blood samples were collected. Blood samples collected in centrifuge tubes were centrifuged at 3000 rpm for 20 min. Serum was stored at -20°C until used for biochemical assays. Level of uric acid in serum was measured by the uricase colorimetric test (Biochromehre kit, Tunisia, ref: 20991).

**Tissue preparation**

Liver and testis were excised, frozen and stored at -80°C until use. Tissue samples were homogenized in 50 mM sodium pyrophosphate buffer (pH 7.4). The homogenate was then centrifuged at 3000 × g for 15 min at 4°C, and aliquots of supernatant were kept at -30°C until used for assays.

**Assessment of oxidative stress markers**

Level of lipids peroxidation in tissues was measured as the amount of thiobarbituric acid reactive substances (TBARS) according to Yagi (1976). 125 µl of supernatant were mixed with 125 µl of trichloroacetic acid (TCA) in order to discard proteins and after centrifugation (1000 × g, 10 min, 4°C), 200 µl of the new supernatant were mixed with 40 µl HCl (0.6 M) and 160 µl of thiobarbituric acid (TBA) 20% in TBS. The mixture was heated at 80°C for 10 min and, after cooling, the absorbance was read at 530 nm. The amount of TBARS was calculated by using an extinction coefficient of 1.56×10^5 M⁻¹ cm⁻¹. Catalase (CAT) activity was measured according to Aebi (1984). 20 µl tissue homogenate (about 1.5 mg proteins) were added to 1 ml phosphate buffer (0.1
M, pH 7) containing 100 mM H₂O₂. Rate of H₂O₂ decomposition was followed by measuring the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated using an extinction coefficient of 0.043 mM⁻¹ cm⁻¹ and expressed in international units (I.U.), that is, in μmoles H₂O₂ destroyed/min/mg protein, at 25°C. Superoxide-dismutase (SOD) activity was assayed by measuring its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) (Beyer and Fridovich, 1987). In this assay, one unit of SOD is defined as the amount required to inhibiting the photoreduction of NBT by 50%. Riboflavin (0.26 mM final concentration) was added to start the reaction and absorbance was recorded at 560 nm for 20 min. The activity was expressed as units/mg protein, at 25°C. Glutathione peroxidase (GPX) activity was measured as previously described (Flohe and Gunzler, 1984). Change in absorbance at 340 nm was monitored for 5 min. A blank control with all the ingredients except the sample was also monitored. The specific activity was expressed as μmoles NADPH consumed per minute per mg protein (that is, U/mg protein).

Protein content was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

**Histopathological studies**

For histological studies, the Bouin-fixed tissue was dehydrated in graded alcohol and embedded in paraffin. Thin sections (6 μm) were stained with routine haematoxylin-eosin solution and examined classically with a photonic microscope to determine histopathological lesions.

**Statistical analysis**

Results are reported as mean ± SEM for at least 6 determinations throughout the study. Results were analysed by one-way analysis of variance (ANOVA) followed by the Duncan’s multiple range test. The software SPSS 11.0 for Windows was used for statistical evaluation. P < 0.05 was accepted as significantly different.

**RESULTS**

**Effect on serum level of uric acid**

As shown in Table 1, oxonate treatment resulted in a hyperuricemia (329 versus 240 μmol/L in controls). Treatment with aqueous extract of *J. phoenicea* reduced the serum uric acid level of oxonate-treated animals to 122 μmol/L (group J1) and 267 μmol/L (group J2), whereas allopurinol treatment decreased the uric acid level to 185 μmol/L. Interestingly, the hypouricemic effect was more pronounced in J1 group than in J2 group. Somewhat similar results were observed about uric acid levels in hepatic tissue. Uric acid concentrations were 680 μmol/g in oxonate-treated rats versus 355 μmol/g in controls.

The values were reduced to 494 and 219 μmol/g in J1 and allopurinol groups, respectively. Surprisingly, no such lowering effect was obtained in the J2 group (710 μmol/g).

In order to analyze the relevance of uricemia reduction, level of lipids peroxidation and activities of main antioxidant enzymes were measured in liver, erythrocyte and testis. The results are reported in Figures 1, 2 and 3. In hyperuricemic (oxonate-treated) rats, the TBARS content in the liver was found to be significantly increased as compared to controls (p < 0.05). The SOD, CAT, and GPX activities in the liver of hyperuricemic rats were significantly increased as compared to normal rats (p < 0.05). In hyperuricemic rats treated with allopurinol or with the *J. phoenicea* extracts (100 or 200 mg/kg bw), an ameliorative effect was observed. *J. phoenicea* (200 mg/kg bw) extract induced a more pronounced decrease of TBARS level, and SOD, CAT, and GPX activities in liver as compared to control rats, hyperuricemic rats and allopurinol-treated rats (p < 0.05). In oxonate-treated rats, the TBARS content in erythrocyte is significantly increased, compared to controls (p < 0.05). Activities of SOD, CAT, and GPX are also increased in these cells as compared to controls (p < 0.05). Values of all these parameters were reduced toward normality by treatment with either allopurinol or *J. phoenicea* extracts, especially when administering the J2 dose. In testis of induced hyperuricemic rats, both TBARS content and SOD, CAT, and GPX activities were significantly increased compared to controls (p < 0.05). Treatment with allopurinol exerts some ameliorative effect. However, treatment with *J. phoenicea* extracts fails to produce any significant effect, suggesting that the active substances do not reach this organ.

**Liver and testicular histopathology**

**Liver histology**

Free radical formation during the metabolism of oxonate by hepatic microsome, cause lipid peroxidation of the cellular membrane leading to the necrosis of hepatocytes. Rats treated with oxonate developed significant hepatic damage as compared to controls (Figure 4a and b). An ameliorative effect was obtained in hyperuricemic rats by oral treatment with either allopurinol (Figure 4c) or *J. phoenicea* extract (Figure 4d and e).

**Testis histology**

Histopathology of testis of control group showed no marked changes. Micro thin sections from these three groups indicated the normal cycle of spermatogenesis. Seminiferous tubules had well preserved sertoli cells and well delineated tubular basement membrane (Figure 5a). The interstitium between tubules and Leydig cells were also intact in these groups. However, in the oxonate-treated groups (Figure 5b), differences were observed in the histology of the testis. Although the tubular basement
membranes of seminiferous tubules were identified in some areas, tubules could exhibit focal or diffuse intermediate necrosis. Treatment of *J. phoenicea* extracts ameliorated the toxic effects of the oxonate, in a dose dependent manner (Figure 5d and e), in particular, in the group treated with low dose of *J. phoenicea* (100 mg/kg bw) (Figure 5d).

**DISCUSSION**

Today, millions of people around the world use medicinal plants as part of traditional medicine for a large range of medical disorders (Namukobe et al., 2011). The use of traditional medicine in developing countries contributes directly to the socio-economic status and benefit of the rural communities (Chiranjib et al., 2006). People, especially herbalists and traditional healers, generate income from medicinal plants. Uganda is one of the developing countries where about 80% of the population depends on herbal medicine for treating various diseases (Tabuti et al., 2003). It is reasonable to consider that a survey of ethnomedical uses of a plant may provide useful clues for drug discovery. In such ethnomedical research, one must identify a clear preferential use of a particular plant and its objective effect upon a specific disease or symptom (Lansky et al., 2008). The disorder of uric acid metabolism in gouty patients is in part attributed to an oxidative stress due to several factors. An over-production of free radicals may exert deleterious effects

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum uric acid (µmol/l)</th>
<th>Liver uric acid (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>240.49±17.68</td>
<td>355.50±18.97</td>
</tr>
<tr>
<td>OxoT</td>
<td>329.16±36.35*</td>
<td>680.17±44.93*</td>
</tr>
<tr>
<td>OxoT+ allopurinol</td>
<td>185.40±17.25**</td>
<td>219.05±38.48**</td>
</tr>
<tr>
<td>OxoT + J1</td>
<td>122.64±15.83***</td>
<td>494.36 ±19.90***</td>
</tr>
<tr>
<td>OxoT+J2</td>
<td>267.94±27.28***</td>
<td>710.50±44.47***</td>
</tr>
</tbody>
</table>

J1 = 100 mg/kg bw; J2 = 200 mg/kg bw. Values are given as mean ± SEM for group of 12 animals each; values are statistically *p < 0.05 for control rats, **p<0.05 for gouty rats, ***p < 0.05 for allopurinol-treated rats.

**Table 1.** Uric acid levels in serum and hepatic tissue after 1 week in oxonate-treated (OxoT) rats given allopurinol or *Juniperus* extracts.

**Figure 1.** Effect of *Juniperus phoenicea* extract (100 or 200 mg/kg bw) and allopurinol (10 mg/kg bw) on TBARS level and activities of SOD, CAT and GPX in liver of oxonate-treated (hyperuricemic) rats after 1 week treatment. Values are the mean ± SEM for group of 6 animals each. Statistically significant: *p < 0.05 as compared to controls; **p < 0.05 as compared to hyperuricemic rats; ***p < 0.05 as compared to allopurinol-treated rats.
on liver, leading to different purines metabolism in this organ. Under our experimental conditions, the oxonate-induced hyperuricemia is accompanied by an oxidative aggression as evidenced by the significant increase in hepatic lipids peroxidation levels and a significant increase of SOD, CAT, and GPX activities in liver.

In order to better evaluate the effects of Juniperus extract, allopurinol, a classical drug for treatment of gout exerting a well documented hypouricemic activity, was used for comparison. Administration of J. phoenicea in hyperuricemic rats appears to be very efficient in reducing uricemia. This action could be consequence of an increased expression and/or activity of antioxidant enzymes; or inactivation of the circulating free radicals and reactive oxygen species. The reactive oxygen species produced by mitochondria and during inflammation process are considered as responsible for many pathological aspects in gouty patients. These reactive oxygen species are present in cells under physiological conditions, producing toxic effects when their production rate increases and exceeds the antioxidant defence capacity of the cells (Glantzounis et al., 2005). Thus, oxidative stress generally means a disturbance in the prooxidant-antioxidant balance in favour of the former. The protection system to prevent and repair the oxidative damage includes enzymes such as superoxide dismutase and glutathione peroxidase, as well as antioxidants and radical scavengers such as vitamin E and the β-carotenes in the lipid portion of the cells, and glutathione, ascorbic acid and uric acid in the aqueous phase (Watanabe et al., 2002). Globally, the beneficial effect of Juniperus extracts could be in large part attributed to the presence of flavonoids and other anti-oxidative molecules.

It is also interesting to underscore the dual property of uric acid. Being a powerful radical scavenger as well as being able to act as chelator of metal ions, such as iron and copper, by converting them to poorly reactive forms unable to catalyse free-radical reactions, uric acid is one of the most important endogenous antioxidants in human biological fluids (Glantzounis et al., 2005). Although plasma uric acid is not as effective as plasma ascorbate in preventing the initiation of lipid peroxidation, it does lower the rate at which lipid peroxidation occurs (Frei et al., 1989). Roughly, it is thought that uric acid contributes to more than 50% of the antioxidant capacity of blood (Glantzounis et al., 2005; Parmar, 2009). The increase in blood uric acid concentration could have enabled the hominids to maintain blood osmotic pressure in times of

*Figure 2. Effect of Juniperus phoenicea extract (100 or 200 mg/kg bw) and allopurinol (10 mg/kg bw) on TBARS level and activities of SOD, CAT and GPX in erythrocyte of oxonate-treated (hyperuricemic) rats after 1 week treatment. Values are the mean ± SEM for group of 6 animals each. Statistically significant: *p < 0.05 as compared to controls; @p < 0.05 as compared to hyperuricemic rats; +p < 0.05 as compared to allopurinol-treated rats.*
low salt ingestion and it has been suggested that this increase in blood pressure from the increase in uric acid could be essential for hominids to maintain their vertical position (Parmar, 2009).

Increased uric acid may be a compensatory mechanism trying to counteract oxidative stress (Ames et al., 1981). After showing that uric acid is an effective antioxidant, Ames et al. (1981) hypothesized that uric acid may be an evolutionary antioxidant substitute for the loss of ability to synthesize ascorbic acid in higher primates. In humans, uric acid exists in blood at concentration close to maximum solubility. Same authors explained that these high levels of uric acid may be the result of the evolution of effective protective mechanisms against oxygen radicals and that this may partly explain the marked increase in life-span and the decrease in cancer rates in the evolution from prosimians to modern humans.

However, uric acid is one of the potential uremic toxins. It is a potent stimulator of the pathological apoptosis in mononuclear cells. It activates the two most important apoptotic pathways while its pro-inflammatory action might represent a further mechanism (Bordoni et al., 2005). Questioning the multifaceted relationship between uric acid and oxidative stress generates a series of directly testable hypotheses with significant implications. One hypothesis is that purines catabolism can be modulated to augment mitochondrial defence in times of oxidative stress, such as in gouty patients. A related hypothesis is that impairing purines catabolism would compromise mitochondria functions when exposed to oxidative stress. Testing these hypotheses will require further investigations. Our study demonstrated a potential and beneficial effect of Juniperus phoenicea in attenuating oxidative stress and enhancing the body’s own antioxidant defences in oxonate-treated rats. As afore presented, most of the measured parameters were restored to values comparable to those of controls. Previous studies have shown that different types of chemical constituents were found in the various parts of Juniperus species. These were mainly flavonoids, coumarins, lignans, sterols (Seca and Silva, 2007), terpenoids (Seca et al., 2008), polysaccharides (Schepetkin et al., 2005) and other aliphatic or aromatic compounds (Seca and Silva, 2007). Among these compounds, diterpenoids such as honokiol isolated from J. polycarpos was shown to exert anti-inflammatory activity (El-Sayed, 1998).

Methodical preparation of medicinal remedies involving precise dosage would determine activity and effectiveness.
Figure 4. Microscopic observation of rat liver; (A) Representative section from control depicting the normal structure of lobule and hepatocytes; (B) section from the oxonate showing the centrilobular degeneration and fatty infiltration in hepatocytes; (C) section from the allopurinol (10 mg/kg bw) group showing the near normal architecture of the lobule and hepatocytes; (D and E) Section from the Juniperus phoenicea extract (100 or 200 mg/kg bw) group showing the architecture of the lobule and hepatocytes. Filled circle: normal hepatocyte, open circle: hyper vacuolation of hepatocytes; filled diamond: infiltration of mononuclear cells, open diamond: Inflammation; filled box: kupffer cell, H&E stain (×400); scale bar, 50 µm.

Our findings regarding the in vivo hypouricemic actions of J. phoenicea indicate that, besides anti-inflammatory agents, xanthine-dehydrogenase and xanthine-oxidase inhibitors can be a viable natural drug included in a cocktail therapeutic approach. Although the mechanism of action of the hypouricemic effect of Juniperus extract is not fully understood, it has the potential to be a viable substitute for allopurinol, regarding the many reported side-effects of allopurinol.

Some species of Juniperus are reported to contain toxic substances or to provide toxic secondary metabolites when ingested. It has been reported that all parts of the plant contain poisonous taxine alkaloids, whose toxic effects are maintained during the year (Alden et al., 1977).
However, previous investigations revealed that different parts of some species of Juniperus present cytotoxic effects on some human cancer cell lines (Jafarian et al., 2003). Therefore, an excessive or prolonged use of this plant should be avoided, even if its content in toxic principles is low. Our study demonstrated a potential and beneficial effect of J. phoenicea in attenuating oxidative stress and enhancing the body’s own anti-oxidant defences in oxonate-treated rats. The increase in uric acid concentration in blood favouring role of in the development of metabolic syndrome (Sanchez-Lozada et al., 2008), acting synergistically, are probably responsible for the strong damages appearing in the liver and genital tract. Our histopathological data substantiate liver dysfunction. There are inflammatory leucocytic infiltrations considered, according to Abdel-Rahman and Zaki (1992), as a prominent response of the body tissue facing injurious impacts. The intrahepatic blood vessels, central and portal veins are congested and their lining epithelia are eroded. Nevertheless, rats treated with oxonate developed

Figure 5. Microscopic observation of rat testis; (A) representative section of control group showing normal architecture of the seminiferous tubules; (B) section from the oxonate group showing deterioration of seminiferous tubules; germinal layers, basement membrane is absent and seminiferous tube is infiltrated with the inflammatory cells; (C) section from the allopurinol (10 mg/kg bw) group showing the near normal structure of seminiferous cells; basement membrane and germinal layers are well developed; (D and E) section from the Juniperus phoenicea extract (100 or 200 mg/kg bw) group showing the architecture of seminiferous cells. Filled circle: Germ cells show normal maturation and spermatogenesis is normal, open circle: atrophy of seminiferous tubules, open diamond: anormal spermatogenesis. H&E stain (×100); scale bar, 50 µm.
significant hepatic damage as compared to controls. An ameliorative effect was obtained in hyperuricemic rats by oral treatment with either *Juniperus phoenicea* extract.

**Conclusion**

In conclusion, this study is the first to demonstrate that *Juniperus phoenicea* (100 or 200 mg/kg bw) possess significant antioxidant and anti-uricemic activities in oxonate-treated rats. This corroborates the fact that there are many traditional uses of *Juniperus* extracts in folk medicine. Further studies on this species may yield fruitful results and isolation of some active constituents may lead to the provision of new drugs for treatment of hyperuricemia and gout.

**ACKNOWLEDGEMENTS**

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Full Length Research Paper

Lavender aromatherapy massages in reducing labor pain and duration of labor: A randomized controlled trial

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Labor pain is a challenging issue for midwives and designing intervention protocols. Aromatherapy is one of the non-pharmacological methods for pain relief and Lavandula has analgesic properties. The aim of this study was to investigate the effect of aromatherapy massage with Lavender oil. This was a prospective, randomized, controlled trial that was conducted in 2008 at Mahdeeh Hospital of Tehran University, Tehran, Iran. The subjects included N=60 primiparous women in 38–42 week gestational age, who were expected to have a normal delivery. They were randomly assigned to two groups. The first group received only massage (n=30) and the second group received aromatherapy massage with Lavender oil (n=30). The intensity of pain was measured with the visual analogue scale (VAS). Results showed that pain intensity before and after intervention were significantly lower in the lavender aromatherapy massage group in the latent and active phase, and they had a lower duration of first and second stage of labor. Hence, aromatherapy massage was helpful, providing pain relief and psychological support during labor. This finding suggests that lavender aromatherapy massage is a cost-effective midwifery intervention that can decrease pain and duration of the first and second stages of labor.

Key words: Aromatherapy, complementary alternative methods/therapy, labor pain, lavender oil, massage.

INTRODUCTION

Labor pain is one of the most severe pains that women experience during their life (Lee et al., 2004). It is viewed as a complex physiological phenomenon that encompasses psychological, emotional, spiritual and physical dimensions. This approach represents a shift from the medical model (Adams, 2006). In a study by Bonica et al. (1990), 77% of primiparous reported severe or unbearable pain during labor and they also found that 35% of primiparous women had an unbearable pain, 37% sever pain and 28% had a moderate pain during their labor and delivery experience (Bonica et al., 1990).

In most maternity hospitals, pharmacological and non-pharmacological methods are used to relieve pain. The aim of all these methods is to reduce or mitigate labor pain without any harmful effects on mother and child (Albert et al., 2009). Non-pharmacological treatments are often simple and inexpensive and can be used as replacement or complementary therapy associated with medications treatment. In non-pharmacological methods, pregnant women are the decision-maker, so they feel strengthened and it is effective on their labor progress (Lowdermilk et al., 2004); (Mckinney et al., 2005). Non-pharmacological methods for pain relief include: hypnosis, massage, heat and cold, aromatherapy, percutaneous nerve stimulation, music therapy and changing the mother status during the labor such as walking, breathing control, and accompanied by a person during labor (Behiraee, 1999; Sindhu, 1996).

Aromatherapy is used to relieve pain, anxiety, depression,
insomnia, fatigue, asthma and even create self-confidence, success and creativity (Carroll et al., 1993) and it is applied using acupressure points, taper, compress, footbath and the oils may be massaged in the skin or inhaled by using a steam infusion or burner (Smith et al., 2011). A systematic review evaluated three studies including 117 women, and showed pain reduction after the massage (Fellowes et al., 2004). The use of aromatherapy in labor was also explored in an observational study undertaken in the UK, involving a sample of 8053 participants (Burns et al., 2000). In this study, all consenting women who were in labor were eligible to participate, with the exception of those with multiple allergies or those in premature labor (<36 weeks of gestation). Irrespective of maternal parity or labor onset (spontaneous versus induced), women consistently reported aromatherapy as a helpful adjunct to their labor experience, and there was a lower epidural rate and opioid injection rate in the aromatherapy group (Burns et al., 2007). Essential oils that are used in aromatherapy can be obtained from various portions of plants. The smelling of these oils sends electrochemical messages through the olfactory nerve to the limbic center in the brain and stimulates the release of neurotransmitters from the hypothalamus (Cavanagh et al., 2002; Clarke et al., 1996). The essence of lavender (Lavandula angustifolium) usually is prepared from the root and stem of the plant. Lavender essence is used in aromatherapy and contains linalyl acetate, which is an analgesic (Cunningham et al., 2005). The root of Lavandula has a strong anticonvulsant effect and its leaves and flower have a pain relieving effect (Zargary, 1997).

Despite new methods of strengthening labor contractions, 41.6% of Iranian women deliveries are through cesarean section (IMCH, 2006). Also, drug administration for reduction of labor pain often has some harmful effects on mother and fetus, such as decreased fetal heart rate variability after using of drug (Hill et al., 2003). During 12 weeks after birth, 67% of women with epidural anesthesia and 29% of those who had used other drugs complained of their insufficient breast milk (Volmanen et al., 2004). Also, drugs have adverse effects on labor contractions and cause prolonged labor and delivery complications, followed by some unnecessary manipulation (Carroll et al., 1993). This study was therefore aimed at investigating the effect of massage aromatherapy on intensity of labor pain and on the duration of labor.

MATERIALS AND METHODS

The study was conducted between March 2007 and June 2008 at Mahdeeh Hospital, Tehran, Iran. Inclusion criteria comprised nulliparous and multiparous women, with a singleton pregnancy of gestation age >36 weeks, singleton pregnancy with cephalic presentation, cervical dilatation ≥ 4 cm and having three uterine contractions in 10 min at least with a duration of 30 s. Exclusion criteria included, third trimester bleeding, intrauterine fetal growth retardation, multiple pregnancy, breech presentation, being athletic, addiction (alcohol and cigarettes), using analgesic during 3 h before and during the intervention, the use of sedative drugs, history of infertility, allergy to essences and use of herbal oil.

This was a prospective randomized controlled trial (RCT) with two arms: comparing lavender aromatherapy massage and massage without aromatherapy groups in labor. Sweet almond was provided as carrier oil for massage. Information was gathered in the form of a short questionnaire to elicit maternal feedbacks about receiving and administering aromatherapy. In the labor ward, randomization was accomplished by taking consecutively numbered, sealed, opaque envelopes that contained the allocation to each arm of the trial. Each envelope was identical in appearance and weight. The randomly generated computer sequence (1:1 ratio) was prepared before the trial started by statistician and was only known to him. Thus, allocation concealment was assured to the point of opening the envelope. Blinding was obviously not possible.

After explanation and obtaining written consent of women, they were randomly assigned to two groups: the first group received only massage (n=30), the second group received massage aromatherapy with 2 drops of Lavender oil dissolved in 50 cc almond oil (n=30). The massage was given to all women in a lateral position by one investigator (second author) as midwife, who had been given theoretical and practical training by a physiotherapist before the study and certified by him. Back massage was done during labor as effleurage (friction), gently with medium pushing and rhythmic in two groups in the latent phase (cervix dilated 3 - 4 cm), active phase (5 - 7 cm) and transitional phase (8 - 10 cm) of labor, for 3×20 min (during and between contractions). Aromatherapy was administered for one of the following reasons: to alleviate pain (reduction of level pain intensity) or to augment contractions and as a means of facilitating the mechanism of labor and reducing labor duration, and its effect on type of delivery. Then women were asked to self-rate their level of pain immediately prior to receiving massage in two groups 30 - 40 min afterwards using a 10-point Likert scale, totally in six times. Neonatal outcome data included Apgar scores at 1 and 5 minute. Data on associated adverse effects were also recorded.

Ethical considerations

This trial was approved by the Research Ethics Committee of Ahwaz Jundishpur University of Medical sciences. Women completed informed written consent form. Each woman was assigned an ID code, ensuring data set anonymity. Women could withdraw from the study at any point.

Statistical analyses

Data were analyzed using SPSS 16.0. P-values of <0.05 were deemed to indicate statistical significance. Two-tailed t test was used to analyze continuous data. For example, a paired t test was performed to assess the pain level before and after receiving massage aromatherapy or massage only. Chi-Square test also was used to assess the intrapartum events and maternal satisfaction. Sample size calculation was carried out using the comparison means formula. Power was set at 0.8; alpha level was set at 0.05 and confidence interval set at 95%. The sample size calculated was 23 for each group, and by adding 30% attrition size it changed to 30 women in each group. Registration number was IRCT201105026364N1.
RESULTS

Sixty women completed the trial as randomized, 30 received massage aromatherapy, and 30 received massages only (Figure 1). We obtained 100% follow up and the massage was completed in all women. At trial entry, maternal characteristics were similar (Table 1). There were significant differences in intrapartum events (Table 2); the mean length of active phase was 4.05 ± 1.95 and 5.21 ± 2.52 h for Lavender aromatherapy and massage only, respectively. Also, in the second stage of labor there were significant differences (Table 2); 29 ± 10.5 min in the lavender aromatherapy group versus 42.4 ± 13.9 min (p=0.001) in massage group, respectively. However, there were no differences in mean length third stage of labor, labor onset and in the manner of rupture of amniotic sac in two groups (Table 2). There were also no differences in type of delivery between the lavender aromatherapy and massage group, and each had the same high spontaneous vaginal birth proportion (83-89%). Additionally, Apgar scores were not different for each group at 1 and 5 min.

The intensity of pain between the two groups was compared in the latent phase (cervix dilated 4 - 5 cm), active phase (5 - 7 cm) and transitional phase (8 - 10 cm). There was a relatively steady increase in pain intensity level as labor progressed. A t-test demonstrated that the lavender aromatherapy group had significantly lower pain reactions in the latent, active and transitional phases (Table 3). There was no different in number and duration of contractions in active phase in two groups. Twenty-seven of the 30 (90%) lavender aromatherapy group subjects reported that massage aromatherapy was helpful, providing pain relief and psychological support during labor versus 60% in massage only group (p=0.014). Subjects in the two groups (100%) tended to have a massage at the next delivery and 96.7% in lavender massage aromatherapy and 93.3% in massage group tended to continue massage during this labor and suggested it to their friends, although there was no significant difference between two groups (Table 4).

DISCUSSION

This is the first RCT to examine the exact effect of massage aromatherapy on labor pain intensity level and a wide range of intrapartum outcomes in Iran. Randomization was successful, and the baseline characteristics were similar between the two groups. No maternal adverse effects associated with lavender aromatherapy application were reported, and there were no related neonatal ill effects. The study achieved its main objective in demonstrating that lavender aromatherapy massage is effective in relieving labor pain, although, in massage only group there was a reduction in pain intensity. Data analyses
Table 1. Participant characteristics at baseline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lavender massage aromatherapy n (%</th>
<th>Massage n (%)</th>
<th>P-value</th>
</tr>
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<tbody>
<tr>
<td>Mean maternal age (SD; years)</td>
<td>22.63 (3.48)</td>
<td>26.66 (3.67)</td>
<td>P=0.67</td>
</tr>
<tr>
<td>Mean gestation at delivery (SD; weeks)</td>
<td>39.36 (0.92)</td>
<td>39.5 (1.04)</td>
<td>P=0.18</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>22.44 (2.55)</td>
<td>22.39 (2.50)</td>
<td>P=0.66</td>
</tr>
<tr>
<td>Prenatal care visits</td>
<td>10.56 (3.01)</td>
<td>10.70 (3.45)</td>
<td>P=0.98</td>
</tr>
</tbody>
</table>

Table 2. Intrapartum events.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lavender massage aromatherapy n (%)</th>
<th>Massage n (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean length of active phase of labor (SD; h)</td>
<td>4.05 (1.95)</td>
<td>5.21 (2.52)</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Mean length second stage of labor (SD; min)</td>
<td>29 (10.46)</td>
<td>42.36 (13.86)</td>
<td>P=0.001</td>
</tr>
<tr>
<td>Mean length third stage of labor (SD; min)</td>
<td>5.66 (2.66)</td>
<td>5.86 (2.52)</td>
<td>P=0.61</td>
</tr>
</tbody>
</table>

Labor onset

- Spontaneous (%) 13 (43.3) 13 (43.3) P=1.0
- Induced (%) 17 (56.7) 17 (56.7)

Membrane rupture

- Spontaneous (%) 9 (30) 13 (43.3) P=0.45
- Artificial (%) 17 (56.7) 21 (70)

Table 3. Pain intensity level in dilatation 4 - 5, 6 - 7 and 8 - 10 cm.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lavender massage aromatherapy (n=30)</th>
<th>Massage (n=30)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain intensity in dilation 4-5 cm (mean)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before intervention</td>
<td>4.56</td>
<td>4.6</td>
<td>P=0.01</td>
</tr>
<tr>
<td>After intervention</td>
<td>3.20</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>Pain intensity in dilation 6 - 7 cm (mean)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before intervention</td>
<td>6.83</td>
<td>7.2</td>
<td>P=0.001</td>
</tr>
<tr>
<td>After intervention</td>
<td>5</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Pain intensity in dilation 8-10 cm (mean)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before intervention</td>
<td>8.16</td>
<td>7.76</td>
<td>P=0.05</td>
</tr>
<tr>
<td>After intervention</td>
<td>6.16</td>
<td>7.53</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Maternal satisfaction and views about massage selection.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Lavender massage aromatherapy %</th>
<th>Massage %</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Satisfaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fully satisfied</td>
<td>27 (90)</td>
<td>18 (60)</td>
<td>P=0.014</td>
</tr>
<tr>
<td>Satisfied</td>
<td>3 (10)</td>
<td>12 (40)</td>
<td></td>
</tr>
</tbody>
</table>

Massage selection

- Continue massage during this labor 29 (96.7) 28 (93.3) P=0.66
- Massage selection in next delivery 30 (100) 30 (100) P=0.66
- Suggest to friends 29 (96.7) 29 (96.7)
analysis showed that massage is effective in the first stage of labor pain. In confirmation of this, Chang et al. (2002) in their study also showed that the massage therapy is effective on pain and fear during labor. The results also showed that mean pain intensity in the first stage of labor before and after massage aromatherapy with lavender has reduced. In confirmation of this study, Burns et al. (2000) also stated that the aromatherapy is used for relief pain, nausea and vomiting and to strengthen uterine contractions in labor.

The mean pain intensity after massage in the first stage of labor was compared in two groups (Table 3). Overall increasing labor pain intensity in two groups after intervention was decreased. This drop in massage group with lavender was more dramatic than massage only group (from 8.16 to 6.16). This was probably due to the sedating effects of linalool acetate in lavender as a narcotic. In a study on 635 patients who complained of perineal pain after childbirth, six drops of pure Lavandula in comparison to lavender synthetic oil as daily bathroom for 10 days resulted in most patients expressing a satisfaction with lavender essence (Dale et al., 1994). In a systematic review by Smith et al. (2011) on 535 women in comparing aromatherapy with placebo for pain management of labor, there was no difference between groups for the pain intensity and the length of labor. The authors, however, concluded that further research is needed before final recommendations (Smith et al., 2011).

Conclusion
Lavender massage aromatherapy can decrease pain and duration of first and second stages of labor and it can decrease a wide range of intrapartum worst outcomes. This method can reduce the suffering of women in labor who may need to use oral or intravenous drug for relief pain, and it can also save the costs that is imposed on the health system to relief pain. Also, partners' participation in massage during labor can positively influence the quality of women's birth experiences.

ACKNOWLEDGMENTS
The authors thank those who commented on the early drafts of this article, particularly Mohammad Hossin Haghighizadeh, MSc. Statistics, Department of Statistics and Mathematics, for his full and helpful comments and in the statistical analyses. This paper was prepared from the thesis of Leila Mohamadkhani, and financial support was provided by Ahvaz Jundishapur University of Medical Sciences.

REFERENCES
Full Length Research Paper

Goldcrest honey and its solvent extracts: A natural product with anti-*Helicobacter pylori* activity

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This study evaluates the anti- *Helicobacter pylori* activity of Goldcrest honey at 10, 20, 50 and 75% v/v concentrations as well as its extracts (n-hexane, diethyl ether, chloroform and ethyl acetate) using the agar well diffusion technique. The minimum inhibitory concentrations (MIC₉₀) of the two most active extracts were determined by the broth microdilution assay and MICs were read by enzyme-linked immunosorbent assay (ELISA) microtitre plate reader at 620 nm. The rate of kill of *H. pylori* strains by the most active extract was determined by viability studies over a period of 72 h. Data were analyzed by one-way analysis of variance (ANOVA) at 95% significance level. Goldcrest honey demonstrated anti-*H. pylori* activity, with the most potent activity at 75% concentration, with zone diameter in the range from 12.9 to 14.4 mm. Clarithromycin recorded a zone diameter of 18.0±7.4 mm not significantly different (P>0.05) from diethyl ether extract, with a zone diameter of 19.9±10.1 mm. MIC₉₀ values of n-hexane and diethyl ether extracts were in the range of 0.039 to 10% and 0.078 to 10% (v/v), respectively. The bactericidal effect of n-hexane extract was highest at 4 × MIC concentration, within 30 to 72 h during which 100% of bacterial cells were killed. Goldcrest honey and its solvent extracts may contain potential lead molecules with anti-*H. pylori* activity. Further studies are therefore needed to determine their phytochemical constituents and activity.

Key words: Goldcrest honey, antibacterial activity, minimum inhibitory concentrations (MIC), time kill assay, *Helicobacter pylori*.

INTRODUCTION

*Helicobacter pylori* (*H. pylori*) has probably been part of the human gastric biota since time immemorial (Blaser, 1997). It is a human pathogen of major public health concern since it is directly associated with many diseases of the upper gastrointestinal tract including acute and chronic gastritis, non-ulcer dyspepsia, peptic ulcer disease (gastric and duodenal ulcers) and gastric cancers (McColl, 2010), which are the major causes of death worldwide. Eradication of this pathogen is now a major step in the therapeutic management of the aforementioned diseases (O'Connor et al., 2011). However, triple therapy which is currently the treatment of choice contains two antibacterials (that is, clarithromycin or amoxicillin with metronidazole), combined with a proton pump inhibitor (Malfertheiner et al., 2012). Treatment of *H. pylori* infection is relatively successful, with approximately 80% of patients exhibiting eradication of the organism (O’Connor et al., 2011). The emergence of *H. pylori* strains resistant to the recommended antibiotics, the associated side effects, inactivation of antibiotics by pH and low patient compliance; are cited as some of the major causes of treatment failure (Bytzer and O’Morain, 2005). As a result, there is a need to seek new, safe and effective anti-*H. pylori* regimens with highly selective antibacterial activity against the pathogen, but without the risk of resistance and untoward effects. Honey is among the attractive sources that has received attention as an alternative treatment for *H. pylori* infections.

Apitherapy or therapy with bee products is an age-old therapeutic practice as recorded by several ancient
civilizations. The therapeutic effects of honey have been ascribed to its antimicrobial, anti-inflammatory and antioxidant properties. It has shown powerful antibacterial effects against pathogenic and non-pathogenic microorganisms, yeast and fungi even against those that developed resistance to many antibiotics (Molan and Cooper, 2000). Honey is a supersaturated sugar solution containing vitamins, minerals, proteins, amino acids and nutrients (Ghelldof et al., 2002; Ansari and Alexander, 2009). Its sugars exert strong osmotic potential attracting water molecules and as such inhibit the growth of bacteria and fungi. In addition, it contains the enzyme glucose oxidase, which acts on glucose in the presence of water, producing hydrogen peroxide and gluconic acid (Ansari and Alexander, 2009).

Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (Molan, 1992). Furthermore, antioxidants and flavonoids that may function as anti-bacterial agents are also present. The pH of honey is low and ranges from 3.2 to 4.5 with the most predominant proton donor being gluconic acid. In light of modern science, several important therapeutic effects of honey have been elucidated (Cooper et al., 2002) and these vary with the quality of the honey produced. The type of honey produced is dependent on the flowers blooming in different seasons in different regions and countries (Ndip et al., 2007). Consequently, there is variation in the chemical composition as well as the physical properties of honey. As a result, microorganisms differ in their sensitivity to honeys collected from different sources, regions and countries.

At present, a number of honeys are sold with standardized levels of antibacterial activity for example, Manuka and Capillano honeys are sold as therapeutic honeys in Australia (Lusby et al., 2005). The search for other honeys from different sources with considerable antibacterial activity continues. South Africa has a large floral biodiversity with many unique plants indigenous to the region and honeys with varying antimicrobial potential are produced from these plants and sold commercially in the country. In the Eastern Cape Province of the country, the ‘Xhosas’ use their honeys to produce “iqhilika” (Mead), which is a cultural wine and is said to be of great medicinal value and can be used in the treatment of coughs, kidney and stomach ailments. In spite of the wide body of research on the antibacterial activity of honey against several medically important pathogens including H. pylori, in different parts of the world, there is paucity of information, on the possible antibacterial activity of South African honeys against H. pylori. This study was designed to evaluate the anti-H. pylori activity of Goldcrest honey and its solvent extracts as well as to determine the minimum inhibitory concentrations (MIC50) of the two most active extracts and the rate of kill of the test strains by the most active extract.

MATERIALS AND METHODS

Bacterial strains

Thirty strains of H. pylori were used which were cultured from gastric biopsy specimen obtained from patients with gastroduodenal pathologies attending the endoscopic unit of Livingstone hospital, Port Elizabeth, South Africa. This was done after informed consent was obtained as per our previously reported schemes (Ndip et al., 2008; Tanih et al., 2010). Confirmed isolates were stored in Brain Heart Infusion broth plus 20% glycerol at -80°C for subsequent bioassays. H. pylori ATCC 43526 was used as the control.

Source and dilution of honey

In this study, Goldcrest honey produced from Citrus limon and Citrus sinensis as the main floral sources was used; it was obtained from within South Africa. It was diluted with sterile distilled water to different concentrations of 10, 20, 50 and 75%(v/v); and sterilized by filtering through a 0.22 μm membrane filter (Al-Somai et al., 1994), into separate sterile bijou bottles.

Antimicrobial susceptibility testing of crude honey

The agar well diffusion method of Dastouri et al. (2008) was used to assess the antimicrobial activity of the crude honey. Brain Heart Infusion (BHI) agar (Oxoid, England) was prepared following the manufacturer’s instructions, supplemented with 7% laked horse blood (Oxoid, England) and Skirrow’s antibiotics (SR 0147E, Oxoid, England). A 0.5 Mc Farland standard was prepared and 5 ml aliquoted into a sterile test tube. An inoculum of each clinical isolate was prepared from subculture of the bacterial suspension. With a sterile wire loop, five colonies of the same morphological type were picked and emulsified in sterile normal saline. The turbidity of the suspension was adjusted to correspond to 0.5 Mc Farland standard containing 1.8 x 10⁸ cfu/ml. The suspension was then inoculated evenly onto the surface of BHI plates in triplicate with sterile calcium alginate swab sticks. The plates were allowed to dry for 3 to 5 min. Using a sterile 6 mm diameter cork borer, five wells were cut in the agar and into each was introduced 100 μl of the different concentrations of the honey solution. Into the remaining well, clarithromycin (0.05 μg/ml) was added as the positive control. The plates were incubated at 37°C for 2 to 5 days under microaerophilic conditions (CampyGen BR0056A, Oxoid, England) and later examined for zones of inhibition. The zones of inhibition (in millimeters) were measured, averaged and the mean values recorded. H. pylori control strain ATCC 43526 was included in all the experiments.

Solvent extraction of crude honey

This was carried out using the method of Zaghloul et al. (2001) with modifications. A 100 g of crude honey was placed in a 500 ml separating funnel, diluted with 150 ml of sterile distilled water and extracted with 150 ml of the different organic solvents (n-hexane, diethyl ether, chloroform and ethyl acetate). This was performed as three successive extractions using 50 ml of solvent each time. The shaking time for each extraction process was 15 min, after which the mixture was allowed to stand to permit the solvent layer to separate. The three successive layers were collected, mixed and concentrated by evaporation under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Italy) at 40°C for n-hexane, 30°C for diethyl ether, 50°C for chloroform and 60°C for ethyl acetate. Water containing solvent extract was removed by filtration over anhydrous Sodium sulphate.
Antimicrobial susceptibility testing of honey solvent extracts

The different extracts of the honey at 75% concentration were tested against the isolates using the method described for crude honey above. The respective pure solvent used for the extraction was tested side by side with its extract. Diameters of zones of inhibition of extracts were measured; averaged and mean values recorded in millimeters.

Determination of MIC

The two most active extracts of the honey were employed in broth microdilution assay to determine their MICs against the test isolates, according to the method of Bonacorsi et al. (2009). Two-fold dilutions were prepared in 96-well round-bottom microtitre plates (Greiner Bio-One, Frickenhausen) in BHI broth (Oxoid, England); the final extract concentration was 0.01 to 10% v/v. Similarly, amoxicillin (0.0012 to 1.25 mg/ml) and metronidazole (0.01 to 10 mg/ml) were two-fold diluted and tested on the same plate with the honey solvent extracts as reference antimicrobials. Control wells were also prepared with culture medium only, culture medium with honey extract and culture medium with bacterial isolate only.

The inoculum of each strain was diluted tenfold in sterile normal saline. 20 µl of the bacterial suspension (108 CFU/ml) was aliquoted into each well. The final volume in each well of BHI broth, honey extract and inoculum was 120 µl. The absorbencies were read using an ELISA micro plate reader (Model 680, S/N 19138, Biorad, Japan) adjusted at 620 nm. The micro plates were sealed and incubated at 37°C under microaerophilic conditions for 2 to 3 days, agitated and the absorbencies were again read at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect any increase or decrease in bacterial growth. The lowest concentration of the extract resulting in inhibition of bacterial growth by 50% was taken as the MIC50.

Time-kill assay of extract

Assay for the rate of kill of H. pylori isolates by the most active extract of Goldcrest honey was determined in accordance with the method of Akinpelu et al. (2008) with modifications. Each isolate was subcultured on CBA (Oxoid, England) plates and incubated at 37°C under microaerophilic conditions for 2 to 3 days. Growth of each isolate was transferred into BHI broth (Oxoid, England) and incubated overnight under the same growth conditions. The turbidity of an 18 h old broth culture of the test isolate was standardized to contain approximately 1.8 × 108 cfu/ml. A 0.5 ml volume of the standardized suspension was added to 4.5 ml of different concentrations of the extracts (1/2 MIC, MIC, 2 × MIC and 4 × MIC). These were incubated at 37°C under microaerophilic condition in an orbital shaker at 120 rpm and the killing rate was determined over a period of 72 h. Exactly 0.5 ml volume of each suspension was withdrawn at 6 h intervals and transferred to 4.5 ml of BHI broth recovery medium containing 3% “Tween 80” to neutralize the effects of the antimicrobial compound carry-overs from the test isolates. The suspension was serially diluted and 100 µl plated out for viable counts. The plates were later incubated at 37°C for 72 h. The control plates contained the bacterial cells without the extract. The emergent bacterial colonies were counted and compared to the counts of the culture control. Time-kill assays were carried out in duplicate.

Statistical analysis

Diameters of zones of inhibition were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) test was used to determine any statistically significant difference by comparing zone diameters of the honey at various concentrations; zone diameters of clarithromycin to different solvent extracts, zone diameters of different extracts as well as the MIC values of these extracts were compared to each other at 95% significance level.

RESULTS

Antimicrobial susceptibility testing of crude honey

At various concentrations (10, 20, 50 and 75% v/v), the honey demonstrated antibacterial activity against H. pylori strains with zone diameters (mean ± SD) that ranged from 12.9 to 14.4 mm, with the most potent activity (20/30, 66.7%) at 75% v/v concentration (Table 1). The least antibacterial activity was observed with 20% concentration (16/30, 53.3%). Our positive control (Clarithromycin) equally demonstrated antibacterial activity against H. pylori strains exhibited by a zone diameter (mean ± SD) of 18.0 ± 7.4 mm. However, no statistically significant difference (P>0.05) was reached comparing the zone diameters of the honey at various concentrations.

Antimicrobial susceptibility testing of solvent extracts

All the solvent extracts demonstrated antibacterial activity against the test strains. The zone of inhibition (mean ± SD) ranged from 15.2 to 19.9 mm (Table 2). The most inhibitory activity was demonstrated by diethyl ether extract (22/30, 73.3%) exhibited by an inhibition zone of diameter 19.9 ± 10.1 mm while the least antibacterial effect was observed with the chloroform extract (17/30, 56.7%), exhibited by an inhibition zone of diameter 15.2 ± 8.7 mm. Both the n-hexane (19/30, 63.3%) and ethyl acetate extracts (18/30, 60%) were equally active with inhibition zones of diameter 17.9 ± 8.7 and 16.7 ± 9.3 mm, respectively. Consequently, the zone diameters of the extracts did not reach statistical significance (P>0.05).

MIC determination

The MICs of the two most active extracts were determined by an ELISA microtitre plate reader at 50% bacterial growth inhibition using broth microdilution. N-hexane and diethyl ether extracts were the two most active extracts, with MIC50 values in the range of 0.039 to 10%v/v and 0.078 to 10%v/v, respectively (Table 3). Contrasting, MIC50 values of the reference antibiotics were in the range; 0.001 to 1.25 mg/ml (amoxicillin) and 2.5 to 10 mg/ml (metronidazole). However, no statistically significant difference (P>0.05) was reached comparing the MIC values of the solvent extracts to each other by One-way ANOVA test at 95% significance level.
Table 1. Antibacterial activity of crude honey (Goldcrest) against *H. pylori* isolates.

<table>
<thead>
<tr>
<th><em>H. pylori</em> isolates</th>
<th>Diameter of zones of inhibition (mm) a</th>
<th>Concentration of honey (%v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>PE 11A</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>PE 11C</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>PE 26A</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>PE 76A</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>PE 84C</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>PE 93A</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PE 102C</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>PE 115A</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PE 162C</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PE 219C</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>PE 252C</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>PE 258C</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>PE 308C</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>PE 369A</td>
<td></td>
<td>18</td>
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<tr>
<td>PE 369C</td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>PE 397C</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PE 406C</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>PE 407C</td>
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<td>25</td>
</tr>
<tr>
<td>PE 411C</td>
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<tr>
<td>PE 430C</td>
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<tr>
<td>PE 435A</td>
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<tr>
<td>PE 436A</td>
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<tr>
<td>PE 462A</td>
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<tr>
<td>PE 462C</td>
<td></td>
<td>16</td>
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<tr>
<td>PE 464A</td>
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<td>15</td>
</tr>
<tr>
<td>PE 464C</td>
<td></td>
<td>17</td>
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<tr>
<td>PE 466A</td>
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<td>15</td>
</tr>
<tr>
<td>PE466C</td>
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<td>0</td>
</tr>
<tr>
<td>PE 473A</td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

Mean ± SD 13.3 ± 7.8, 12.9 ± 7.9, 14.4 ± 9.1, 13.7 ± 10.% Susceptibility 60, 53.3 60 and 66.7%.
Zone diameter of sensitive strain ≥14 mm. aMean zone diameter after triplicate assay.

**Time-kill assay**

The bactericidal effect of n-hexane extract on test strain was determined over a period of time ranging between 6 to 72 h. As shown in Figure 1, the lag phase of *H. pylori* strain PE 252C was between 6 and 12 h as there was no growth on the control plate as well. At a concentration of 40%v/v (4 × MIC), 100% bacterial cells were killed within 30 to 72 h. Overall, there was growth of bacterial cells at the concentrations of ½ MIC, MIC, 2 × MIC within 12 to 72 h.

**DISCUSSION**

In the preliminary screening, it was revealed that the test strains were sensitive to honey concentration as low as 10%v/v. This result corroborates the finding of Tajik et al. (2008) who reported the antimicrobial efficacy of natural Urmia honey against Gram negative and Gram positive bacteria. There are many reports about the antibacterial properties of natural honey. Its antimicrobial activity has been ascribed to high content of reducing sugars, low pH, low water activity (Aw), low protein content, lysozyme, and hydrogen peroxide and non-peroxide components (phytochemicals) as investigated by several authors (Molan and Cooper, 2000). Honey is known to contain phytochemicals including tetracycline derivatives, peroxides, amylase, fatty acids, phenols, ascorbic acid, flavonoids, streptomycin, sulfathiazole, terpenes, benzyl alcohol and benzoic acids (Heering et al., 1998). They may
be of plant origin and could be extracted with organic solvents. The amount of these could be small or diluted in the honey but become concentrated and exhibit more activity after extraction (Yao et al., 2004). These components could act as natural anti-oxidants as they are reported to scavenge for free superoxide and other reactive oxygen metabolites liberated during respiratory burst in H. pylori induced mucosal damage (Li et al., 2001). However, Obaseiki-Ebor et al. (1983) isolated volatile substances from honey with antibacterial activity. Other researchers found the non-peroxide components of honey extractable by organic solvents (Oka et al., 1987). In this study, organic solvents of varying polarities namely n-hexane, diethyl ether, chloroform and ethyl acetate were used to extract the antimicrobials in honey. All the solvent extracts demonstrated antibacterial activity against the test strains. Most of the strains were sensitive to diethyl ether extract (22/30, 73.3%). This is in contrast with the finding of Khalil et al. (2001) that showed a better activity of chloroform extract against their isolates. Furthermore, the solvent extracts demonstrated a greater inhibitory effect than the crude honey as evidenced by an increase in the zone diameter of inhibition and percentage susceptibilities of the test strains. This may suggest that the putative antimicrobial components might have been concentrated in the honey after extraction. In addition, five out of seven test strains that were resistant to the positive control (23/30, 76.7%) were remarkably sensitive to the extracts producing appreciable zones. Moreover, no statistically significant difference (P>0.05)

Table 2. Antibacterial activity of solvent extracts of Goldcrest honey against H. pylori isolates.

<table>
<thead>
<tr>
<th>H. pylori isolates</th>
<th>Diameter of zones of inhibition (mm) a</th>
<th>Solvent extracts of Goldcrest honey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n-Hexane</td>
</tr>
<tr>
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<td>32</td>
<td>33</td>
</tr>
<tr>
<td>PE 11C</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>PE 26A</td>
<td>24</td>
<td>28</td>
</tr>
<tr>
<td>PE 76A</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>PE 84C</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>PE 93A</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>PE 102C</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>PE 115A</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>PE 162C</td>
<td>21</td>
<td>25</td>
</tr>
<tr>
<td>PE 219C</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>PE 252C</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>PE 258C</td>
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<td>PE 473A</td>
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Mean ± SD 17.9 ± 8.7, 19.9 ± 10.1, 15.2 ± 8.7 and 16.7 ± 9.3. Susceptibility 63.3, 73.3, 56.7 and 60%. Zone diameter of sensitive strain ≥14 mm. a Mean zone diameter after triplicate assay.
Table 3. MIC values of solvent extracts and classical antibiotics at 50% bacterial growth inhibition.

<table>
<thead>
<tr>
<th>H. pylori isolates</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; values in different concentrations</th>
<th>Solvent extracts of Goldcrest (%)v/v</th>
<th>Antibiotics (mg/m)</th>
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<tr>
<td></td>
<td>n-Hexane</td>
<td>Diethyl ether</td>
<td>Amoxicillin</td>
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<td>PE 76A</td>
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<td>2.5</td>
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MIC<sub>50</sub> values after triplicate assay; -: MIC<sub>50</sub> values not within susceptible range.

was recorded comparing the zone diameters (mean ± SD) of the extracts to the positive control. Suggesting these extracts may contain antimicrobial agents whose therapeutic potential are comparable to clarithromycin (positive control), which is recognized as a key antibiotic against H. pylori infections (Ndip et al., 2008).

In the assay to determine the MIC<sub>50</sub> values of diethyl ether and n-hexane extracts against the test strains, we employed amoxicillin and metronidazole that have been reported as the most sensitive and resistant antibiotics respectively against H. pylori in our environment (Tanih et al., 2010). The antibiotics served as positive controls to test the validity of the assay as well as to control the experiment. This is in accordance with the work of Kňazovicka et al. (2009). The assay indicated that the MIC<sub>50</sub> values varied with extracts and the test strains. This variation could be attributed to different putative components present in the different extracts. This result is in contrast with the work of Aljadi and Yusoff (2003). The bactericidal activity of the n-hexane extract was determined using viability studies. The periods 6 to 12 h could be considered as the lag phase of the isolate since there was no growth on the negative control plate as well as on plates inoculated with sample from extract constituted broth. The bactericidal activity was highest at 4 × MIC concentration within 30 to 72 h. This may suggest that with a further increase in the extract concentration, better results would be obtained. There was recurrent growth of bacterial cells from 48 to 72 h after the cells were being killed at 42 h at the lowest concentration (1/2 MIC). In addition, at MIC, there was growth of bacterial cells from 12 to 72 h showing they were...
inhibited as expected but very little growth of bacterial cells at 2 × MIC from 12 to 48, 60 to 72 h, suggesting the n-hexane extract at these concentrations could be bacteriostatic.

In conclusion, it is worth mentioning that Goldcrest honey and its solvent extracts presented anti- H. pylori activity. It is a promising observation thus indicating compounds of antibacterial potential could be isolated by fractionation from the extracts and harnessed in a bid to provide potential lead molecules with anti- H. pylori activity. Moreover, the honey could complement/supplement standard triple therapy in order to alleviate unpleasant side effects posed by antibiotics as well as improve patients compliance -which are pivotal factors affecting the effectiveness of treatment regimen (Bytzer and O’Morain, 2005). Further studies are therefore needed to determine their phytochemical constituents and activity.

ACKNOWLEDGEMENTS

The authors extend their sincere appreciation to the Govan Mbeki Research and Development Centre, University of Fort Hare, South Africa for funding. We also thank Dr Naidoo N and staff of the GIT unit, Livingston Hospital Port Elizabeth, Dr. Tanih NF and Mr. Okeleye BI for technical assistance.

REFERENCES


Full Length Research Paper

Influence of naringin on the myocardial ultrastructure and NF-κB expression in rats with diabetic cardiomyopathy

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Accepted 24 December, 2012

We investigated the effects of naringin on myocardial nuclear factor-kappa B (NF-κB) expression in rats with diabetic cardiomyopathy (DC), and its protective effect on DC. Wistar rats were randomly assigned into a normal control group, DC group and naringin intervention group. The DC rat model was established using high-sugar, high-fat feeding and streptozotocin (STZ) injection. The ultrastructural changes in the myocardial tissues were observed under electron microscopy; NF-κB expression in the myocardia was detected using immunohistochemistry. The electron microscopy results showed that the myocardial arrangement in the control group and the naringin intervention group were better than in the diabetic group, with little fibrosis. Compared with the control group, the myocardial NF-κB expression was significantly increased in the diabetes group and the intervention group (p < 0.05), and the NF-κB expression in the intervention group was significantly lower than in the diabetic group (p < 0.05). Naringin reduces the NF-κB expression in myocardial cells, which prevents structural changes, and protect them from DC.

Key words: Diabetes, cardiomyopathy, naringin, nuclear factor–kappa B (NF-kB), inflammation.

INTRODUCTION

Clinical and experimental studies have shown that inflammatory reactions possibly play an important role in the development of diabetic cardiomyopathy (DC) (Dasu et al., 2008; Jiao et al., 2009; Van Linthout et al., 2007; Westermann et al., 2009; Lorenzo O et al., 2011). Nuclear factor-kappa B (NF-kB) is the key factor in the inflammation signal pathway. The NF-kB family includes five subsets: NF-κB1 (p50/p105), NF-κB2 (p52/p100), P65 Rel A, Rel B and c-Rel 5. Among these subsets, the P50/P65 heterodimer is the most common (Neurath et al., 1998). NF-kB occurs nearly in all types of tissues and cells, and it participates in the signal transduction of many inflammatory reactions. Numerous studies have investigated the effectiveness of anti-inflammatory drugs as myocardial protectants for preventing. However, drugs that effectively prevent DC and inhibit inflammation have not been developed (Adeghate et al., 2008; Aneja et al., 2008; Boudina and Abel, 2007, 2010; Opie et al., 2011). Therefore, finding potential anti-inflammatory drugs, especially those derived from anti-inflammatory traditional Chinese medicine, and under-standing their mechanisms of action would be academically and clinically significant.

Naringin is the main effective component of the Herbal drugs such as Rhizoma Drynariae, Fructus Aurantii Immaturus, Fructus Aurantii and Exocarpium Citri Rubrum. Its glycoside ligand is 4′,5,7-hydroxy flavone, and its saccharide constituent is rhamnose β-1,2-glucose. In recent years, many animal studies showed that naringin helps regulate glycolipid metabolism, has anti-inflammatory anti-oxidative stress, myocardial preservation effects (Rajadurai and Prince, 2007a, b, 2009).

In this study, we established a DC model in rats to observe changes in the expression of the key inflammatory factor NF-kB in myocardial tissues and the ultrastructure of myocardial cells. We used naringin from medicinal plants as an inflammation inhibitor to determine its effect on the pathogenesis of DC. The results provide an experimental basis for DC prevention and treatment.

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MATERIALS AND METHODS

Animal grouping and establishing DC model

Specific pathogen-free (SPF) grade male Wistar rats, weighing 150 ± 180 g, were provided by the Experimental Animal Centre of Southern Medical University. The blood was collected from tail veins to ascertain whether the blood glucose level is normal or not. Thirty Wistar rats were fed adaptively for two weeks and divided into three groups: the normal control group (NC group, n = 10); the DC group (DC group, n = 10); and the naringin intervention group (NC+ naringin group, n = 10 and DC+ naringin group, n = 10). The rats in the NC group and NC+ naringin group were fed basic feed, whereas the rats in the DC group and DC+ naringin group were fed high-fat, high-sugar feed (20% sugar, 10% lard, 2.5% cholesterol and 67.5% basic feed). During the 6th week, 1% streptozotocin (STZ, U.S. Sigma Company) at 30 mg/kg dose (pH = 4.5, freshly prepared at 4°C) was injected once intraperitoneally. After 72 h, the fasting blood glucose levels of the rats were determined. The rats with fasting blood glucose levels exceeding 11.1 mmol/L were considered successful type 2 DM rat models. They were continuously fed high-fat, high-sugar feed until the 12th week. Transmission electron microscopy revealed that myocardial cells developed apparent pathologic changes. Therefore, the rat DC model was successfully established.

In addition, naringin powder (98% purity; Guangdong Meiyian Cyanobacteria Company) (40 mg/kg) was administered intra gastrically to the NC+ naringin group and DC+ naringin group. Furthermore, distilled water was similarly administered to the NC and DC groups according to bodyweight. The rats were sacrificed at the end of 12th week.

Ultrastructure of the myocardial cells

The hearts of the sacrificed rats were rapidly taken out. A 1 mm3 sample of the left ventricular myocardium was taken, fixed with 3% glutaraldehyde, and examined under a transmission electron microscope. The myocardium was washed with phosphate-buffered saline (PBS), fixed with 1% osmic acid, progressively dehydrated with ethanol and acetone, replaced, embedded, polymerized, sliced and stained. Finally, Japan Hitachi H-600 transmission electron microscope was used for observation.

Immunohistochemistry

The Streptavidin-Peroxidase (SP) method (SP kit was purchased from Beijing Zhongshan Jinqiao Biotechnology Co., Ltd.) was used. The specimen was embedded with paraffin wax and sliced into 4 μm sections. The sections were conventionally dehydrated and washed with water. Then, 3% hydrogen peroxide (H2O2) was used to clear endogenous peroxidase. Subsequently, the sections were soaked in 0.01 M citrate buffer solution with a pH of 6.0 and heated under high pressure for 3 min. After they were sealed with 10% normal goat serum, an NF-kB polyclonal antibody kit (Beijing Biosynthesis Biotechnology Co., Ltd.), secondary antibodies, and tertiary antibodies were added in proper order. Benzidine (DAB) staining was regulated under a microscope. Brown granules represented positive markers, and the samples were subjected to hematoxylin staining, dehydration and vitrification. At the same time, PBS was used to replace the first antibody as a negative control.

Optical microscopy was performed at 400× magnification. For each section, 10 visual fields were randomly selected to acquire images for saving and analysing. In addition, Motic 6.0 Digital medical image analysis system was used to determine the gray value and positive optical density.

Statistical analysis

All data were processed using the SPSS 17.0 statistical software, and the measurement data were expressed as ± x. For comparison between groups and among groups, t test or variance analysis of completely random grouping was carried out. Differences with p < 0.05 were considered significant.

RESULTS

Success rate of the rat DC model and death situations

After the rats were fed high-fat, high-sugar feed for 12 weeks and intraperitoneally injected once with 1% STZ (30 mg/kg), the rat DC model was successfully established, with a 100% success rate. Only two rats died during the experiment, one in the DC group and one in the DC+naringin group. The deaths were possibly caused by excessively high blood glucose that caused diabetic ketoacidosis, infection, or other diabetic complications.

General data

Body weight, heart/body index, haemoglobin Alc (HbAlc), free fatty acids (FFA), and the homeostasis model assessment–insulin resistance index (HOMA-IR) of the DC group were significantly higher than those of the NC group (p < 0.05). These indicators for the DC+ naringin group were significantly lower than those of the DC group (p < 0.05). No significant difference was observed between the NC group and the NC+ naringin group (p > 0.05) (Table 1).

Ultrastructure of myocardial cells

The rat myocardial cells in the NC and NC+ naringin groups had numerous regularly arranged myofilaments. Light and dark bands were clear and visible; mitochondria were normally round or oval, with closely arranged developed ridges. Regularly arranged intercalated discs connected the myocardial cells. In the DC group, the myocardial intracellular myofibril content of the rat clearly decreased, while the myofilaments were arranged irregularly and sparsely. The myofilaments were partially fractured, distorted, and presented local dissolution and loss. Furthermore, the sarcomeric lengths were inconsistent, and the periodic sarcomeric structures disappeared. Light and dark bands were unclear, and the mitochondria were irregularly arranged. Some of the mitochondria were apparently swollen, with cristae that were broadened, fractured, and some even disappeared. The mitochondrial matrix density decreased, and vacuoles formed in the matrix. The regional gaps in the myocardial intercalated disc were broadened, and some were irregularly connected or fractured. Meanwhile, the sarcoplasmic
reticula were clearly loose and vacuoles formed within them.

In the DC+ naringin group, the myofibril content of the myocardial cells clearly increased more than in the DC control rats, and it was close to the normal level. In addition, the myofilaments were almost regularly arranged, the sarcomeres were apparent, the light and dark bands were clear, and the intercalated disc structure was nearly close to normal. Moreover, cell gap widening and mitochondrial swelling were not obvious. The cristae gaps were not broaden, and the cristae were orderly arranged. No fusion and fractures were observed and no vacuoles formed (Figure 1).

### Myocardial NF-κB expression

The rat myocardial tissues in the NC and NC+ naringin groups exhibited slight NF-κB expression, and no significant difference was observed between the NC group and the NC+ naringin group (p > 0.05). The NF-κB expression in the DC group was significantly higher than that in the NC group (p < 0.05). The NF-κB expression in the DC+ naringin group was significantly lower than in the DC group (p < 0.05) (Figure 2).

### DISCUSSION

DC is a lesion specific for diabetic cardiomyopathy. Rubler et al. (1972) observed a specific cardiomyopathy in diabetic patients without apparent coronary atherosclerosis. Hamby et al. (1974) initially proposed the DC concept in a further study. Although, theoretical and clinical studies have been carried out for more than 30 years, the pathogenesis of DC is still unclear. Furthermore, glycolipid metabolism disorder, insulin resistance, oxidative stress, cell apoptosis, microvascular lesions and myocardial fibrosis have all been linked to DC. The main pathologic changes in DC include myocardial hypertrophy, myocardial fibrosis, microvascular lesions, and so on. Inflammatory reactions are involved in the pathogenesis and progression of DC. NF-κB is one of the key transcription factors in B lymphocytes discovered by Sen and Baltimore in 1986. NF-κB specifically binds the enhancer sequences of immunoglobulin κ light chain gene (Neurath et al., 1998) and participate in many pathologic and physiologic processes, including the immune response, cell apoptosis, carcinogenesis and inflammatory reactions. NF-κB is involved in the "gene switching" of inflammatory reaction chain, which participates in regulating the expression of many cytokines. High blood glucose induces AGE formation and protein kinase C (PKC) activation, thereby activating NF-κB to cause microcirculation lesions (Picchi et al., 2010).

Furthermore, Mohan and colleagues (Dasu et al., 2008) proved that high blood glucose rapidly activates PKC and NADPH oxidase to induce the expression of toll-like receptors, thereby promoting intracellular reactive oxygen species (ROS) generation and NF-κB activation to cause inflammation. In addition, several studies reported that NF-κB activation increases oxidative stress and induces mitochondrial dysfunction and cardiac insufficiency in rats with type 2 DM (Mariappan et al., 2010). In this experiment, NF-κB expression in the myocardial tissues of the DC model rats was significantly higher than in the NC group (p < 0.05), and changes in the myocardial ultrastructure were indicative of significant cardiomyopathy. Therefore, the activated NF-κB–mediated inflammatory reaction during diabetes is possibly involved in the pathological damage to myocardial cells in DC.

In China, Xiong et al. (2010) reported that naringin suppresses the ROS generation induced by high blood glucose to inhibit NF-κB activation. Naringin further inhibits the expression of intercellular adhesion molecules and vascular endothelial cell adhesion molecules, thereby inhibiting inflammatory reactions. This study indicated that both the regular arrangement and structural integrity of myocardial myofilaments and mitochondria in the DC+ naringin group were better than those in the DC group, and the extent of fibrosis was milder. In addition, NF-κB expression in the myocardium of the DC+ naringin rats was lower than that of the DC group. Hamid et al. (2009) confirmed the feasibility of reducing apoptosis by suppressing NF-κB. Sandeep and colleagues (Kumar et al., 2011) showed that the NF-κB inhibitor PDCT reduces the deposition of extracellular matrix and the expression of cell adhesion molecules by suppressing NF-κB pathways, thereby relieving myocardial fibrosis. Thus, naringin reduces the myocardial damage in DC by inhibiting the inflammatory reactions mediated by NF-κB.
Therefore, it can be suggested that naringin delays the occurrence and progression of DC and protects the myocardium.

ACKNOWLEDGMENT

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Full Length Research Paper

Function of human insulin-like growth factor-1 (hIGF-1) transgene towards the regeneration of peripheral nerves in vivo

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The aim of this study was to investigate the function of human insulin-like growth factor-1 (hIGF-1) transgene in vivo towards the regeneration of peripheral nerves. Thirty (30) male Wistar rats were randomly grouped after the sciatic nerve regeneration chamber model was set up. The hIGF-1 Treatment Group (TG) was injected the mixed solution of pcDNAhIGF-1 and LipfectAmine (hIGF-1 DNA was 4 ug) immediately at the spot of crush injury. The Treatment Model Group (MG) was injected 10 ul of the mixed solution of pcDNA3.1, LipfectAmine and physiological saline, while the control group (CG) was not injected any solution. The functional indexes of sciatic nerve were tested after 8 weeks of the injection. At the same time, electrophysiology of the regenerated nerve fibers, histology, morphology and ultrastructural organization observation were carried out. Compared with MG and CG, TG showed significant improvements in the fields of nerve conductive velocity of the regenerated nerve fibers, the greatest amplitude and electric potential of complex musculation electric potential (P<0.01). The axon diameter, medulla sheath thickness and medullated nerve fibers counts of the regenerated nerve fibers of TG were also higher than those of MG and CG (P<0.01). The degree of maturity of the regenerated nerve fibers of TG was better in the ultrastructural organization observation. Furthermore, other functional indexes were improved. hIGF-1 transgene in vivo could promote the regeneration of the injured peripheral nerves.

Key words: Peripheral nerve, insulin-like growth factor-1, electrophysiology, histology.

INTRODUCTION

The axonotmesis caused by the injury of peripheral nerves (IPN) would subsequently induce distal Fahrenheit Degeneration and proximal Retrogressional Degeneration of axonal injury, which would further lead to the interruption of axoplasmic transportation. If this situation could not be repaired, the atrophy of skeletal muscle, especially the degeneration of motor end-plate and sensory end-organ, and the death of spinal neuron cells would unavoidably happen. While because of the anatomical and functional specialties of peripheral nerves, the recovery of the nerve function could not reach an ideal status. So, how to find a new solution for the improvement of the speed and quality of the regeneration of IPN is still a very urgent problem. A large number of studies have reported the effect of neurotrophic factor in this field, whereas the effect of insulin-like growth factor on peripheral nerves needs more attention. Therefore, this study was designed to investigate the regeneration effect of human insulin-like growth factor-1 on peripheral nerves with the method of transgenesis.

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the research of IPN. Within 30 years, the basic research has made people realize many nutrilites related to the nerve regeneration, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and insulin-like growth factor (IGF), etc, among which IGF was discovered in the latest 10 years. IGF is a kind of multi active bio-polypeptide, showing neurotrophic and neuroregulating activities in the center and periphery (Nguyen et al., 2012; Zheng et al., 2000), while the researches of IGF are mainly in the center, the reports about IGF in periphery are relatively rare. In this study, human insulin-like growth factor-1 was transgenically used to the regeneration chamber mode after injury of peripheral nerves in rats. The effect of human insulin-like growth factor-1 in the promotion of nerve regeneration, protection of spinal motor neurons, inhibition of denervation muscle atrophy were observed through the experimental results.
2. According to the theory of Neurotization Interstice, namely a suitable interspace, based on the trophicity and chemotaxis of the regeneration of nerves, could realize more accurate opposing connection (D’Ercole et al., 2002; Kou et al., 2011). In this study, the regeneration chamber mode of sciatic nerve crush injury was firstly set up, then sciatic nerve functional indexes (SFI), electrophysiology, axon diameter of medullated nerve fibers, thickness of myelin and transmission electron microscope observation were carried out for the investigation of the regeneration function and mechanism of hIGF-1 transgene in vivo to peripheral nerves in different spaces and periods.

MATERIALS AND METHODS

Animals

Thirty (30) male white Wistar rats were provided by Animal Experiments Center of Jilin University, body weight 200~250g. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Clinic Medical Academy of Yangzhou University.

Animal models and grouping

The rats were randomly divided into 3 groups, namely hIGF-1 Treatment Group (TG, 10), Treatment Model Group (MG, 10) and Control Group (CG, 10). After the anesthesia with intraperitoneal injection of 5% Ketamine Hydrochloride (130 mg/kg), the right sciatic nerve was exposed under sterile situation. Five millimeter (5 mm) of lower margin of piriform muscle was then clipped with special forceps clip 3 times, 10 s each with extruding width at 3 mm, which would appear to be film status under the 6x Operation microscope. TG was then injected the mixed solution of pcDNAhIGF-1 and LipfectAmine 10 μl immediately at epineurium. MG was injected the mixed solution of pcDNA3.1, Lipfect Amine and 10 μl of distilled water, while CG was injected nothing. Each rat was fed in a separate cage after the operation.

Observation items and methods

Sciatic nerve functional index (SFI)

According to the referenced method (Silva-Couto et al, 2012), SFI was calculated by determining the walk print of the targets in different time spots.

Electrophysiology

Eight weeks after the operation, rats were anesthetized and fixed, then sciatic nerve and triceps surae muscle of the operational side were exposed to the stimulating electrode and recording electrode, respectively to record the conductive velocity of the regeneration nerve and the greatest amplitude of the complex musculcation electric potential.

Histology and photo analyses

After the check of nerve electrophysiologic study, 5 injured nervous tissues of each group, 10 mm each, within the injured spots were made to paraffin section of cross section, with thickness of 4-6 μm, Marsland and LFB double staining was used after the tissues were fixed with toasting method. HPIAS-1000 High Resolution Color Pathological Photo Analysis System was then used to determine the axon diameter of medullated nerve fibers and thickness of myelin in 5 random fields of vision. At the same time, nerve fibers counts should also be recorded in each vision field.

Transmission electron microscope observation

After the check of nerve electrophysiologic study, 5 injured nervous tissues of each group, 10 mm each, within the injured spots were selected, then solidified with 2.5% glutar for the former end and 1% osmic acid for the latter end, respectively; then dehydrated with ethanol and entrapped with Epon812 epoxide resin. LKB-III ultramicrocuts were then performed and double stained with uranyl acetic acid and lead citrate. The counts of the regeneration nerve, degree of maturity of myelin, integrality of perimysium and hyperplastic Schwann Cell were observed and photoecl with JEM-1200EX Transmission Electron Microscope.

Statistic analysis

SPSS software (version 11.5) was used for the statistic analysis, the credible range of multi group mean analysis of variance was set at 95%, and the data were shown in the form of S.

RESULTS

SFI

SFI values got better as time passed by, which were much more obvious after 24 days, and in every time spot, the SFI values of TG were better than the other two
groups (Figure 1).

Electrophysiology

Fifty-six days after operation, the regenerating motor nerve conduction velocity, the maximum amplitude and latency of compound muscle action potential of the right sciatic nerve in hIGF-1 TG were significantly improved than those of MG and CG (P<0.01). There was no significant difference between MG and CG (P>0.05) (Table 1).

Compared with CG, electrophysiology results of MG showed no significant difference (P>0.05), while TG showed significant difference (P<0.01). Related data are shown in Table 1.

Histology and photo analyses

Regenerating sciatic nerve fibers was stained with Marslan and LFB. Axon was black and myelin was blue. The number of myelinated nerve fibers, axon diameter and the thickness of myelin regeneration were measured in each group with sciatic nerve injury regeneration by the Pathological Photo automated analysis system microscope (×600) (Table 2, Figure 2). Compared with MG and CG, IGF-1 TG showed a significant difference (P<0.01). There was no significant difference between MG and CG (P>0.05). Compared with CG, there were no significant difference in diameter of regenerating neurite, myelin thickness and nerve fibers counts in MG (P>0.05), while TG showed significant difference in the above mentioned items (P<0.01). The photo analysis results are shown in Table 2 and Figure 2.

Transmission electron microscope observation

Intramedullary spinal cord neuropil and regenerating sciatic nerve fibers were observed with transmission electron microscope 56 days after operation. The structure of intramedullary spinal cord neuropil in IGF-1 TG was normal, regenerating sciatic nerve fibers were abundant and matured. Nerve fibers were thick and myelin were thick and evenly. In MG, the gap of neuropil protruding was large, mitochondria had a dense formation in axons, and myelin of myelinated nerve was loose. Regenerating sciatic nerve fibers were relatively thinner, less matured than those in TG, in addition that myelin thickness was less and more different than those in TG. In CG, there were vacuoles in the neuropil. Regenerating sciatic nerve fibers were small and immature. The myelin thickness was thin and irregular.

There were more and more mature regenerating sciatic nerve fibers of TG: thicker nerve fibers, thicker myelin sheath. MG showed slimmer and less matured regenerating sciatic nerve fibers with less thick myelin sheath and bigger difference. CG showed small regenerating sciatic nerve fibers, immature, and the thickness of myelin sheath was slim and irregular (Figure 3).

DISCUSSION

Whether the peripheral nerve could be regenerated successfully after injuries mainly depends on the activities of central neuron and suitable regenerating microenvironment. Nerve Growth Factor (NGF) is one of the most important factors in regenerating microenvironment. Many researchers applied exogenous stimulating NGFs for the regeneration of IPN, while the half lives of these factors are short, which need repeated injection of these giant molecule substance if a traditional injection method is used, and still hard to reach the specified place and maintain effective concentration. With the development of Molecular biology, applying genetic engineering could solve the problem. It theoretically needs occasional, even once, application to reach the therapeutic purpose if a certain protein gene with therapeutic activity is transferred into specified tissue. Furthermore, gene therapy could limit the adverse effects, and show unexampled advantage.

IGF-1, a single-stranded polypeptide with 70 amino acids composition, belongs to the family of Insulin-Like Growth Factor and shows endocrine, autocrine and paracrine activities. Its molecular weight is about 7649 Kbp. Since the first report from Salmon and Daughaday,
Table 1. Electrophysiology results of right sciatic nerve of three groups rats after 56 days (X ± S).

<table>
<thead>
<tr>
<th>Group</th>
<th>Motor neural latency (ms)</th>
<th>Motor neural amplitude (mV)</th>
<th>Neural conduction speed (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>2.65±0.55</td>
<td>6.60±0.59</td>
<td>29.22±3.20</td>
</tr>
<tr>
<td>MG</td>
<td>2.55±0.36</td>
<td>6.67±0.69</td>
<td>29.57±4.06</td>
</tr>
<tr>
<td>TG</td>
<td>2.14±0.22</td>
<td>7.81±0.84</td>
<td>36.91±4.37`</td>
</tr>
</tbody>
</table>

Note: p < 0.01 vs CG.

Table 2. Pathological Photo Analysis of regenerating nerve fibers (X ± S).

<table>
<thead>
<tr>
<th>Group</th>
<th>Axon diameter (μm)</th>
<th>Myelin thickness (μm²)</th>
<th>Medullated nerve fiber count (piece)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG</td>
<td>2.18±0.29</td>
<td>1.03±0.15</td>
<td>68.60±8.55</td>
</tr>
<tr>
<td>MG</td>
<td>2.28±0.33</td>
<td>1.08±0.18</td>
<td>71.80±8.25</td>
</tr>
<tr>
<td>TG</td>
<td>3.03±0.35</td>
<td>1.65±0.24`</td>
<td>88.20±8.82</td>
</tr>
</tbody>
</table>

Note: ` P < 0.01 vs CG.

Figure 2. Regenerating sciatic nerve fibers (double stained with Marsland and LFB, ×600). a: hIGF-1 TG, b: MG, c: CG.

Figure 3. Transmission Electron Microscope Observation Results 8 weeks after the operation (×6000) a: hIGF-1 TG, b: MG, c: CG.
this factor, related closely to growth hormone (GF), has attracted researches in many fields, and the appearance of bio products of recombination of IGF-1 further has opened the research visions in the fields of disease diagnosis and therapy (Gehrig et al., 2012; Mu et al., 2012; Yashida et al., 2011). During this experiment, a 3 mm neurotization chamber was successfully set up with sciatic nerve clip. This suitable interstice could solve the problem of nerve dislocation growth through the neurotrophy and chemotaxis and realize much more accurate opposing connection. Many research have reported that 2–3 mm is best for regeneration (Buti et al., 1996; Zhang et al., 2009), and some researchers also reported that the crevice should not be over 10 mm. Compared with viral vectors, plasmid vectors could hold Klenow fragment DNA. Furthermore, when the plasmids are transferred into the target cell, they would appear to be circular DNA, which could not be integrated and copied, so that they could not generate replication-competent viruses, and show no potential possibilities of viral infection and carcinogenic ability to the host. At the same time, plasmid vectors with suitable purities could avoid the target cell death caused by the immune-reactions to exogenous genes. In this study, the axon diameter of the regenerating neurite, diameter of myelin, nerve fibers counts and degree of maturity of the regenerating nerve under the degree of maturity of TG were all significantly better than those of CG, and the conduction velocity of regenerating nerve and amplitude of target compound muscle action potential were also obviously higher than CG, which revealed that IGF-1 could promote the regeneration of peripheral nerves.

Previous researches working on IGF-1 concerned more about diabetic perineuropathy (Godinez-Gomez et al., 2006; Zeng et al., 2005), while few about the treatment of IPN. In recent years, some researchers reported the IGF-1 experiments of IPN in animals (Emel et al., 2011; Hu et al., 2004; Kryakova et al., 2010), such as facial nerve and sciatic nerve. Some scientists also applied IGF-1 in the clinical treatment of Human Hypogonadism (Hayes et al., 2001). To solve the problem of short half life of IGF-1, the experiment applied the method of gene engineering, which came up with significant results. The data of the present study showed that the histology and ultrastructural organization of the regeneration nerve treated with hIGF-1 were better than the other groups after 8 weeks of operation, and the expression of hIGF-1 in dynamoneur cell in TG was obviously higher than those in MG and CG after 4 weeks of operation.

As for the transferring mechanism, protection and trophism activities of hIGF-1 towards the nerve tissue, it was still unclear. In central nervous system, ecotogenic IGF-1 mainly acts through the action on IGFBP-2 target neuron (Govoni et al., 2011; Sizonenko et al., 2001), and Nagano et al. (2003) considered that the phosphoinositide-3 kinase pathway might be the explanation, while Zhang et al. (2004) thought exogenous plasmid DNA was taken for some external stimulation, such as wound, which would further improve the cell membrane permeability. The above theories still need further investigation.

The experiment proved that hIGF-1 did have some effects on the regeneration of nerve fibers after IPN, and just as the regeneration of nerve after IPN was connected with age, the results might be related with the following factors:

1. Rats at different ages might affect the results (Apel et al., 2010).
2. The experimental results might change owing to the different counts and effects of hIGF applied in the experiment.
3. Different administration methods: the half life of plasmid is short and would have been degraded soon in vivo, which therefore could not maintain the effective concentration. So, the effects of multi administration of hIGF-1 plasmid to a specific local with a certain time interval would be better than one administration. Moreover, the effects might be different if local administration methods were different, such as nerve pars affecta administration, target muscle injection, pars affecta pump administration, or the combination of two methods.

Transgene in vivo with human Insulin-like growth factor-1 can promote nerve regeneration after peripheral nerve injury.

REFERENCES


Preparation and characterization of toltrazuril polyethyleneglycol 6000 solid dispersions with improved solubility

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The current study investigated toltrazuril /polyethyleneglycol 6000 solid dispersions (toltrazuril/PEG6000 SDs) with improved solubility. The SDs were prepared by solvent-melting method with PEG 6000 as carrier. It was validated by differential thermal analysis (DTA) and cumulative dissolution rate. The solubility of toltrazuril, physical mixture and SDs were measured. The ultra violet (UV) spectrophotometer method was developed for the determination of toltrazuril. It was found that the spectra of cumulative dissolution rate, DTA of the SDs were different from the toltrazuril and physical mixture. Solubility of toltrazuril was enhanced for the formation of SDs. The calibration curve was linear with a correlation coefficient $r = 0.9998$ in range of 4.0~20.0 µg/mL. The method was simple and practical in preparation and determination the toltrazuril SDs.

Key words: Toltrazuril, polyethyleneglycol 6000, solid dispersions, solubility.

INTRODUCTION

Toltrazuril chemically, 1-methyl-3-[3-methyl-4-[4-(trifluoromethylthio)phenoxy]phenyl]-1,3,5-triazinane-2,4,6- trione, is a symmetrical triazinetrione broad-spectrum anticoccidial and antiprotozoal agent (Figure 1). It is widely used in poultry and swine for the prevention and treatment of coccidiosis. Toltrazuril may have clinical application in the treatment of Neospora caninum and other protozoal infections in cattle (Dirikolu et al., 2009; Martinez-Villalb et al., 2010). Toltrazuril is effective in vivo against Eimeria species in avians, in vitro against Toxoplasma gondii, and in vivo against intestinal and hepatic coccidiosis in rabbits (Peters and Geeroms,1986; Chapman,1987; Ricketts and Pfefferkorn,1993; Reynaud et al., 1999; Ai et al., 2011). However, due to toltrazuril’s relatively poor water-soluble and low dissolution in gastric fluids, it is not well absorbed from the preparations. It shows variation in bioavailability. It is necessary to enhance the solubility and bioavailability of toltrazuril through the preparation technology. Solid dispersions (SDs) technology is one of the effective and widely used techniques for dissolution enhancement in the field of pharmaceutical preparation technology (Win and Sidney, 1971; Nemanja, 2012). Drugs in the SDs systems may exist as an amorphous form in polymeric carriers, and improve the solubility and dissolution rate compared with crystalline material. The basic procedure used to prepare SDs is solvent-melting techniques. It is very easy and less expensive for preparation of SDs (Hao et al., 2009).

Polyethyleneglycol 6000 (PEG6000) is semicrystalline polymer that has been used extensively in the SDs preparation (Craig, 1990). The advantages of PEG6000 for the formation of SDs are that it has good solubility in many organic solvents and lower melting point. Additional attractive features of PEG6000 include their ability to solubilize some compounds and improve compound wettability (Madhuri et al., 2008).

The purpose of this research was to choose PEG6000 as a suitable polymer for the preparation of toltrazuril polyethyleneglycol 6000 SDs. SDs were then evaluated by dissolution rate and differential thermal analysis (DTA).
Toltrazuril was purchased from Zhengzhou Vet Chemp Pharm Co., Ltd, Zhengzhou, China. PEG6000 and other reagents of analytical grade were purchased from Aoboxing Chemicals Co. Ltd, Beijing, China. The instruments employed were Spectrophotometer (Shimadzu UV-1800, Kyoto, Japan), dissolution apparatus (ZRC-6FT, Tianjin Chuangxin Electronic Equipment Manufacture, Tianjin, China) and DTA instrument (CRY-32P, Shanghai Precision and Scientific Instrument Co. Ltd, Shanghai, China).

Preparation of SDs
Toltrazuril/PEG6000 SDs at three different mass ratios (1:10, 1:15 and 1:20) were prepared by solvent-melting methods. The PEG 6000 was placed in a porcelain dish and allowed to melt by heating up to 80°C Toltrazuril was dissolved in an appropriate amount of diethyl carbonate to its saturation solubility. After complete dissolution of toltrazuril, solution was added to the melted mass. The mixture was stirred constantly until homogenous dispersion was obtained. The resultant solution was removed and cooled in an ice bath, and then it was stored in desiccators for 24 h for rapid solidification. The SDs were then scrapped, pulverized and passed through a 100-mesh sieve. Then the prepared SDs were filled in glass bottles, sealed and stored in desiccators until further use.

Preparation of physical mixtures
Physical mixtures of toltrazuril and PEG6000 at three different mass ratio (1:10, 1:15 and 1:20) were prepared in a glass mortar by simple blending for 20 min. The mixtures were passed through a 100-mesh sieve. Then they were filled in glass bottles, sealed and stored in desiccators until further use.

UV absorption spectrophotometry
Spectrophotometry was performed with a Shimadzu UV-1800 spectrophotometer. Standard solutions of toltrazuril was prepared with acetonitrile/water (60/40); working solutions were prepared by diluting stock solutions with acetonitrile/water (60/40). Calibration standard solutions were prepared at concentrations of 4.0, 8.0, 10.0, 12.5, 15.0 and 20.0 μg/mL for toltrazuril and assayed in replicates of three. Complete spectrophotometric scans between 200 and 400 nm were performed to monitor any changes in the UV spectra of the toltrazuril. The absorbance maximum 243 nm of toltrazuril was selected to quantify its concentration. The certain absorbance value was regressed with the certain concentration to calculate the calibration equation.

MATERIALS AND METHODS

Drug content
The drug content in each SDs and physical mixture was determined by the UV-spectroscopic method. An accurately weighed quantity of 50 mg sample was transferred to 100 mL volumetric flasks containing water and dissolved, the solution was filtered, diluted and assayed spectrophotometrically at 243 nm, the contents of toltrazuril were calculated from the regression equation generated from standard data.

Saturation solubility study
The saturation solubilities of toltrazuril, physical mixture, SDs were carried out in water at room temperature. Pure toltrazuril (25 mg), a quantity of toltrazuril/PEG6000 SDs and the physical mixtures (mass ratio 1:10, 1:15 and 1:20) equivalent to 25 mg of toltrazuril were weighted into sealed vials and stirred vigorously in a water bath shaker at 25±0.5°C with water (10 mL) for 24 h. The samples were then centrifuged and filtered through 0.45 μm cellulose acetate membrane filters. After suitable dilution, the absorbance was assayed spectrophotometrically at 243 nm.

Dissolution rate studies
In vitro dissolution studies of toltrazuril, SDs and the physical mixtures (mass ratio 1:10, 1:15 and 1:20) were carried out in a dissolution apparatus using the second method described in Chinese Pharmacopoeia at 37±0.5°C, rotating at 50 rpm. Hundred (100) mg toltrazuril or its equivalent in physical mixture or SDs was added to 900 mL distilled water, 5 mL dissolution medium was withdrawn at 5.0, 10.0, 20.0, 30.0, 45.0 and 60.0 min with a pipette. The samples were immediately filtered and assayed spectrophotometrically at 243 nm. Equivalent amount of fresh water pre-warmed to 37±0.5°C was replaced after each sampling. The cumulative percentage of toltrazuril dissolved was calculated from the regression equation generated from standard data.

Differential thermal analysis
DTA curves of toltrazuril, PEG6000, physical mixtures and SDs (mass ratio 1:10) were measured with a DTA instrument. Each sample (10mg) was accurately weighed and heated in an hermetically aluminum pan at a rate of 10°C/min between 40 and 300°C temperature range under an air flow. An empty aluminum pan was used as a reference. The DTA curves were compared with one another regarding to peak position, peak shifting, and the presence or lack of peaks in certain temperature values.

RESULTS

UV absorption spectrophotometry
The response fitted a linear regression model, the calibration equation is $A = 0.0466C - 0.0085$ in the concentration range of 4.0~20.0μg/mL and the correlation coefficient is 0.9998. Additionally, the presence of PEG6000 did not interfere the UV absorbance of toltrazuril at 243 nm.

Figure 1. Chemical structure of toltrazuril.
Table 1. The results of solubility test (mg/mL).

<table>
<thead>
<tr>
<th>NO</th>
<th>Toltrazuril</th>
<th>1:10</th>
<th>1:15</th>
<th>1:20</th>
</tr>
</thead>
<tbody>
<tr>
<td>toltrazuril</td>
<td>0.0041</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>physical mixture</td>
<td>-</td>
<td>0.0054</td>
<td>0.0054</td>
<td>0.0058</td>
</tr>
<tr>
<td>SDs</td>
<td>-</td>
<td>0.7914</td>
<td>0.8153</td>
<td>0.8601</td>
</tr>
</tbody>
</table>

Figure 2. Dissolution curves of toltrazuril, physical mixtures and SDs (mass ration 1:10, 1:15 and 1:20).

Saturation solubility study

The solubility data were presented in Table 1. It showed that the PEG6000 enhanced the solubility of toltrazuril in SDs formulations. Solubility of toltrazuril was 0.0041, 0.8601, 0.0058 mg/mL from toltrazuril, 1:20 (w/w) SDs and 1:20 (w/w) physical mixtures, respectively. It was also proved that the solubility of toltrazuril increased with the increment in ratio of PEG6000 in SDs.

Dissolution rate studies

The dissolution rate tests are shown in Figure 2, enhancement of toltrazuril dissolution rate was achieved. The dissolution rate of toltrazuril from the physical mixture was improved as compared to that with crystalline toltrazuril and can be ascribed to the solubilizing effect of PEG6000 (Doshi et al., 1997; Moneghini et al., 2001). Furthermore, SDs had faster dissolution rates than the pure drug and physical mixture. For example, at the end of 60 min, approximately 8.90, 37.86, 56.74, 58.03, 85.25, 91.78 and 97.87% of toltrazuril was released from crystalline toltrazuril, physical mixtures and SDs (mass ratio 1:10, 1:15 and 1:20), respectively.

Differential thermal analysis

The DTA thermograms of toltrazuril, PEG6000, physical mixture and SDS are shown in Figure 3. The thermogram of toltrazuril exhibited an endothermic reaction and its melting peak was at 193.5°C (a). The thermal behavior of PEG6000 exhibited a sharp but slightly broad endothermic peak at 65.7°C owing to its amorphous nature (b). The DTA thermograms of physical mixture exhibited the comprehensive characteristic of toltrazuril and PEG6000. Complete peaks appearance of toltrazuril and PEG6000 were observed in physical mixture (c). The peaks disappearance of toltrazuril and PEG6000 observed in SDs indicated the interaction between toltrazuril and PEG6000, and it attributable to complete miscibility of the drug in the melted carrier (d).

DISCUSSION

The described solvent melting method in preparation of SDs appeared to be suitable for improving toltrazuril solubility. It is the common method for preparation SDs. The method involves melting the carrier followed by addition of the toltrazuril solution, evaporation of the solvent, and cooling to obtain the product. The uniformity was influenced by the different ways of toltrazuril adding to the PEG6000. Ultimately it affected the dissolution rate of toltrazuril.

The solubility study indicated that PEG6000 as the carrier in SDs leads to an improvement in the solubility of toltrazuril. The solubility increase observed for SDs may be attributed to the presence of an optimum hydrophilic
environment and finer distribution of toltrazuril in PEG6000 as the SDs corresponds to its eutectic composition. Enhancement of toltrazuril dissolution rate was achieved, but the full mechanism behind the improved dissolution rates for amorphous drug compounds stabilized by a hydrophilic carrier is still not fully understood (Leuner and Dressman, 2000). This dissolution has been suggested to either be carrier-controlled or drug-controlled. For the carrier controlled, the dissolution is dominated by the properties of the carrier, whereas for the drug controlled, drug properties such as particle size and physical form can be linked to the dissolution rate. The possible reasons for solvent-melting method, synergistic effect of trituration and solubilization of used solvent reduces crystallinity leading to improvement in dissolution rate. The other reason may be due to availability of increased surface area of particles PEG6000 and dispersing uniformity.

DTA provided the evidence that SDs were formed. When toltrazuril changed into another crystal lattice, it’s melting, boiling, or sublimation point generally shifted to a different temperature or disappears within the temperature range where PEG6000 decomposes.

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REFERENCES


Antioxidant, anti-cholinesterase and antibacterial activities of the bark extracts of *Garcinia hombroniana*

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This study reports an antioxidant, anti-cholinesterase, antibacterial activities and total flavonoids content of different extracts (water, methanol, ethyl acetate, dichloromethane and n-hexane) of the bark of *Garcinia hombroniana*. The antioxidant activity was evaluated using radical scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP) and Folin Ciocalteu assay (FCA). Ethyl acetate extract showed the highest antioxidant activity (DPPH = 8667.7 ±166.6 μmol TE/g (trolox equivalent), ABTS (IC50 = 3.03 μg/ml), FRAP (5579.8 ± 117.7.7 μmol TE/g) and FCA = 3320.4 ± 98.3 μmol GAE/g (gallic acid equivalent)). The flavonoids content of ethyl acetate extract is 2385.7 ± 87.3 RE/g (rutin equivalent) and 3317.6 ± 131.0 QE/g (quercetin equivalent) while the methanol extract is 2234.0 ± 90.0 RE/g and 3090.0 ± 135.1 QE/g). Both extracts are statistically close to each other. In anti-cholinesterase study, ethyl acetate extract showed the highest activity against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes with IC₅₀ of 13.73 ± 1.56 and 32.17 ± 0.36 μg/ml, respectively. Dichloromethane and n-hexane extracts showed good antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results of this study showed that the bark extracts of *G. hombroniana* have valuable pharmacological activities.

Key words: *Garcinia hombroniana*, bark extracts, antioxidant, anticholinesterase, antibacterial.

INTRODUCTION

*Garcinia* is a genus of plant belonging to the family Clusiaceae. These plants grow in the form of medium height trees or shrubs and are native to the regions of Asia, Northeast Australia, Madagascar, West Polynesia, Southern Africa and Tropical America (Patil, 2005; Xiwen et al., 2007). Many parts of the plant species of *Garcinia* such as the fruits, leaves, flowers, stem and bark have been used traditionally to treat various diseases including abdominal pain, dysentery, diarrhea, suppuration, infected wound, leucorrhoea, chronic ulcer and gonorrhea. Various biological and pharmacological activities have been reported on the species of *Garcinia*. These include anti-HIV (Chen et al., 1996), anticancer (Liu et al., 2010), antioxidant (Chiang et al., 2003), anti-tuberculosis (Lin et al., 2001), antifungal (Selvi et al., 2003), antibacterial (Negi et al., 2008) and the ability to prevent the formation of acute ulcer (Yamaguchi et al., 2000). The bioactive compounds reported from the plants are xanthones (Bennett and Lee, 1989), flavonoids (Kaikabo et al., 2009) and benzophenones (Williams et al., 2003). Among

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the many species of *Garcinia, Garcinia hombroniana* appeared to be the one which is rarely studied. The local name of the plant is "manggis hutan", translated as "jungle mangosteen". The fruits are edible, while the roots and leaves are used to relieve itching (Gimlette and Burkill, 1930). There has been an account on the low-density lipoprotein (LDL) antioxidant and antiplatelet aggregation activities of the compounds isolated from the twigs of *G. hombroniana* (Saputri and Jantan, 2012). Other reports on the randomly isolated compounds from the plant did not show any significant biological activities (Rukachaisirikul et al., 2000, 2005). In view of the limited amount of data concerning the biological and pharmacological potential of the plant, the present study is carried out to evaluate the antioxidant, anti-cholinesterase and antibacterial activities of the bark extracts of *G. hombroniana*.

**EXPERIMENTALS**

**Chemicals**

All the chemicals used in the antioxidant and antibacterial assays were purchased from Sigma Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) companies. Acetylthiocholine iodide (ATCI), acetylcholinesterase from electric eel (ACHE), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), butyrylcholinesterase from equine serum (BChE), S-butyrylthiocholine chloride and physostigmine for anti-Alzheimer's activity were purchased from Sigma Chemicals (St. Louis, MO, USA). All the chemicals were of analytical grade.

**Plant collection and extraction**

Plant was collected from the Botanical Garden, Penang where a voucher specimen (PBGK12) was deposited. The air dried bark of *G. hombroniana* was extracted using Soxhlet with solvents of increasing polarity in a successive manner. The solvents used were *n*-hexane, dichloromethane, ethyl acetate and methanol. The extracts obtained were concentrated *in vacuo* at 40°C prior to drying with gaseous nitrogen.

**Antioxidant assays**

**DPPH radical scavenging capacity assay**

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay was carried out according to the method described by Brand-Williams et al., (1995) and modified by Thaipong et al., (2006). In brief, DPPH stock solution was prepared by dissolving 24 mg DPPH in 100 ml methanol. Prior to conducting the assay, a working solution with an absorbance of approximately 1.1 at 515 nm was prepared by diluting 10 ml stock solution with 45 ml methanol. A series of Trolox standard solutions in the concentration range of 50 to 800 μM was prepared. For the assay of plant extracts, samples were prepared at a concentration of 100 μg/ml. An aliquot of 150 μl of each standard solution (trolox) and samples (extracts) was added to 2850 μl of the DPPH solution and the mixture was allowed to incubate in the dark at room temperature for 24 h. The absorbance was then taken at 515 nm. A vehicle blank was prepared using methanol to serve as the control. The inhibition percentage was calculated according to the following formula:

\[
\text{% inhibition} = \frac{A_{\text{sample}} - A_{\text{control}}}{A_{\text{control}}} \times 100
\]

The capacity of the extracts to reduce DPPH was obtained from the standard curve and the results are expressed as μmol trolox equivalent (TE)/g of extract.

**ABTS radical scavenging assay**

ABTS radical scavenging assay was carried out in accordance to the method described by Thaipong et al., (2006) with some modifications. Stock solutions of 7.4 mM ABTS and 2.6 mM potassium persulfate were prepared. Prior to the assay, both solutions were combined at equal volume and the mixture was allowed to react in the dark for 16 h to produce ABTS radical cation. Then, 1 ml of the solution was diluted with deionized water until an absorbance reading of approximately 0.73 was attained at 734 nm. A 150 μl aliquot of each sample with the concentration of 0.25, 1.5, 2.5, 5, 10, 15 and 25 μg/ml were added to 2850 μl of the diluted ABTS solution and the absorbance was measured at 734 nm after 6 min. Standard solutions of trolox and gallic acid were also prepared for comparison. A dose-response curve was constructed by plotting percentage inhibition against the concentration of the samples as standard. The concentration required to scavenge 50% of the free radicals (IC50) was calculated using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, USA).

**Ferric ion reducing antioxidant power (FRAP) assay**

FRAP assay was done according to the method described by Benzie and Strain (1996) with some modifications. Working solution of FRAP was prepared by mixing 25 ml of 300 mM acetate buffer (pH 3.6), 2.5 ml of 10 mM FeCl3.6H2O. The freshly prepared mixture was warmed to 37°C prior to use. Standard solution of trolox was prepared in the concentration range of 50 to 800 μM. The samples were prepared at 100 μg/ml. 150 μl aliquot of each standard solution and samples was then added to 2850 μl of the FRAP reagent and the mixture was allowed to react in the dark at room temperature for 2 h. The absorbance of the product was then measured at 593 nm. Results were expressed in μmol TE/g of extract.

**Folin-Ciocalteu assay (Total phenols assay)**

The total phenols assay was performed according to the method described by Swains and Hillis (1959) and modified by Thaipong et al., (2006). Gallic acid standard solution in the concentration range of 50 to 1000 μM was used to construct the calibration curve. The samples were prepared at 100 μg/ml. A volume of 150 μl of each standard solution and samples was combined with 2400 μl of deionized water and 150 μl of 0.25 N Folin-Ciocalteu reagent and the mixture was allowed to react for 3 min. Then, 300 μl of 1 N Na2CO3 solution was added and the mixture was allowed to incubate for 2 h at room temperature (25°C) in the dark. Absorbance was then measured at 725 nm. The results were represented as μmol gallic acid equivalent (GAE)/g of extract.

**Total flavonoids content analysis**

The flavonoids content of the extracts of *G. hombroniana* was determined using the colorimetric assay described by Zhishen et al., (1999). In brief, a series of rutin and quercetin standards were prepared in the concentration range of 50 to 1000 μM. The extracts
have a concentration of 500 µg/ml. A volume of 0.5 ml of each standard and extracts was added to 4.5 ml of distilled water in a 10ml vial. 0.3 ml of 5% (w/v) NaNO₂ was added to the vial and mixed well. After 5 min, 0.6 ml of 10% (w/v) AlCl₃ was added followed by a waiting time of another 6 min. 2 ml of 1 M NaOH was then added and the solution was made up to the volume of 10 ml with distilled water. The absorbance was measured at 510 nm. The total flavonoids content of the extracts was expressed as µmol RE/g and µmol QE/g extract.

Cholinesterase enzymes inhibitory assay

Cholinesterase enzymes inhibitory potential of the test samples were determined by Ellman's microplate assay (Ellman et al., 1961) with some modification. Physostigmine was used as positive control. The test samples and physostigmine were prepared in DMSO at the initial concentration of 1 mg/ml. The concentration of DMSO in final reaction mixture was 1%. At this concentration, DMSO has no inhibitory effect on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes.

AChE inhibitory assay

In this assay, 140 µl of 0.1 M sodium phosphate buffer (pH 8) was added to 96 wells microplate followed by 20 µl of test samples and 20 µl of 0.09 units/ml AChE enzyme. Then, 10 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was added into each well followed by 10 µl of 14 mM of acetylthiocholine iodide. Absorbance of the colored end product was measured using Tecan Infinite 200 Pro Microplate spectrometer at 414 nm, 30 min after initiation of the enzymatic reaction.

Butyrylcholinesterase (BChE) inhibitory assay

BChE inhibitory assay adopt the same procedures as described earlier with BChE from equine serum as enzyme and S-butyrylthiocholine chloride as substrate. Absorbance of the test samples was corrected by subtracting the absorbance of their respective blank. A set of five concentrations was used to estimate the 50% inhibitory concentration (IC₅₀).

Antibacterial assay

Bacteria culture and preparation

The antibacterial activities of G. hombroni ana extracts were evaluated on four types of bacteria, namely, Staphylococcus aureus (ATCC 29213), Bacillus subtilis (ATCC 16859), Pseudomonas aeruginosa (ATCC 17588) and Escherichia coli (ATCC 25922). Prior to the assay, an inoculum of bacteria was prepared in Muller-Hinton broth from the bacteria culture and the inoculum was diluted to the turbidity of 0.5 McFarland Standard. The standardized bacteria inoculum was then used for both disc diffusion and minimum inhibitory concentration (MIC) assays.

Disc diffusion method

Approximately 400 µl of the bacteria inocula was pipetted and spread onto the agar prepared in a Petri dish. Separately, an aliquot of 10 µl extract with a concentration of 3 mg/ml was added to filter paper discs of 6 mm diameter. The discs were then placed on the inoculated agar. The Petri dish was incubated at 37°C for 24 h. Diameters of the clear inhibition zones were then measured.

Vancomycin and streptomycin were used as positive control while the carrier solvent was used as the negative control.

MIC assay

Plant extracts prepared in DMSO were diluted in the culture broth prior to transferring into a 96-well plate. The final concentration of the samples were 62.5, 125, 250, 500, 1000 and 2000 µg/ml. The 96-well plate was then allowed to incubate at 37°C for 24 h. At the end of the incubation period, 50 µl of a freshly prepared solution of para-iodonitrotetrazolium (INT) with the concentration of 200 µg/ml was added to all wells and the plate was returned to incubation for another 30 min. Bacteria inhibition was evaluated by observing the colour changes in each well. MIC of the plant extract was regarded as the lowest concentration that inhibits bacteria growth. The inhibition results were compared with Vancomycin and Streptomycin.

Statistical analysis

All data were analyzed and expressed as means ± standard deviation of three replicates (n = 3). The differences between the assayed values of the various extracts were analyzed using one-way analysis of variance (ANOVA) which is a parametric test, followed by Tukey’s honestly significant difference (HSD) Test at 95 and 99% confidence interval. Results with p < 0.05 were considered significant, while those with p < 0.01 were regarded as very significant. This analysis was carried out using Statistical Package for Social Sciences (SPSS) software, version 18.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

Antioxidant activities and total flavonoids content

Antioxidants are gaining an increasing amount of attention in the recent decades as clinical trials and epidemiological studies have found a close correlation between the intake of fruits and vegetables rich in antioxidants to the reduction of risk in several chronic diseases, such as cancer, inflammation and atherosclerosis (Kris-Etherton et al., 2002; Gosslau and Chen, 2004; Podsędek, 2007; Asgarpanah and Ramezanloo, 2012). Although, several synthetic antioxidants are available to date such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), their applications are limited due to controversies over their tumour promoting properties (Hocman, 1988). As such, recent efforts have been concentrating on searching for safer antioxidants, particularly those of natural product origin.

In the present study, the bark extracts of G. hombroniana were evaluated for their antioxidant potential in vitro. Three assays, namely the DPPH, FRAP and Folin-Ciocalteu assays indicated that all polar extracts possessed antioxidative properties. The order of the antioxidative capacity of the extracts was found to be: ethyl acetate extract > methanol extract > water extract (Table 1). The non-polar extracts, such as the n-hexane and dichloromethane extracts, were found to have poor antioxidative effect. The ability of the plant extracts to
Table 1. Antioxidant activities of the various extracts of *G. hombroniana*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>DPPH assay (μmol TE/g)</th>
<th>FRAP assay (μmol TE/g)</th>
<th>Total phenols assay (FCA) (μmol GAE/g)</th>
<th>Total flavonoids assay (μmol RE/g)</th>
<th>μmol QE/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>7220.1±19.8*</td>
<td>4127.5±74.7</td>
<td>2312.9±94.0</td>
<td>1716.1±27.3*</td>
<td>2313.1±40.9</td>
</tr>
<tr>
<td>Methanol</td>
<td>7859.2±69.6*</td>
<td>4709.6±88.0</td>
<td>3320.0±88.8</td>
<td>2234.0± 90.0</td>
<td>3090.0±135.1</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>8667.7±166.6*</td>
<td>5579.8±117.7*</td>
<td>3320.4±98.3</td>
<td>3317.6±131.0</td>
<td></td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>&lt;500</td>
<td>&lt;2000</td>
<td>&lt;600</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>&lt;500</td>
<td>&lt;2000</td>
<td>&lt;600</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Results are mean values of 3 replicates ± SD. *The mean difference is statistically significant (p < 0.05).

inhibit ABTS$^+$ free radicals were further evaluated at seven different concentrations (Figure 1). The ethyl acetate, methanol and water extracts were found to have a comparable inhibition profile with IC$_{50}$ of 3.03, 3.43 and 3.77 μg/ml, respectively as compared to 3.17 μg/ml for trolox. Complete inhibition of the ABTS$^+$ free radical was attained with 10 μg/ml of ethyl acetate and methanol extracts, and 15 μg/ml of water extract, while a slightly lower concentration of trolox 6.25 μg/ml was needed to inhibit all free radicals. On the other hand, all three extracts were found to have much lower antioxidative capacities as compared to gallic acid (IC$_{50}$ = 0.58 μg/ml).

The polar extracts which showed strong antioxidative capacities were further evaluated for total flavonoids content (TFC). The methanol and ethyl acetate extracts were found to have almost equal content of flavonoids (Table 1). Statistically, the differences between the results observed for these two extracts were not significant (p > 0.05). As such, the strong antioxidative effect observed in all these three extracts may be correlated to their total flavonoids content.

Cholinesterase enzymes inhibitory assay

Alzheimer Disease (AD) is a chronic and progressive neurodegenerative disorder of the brain which is characterized clinically by deterioration in activities of daily living, behaviours and cognition (Grossberg, 2003). According to the cholinergic hypothesis, memory impairment in patients suffering from AD resulted from decreased level of neurotransmitter acetylcholine. Thus, attempts of researchers are to discover cholinesterase inhibitors in order to sustain the concentration of acetylcholine in the synaptic cleft and prolong its effect (Francis et al., 1999). Table 2 summarizes the IC$_{50}$ and the selectivity index of examined bark extracts and standard drug, physostigmine. Among all the examined extracts, ethyl acetate extract demonstrated the strongest inhibitory activity against AChE and BChE (13.73 ± 1.56, 32.17 ± 0.36 μg/ml), in which AChE is approximately three times more potent than BChE. The dichloromethane extract showed a reasonable inhibition against BChE only (22.35 ± 1.4 μg/ml), while methanol and water extracts
The study showed that ethyl acetate extract were weak cholinesterase inhibitors. The ethyl acetate extract was found to inhibit the growth of *S. aureus* with a MIC value of 62.5 µg/ml. The ethyl acetate extract was able to inhibit the growth of *B. subtilis* with an inhibition zone of 7.5 to 9.5 mm. Further evaluation revealed that the dichloromethane extract was found to be inactive in all three biological studies. This study pointed out the potential of *G. hombroniana* as a new source of phytomedicine for treatment of Alzheimer’s disease, bacterial infection and other diseases which occur from oxidative stress. Further work to isolate and characterize the constituents responsible for the biological activities of the plant is currently ongoing in our laboratory.

**Antibacterial activity**

The various extracts of *G. hombroniana* were tested for antibacterial activities *in vitro* using the disc diffusion technique and MIC assay. Results in Table 3 show that *n*-hexane extract (3 mg) was able to inhibit the growth of *S. aureus, B. subtilis* and *P. aeruginosa* on agar plates with an inhibition zone of 7.5 to 9.5 mm. Further evaluation of the *n*-hexane extract showed MIC values of > 100 µg/ml, indicating weak antibacterial activity. The dichloromethane extract (3 mg) was found to be active against all the four types of bacteria by disc diffusion, with inhibition zones between 8 and 9.5 mm. Further evaluation revealed that the dichloromethane extract was able to inhibit the growth of *B. subtilis* and *P. aeruginosa*, with a MIC value of 62.5 µg/ml. The ethyl acetate extract was found to inhibit the growth of *S. aureus* and *E. coli* with an inhibition zone of 7 and 8.5 mm in disk diffusion method. However, in MIC method the antibacterial effect was found to be very weak. The water and methanol extracts were inactive against all the four bacteria strains.

**Conclusion**

The various extracts of the bark of *G. hombroniana* were evaluated for antioxidant, anti-cholinesterase and antibacterial activities. The study showed that ethyl acetate extract has good antioxidant and anti-cholinesterase activities, while the dichloromethane extract has good antibacterial activity. However, the methanol and water extracts showed lower antioxidative capacities and they were weak cholinesterase inhibitors. The *n*-hexane extract was found to be inactive in all three biological studies. This study pointed out the potential of *G. hombroniana* as a new source of phytomedicine for treatment of Alzheimer’s disease, bacterial infection and other diseases which occur from oxidative stress. Further work to isolate and characterize the constituents responsible for the biological activities of the plant is currently ongoing in our laboratory.

**ACKNOWLEDGEMENTS**

This study was supported by the Short Term Grant(230/PKIMIA/6711179), Research University Grant (RU1001/PKIMIA/811129) provided by Universiti Sains Malaysia (USM) and the Fundamental Research Grant Scheme (203/PKIMIA/6711227) provided by Ministry of Higher Education (MOHE). We would like to thank Penang Botanical Garden for providing the sample (bark) for this research work. Jamila Nargis was sponsored by TWAS-USM PG fellowship.
Full Length Research Paper

Effects of leptin on colorectal cancer cell line HT-29

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The purpose of this study was to investigate the effect of leptin on the growth of HT-29 colorectal cancer cell line. HT-29 cells were treated with leptin at several concentrations of 0, 5, 50, 100, 200 ng/ml, respectively. Epidermal growth factor (EGF) was taken as the positive control. The effect of leptin on the growth, proliferation and apoptosis of HT-29 colorectal cancer cell line was assayed through drawing growth curve and by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method and flow cytometry (FCM). According to the growth curve, the cell counts of HT-29 cell line increased in proportion to leptin concentration, varying from 5 to 200 ng/ml at a dose-dependent manner. However, the cell proliferation amounts of 200 ng/ml leptin group were still less than those of the positive control group (EGF). The MTT results were similar to those from cell counting method. Leptin at different concentration levels promoted the growth of HT-29 cell lines in a time- and dose-dependent manner, and the proliferation rate varied from 5.1 to 67.9%. FCM analysis showed that leptin can significantly affect cell cycle of HT-29 cells. With increasing concentrations, G0/G1 phase cells decreased gradually, and S and G2/M phase cells rose gradually, but no significant effect of leptin on apoptosis of HT-29 cells was observed. In vitro, leptin could promote growth and proliferation of HT-29 cell line according to the concentration and experimental time, but there was no significant correlation in apoptosis.

Key words: Leptin, colorectal neoplasms, cell proliferation, apoptosis, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, flow cytometry.

INTRODUCTION

Colorectal cancer is a very common gastrointestinal malignancy. In western countries, it is the third most common malignancy in both males and females, and the second leading cause of cancer-related death (Song et al., 2012). In China, it is the fourth common malignant tumor and is the fifth key cause of cancer-related death (Hu et al., 2012). The prognosis is closely related with tumor TNM stage. It is generally believed that the development of colorectal cancer is closely related to dietary habits, genetic factors and colorectal adenoma (Zandonai et al., 2012; Parkin and Boyd, 2011; Di Gregorio et al., 2012; Mates et al., 2012). However, the pathogenesis of colorectal cancer is not clear yet. Leptin as the obese gene product, produced by adipocytes, is an important regulatory molecule in energy regulation and food intake (Kishali, 2011; Türkmen, 2011; Gisou et al., 2009). The present study found that leptin was a growth factor for a wide variety of tumor. It promoted cell proliferation, angiogenesis, tumor invasion and metastasis, and inhibited cell apoptosis (Chen et al., 2007; Guo and Gonzalez-Perez, 2011; Zhou et al., 2011). In this study, the role of leptin on the growth and apoptosis of colorectal cancer cell line HT-29 was investigated, so as to provide a new theoretical basis for the pathogenesis of colorectal cancer.

MATERIALS AND METHODS

Cell line

The human colorectal cancer cell line HT-29 was obtained from the Cancer Research Institute, Xiangya School of Medicine, Central South University (Changsha, China). Cells were adherently cultured...
in RPMI1640 medium (Gibco) supplemented with 10% calf serum (Gibco), 50000 IU/L benzylpenicillin and 50 g/L streptomycin, and were incubated at 37°C in a humidified incubator containing 5% CO2. Cells were digested and passaged with 0.25% trypsin (Sigma) every three days. Normally, HT-29 was epithelioid and grown adherently in a single layer. Before every experiment, cells were stained by trypan blue to ensure that the rates of living cells were greater than 98%. Cell Quest software was used for the analysis.

Detection of the effect of leptin on the growth of HT-29 by cytometry

Cells were dispersed with trypsin (Sigma). After cell counting, cells were seeded in a 96 well plate at 8×104 cells per well. After cultured for 24 h, cells were treated by increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) respectively. Adding just culture solution was the negative control group (0 ng/ml) and adding 100 ng/ml EGF was the positive control group. Then cells were cultured in the incubator for 24, 48, and 72 h. After incubation, cell counting was performed. Cellular growth curve was drawn according to incubation time and cell numbers.

Detection of cellular proliferation by MTT assay

Cells were plated in a 96 well plate at 1×104 cells per well in triplicate for five treatment groups and cultured at 37°C in a humidified incubator containing 5% CO2 for 24 h. Then cells were treated in the presence of increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) for a sequence of time incubating for 24, 48, and 72 h, respectively. Twenty microlitres of 0.5% MTT reagent (Gibco) was added into each well and cells were cultured for additional 4 h. After that, the supernatant fluid was discarded, and the remaining formazan crystals were dissolved by adding 200 µl DMSO (Gibco) into each well. After concussion for 10 min, optical densities at 492 nm were measured using automatic microplate reader. The average results were reported. The growth rates of cells were calculated using the following formula: growth rate = (1 - average value of untreated control well/average value of experimental well) × 100%.

Cell cycle analysis by flow cytometry

Cells were seeded into several culture flasks of 75 ml. Five millilitre RPMI1640 culture solution was added to each culture flask. After incubation for 24 h, cells were treated in the presence of increasing concentrations of leptin (0, 5, 50, 100, 200 ng/ml) and then were cultured in incubator. After 3 days, cells were harvested by trypsin (Sigma), and washed twice with PBS. Then cells were fixed and passaged with 0.25% trypsin (Sigma) every three days. Normally, HT-29 was epithelioid and grown adherently in a single layer. Before every experiment, cells were stained by trypan blue to ensure that the rates of living cells were greater than 98%. Cell Quest software was used for the analysis.

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RESULTS

Detection of the effect of leptin on the growth of HT-29 by cytometry

After incubation with different concentrations of leptin, cellular growth curve was drawn as shown in Figure 1. The results show that after intervention, cells in the negative control group grew normally, and when the concentrations of leptin were more than 50 ng/ml, cellular growth curve obviously moved up. In the same period, cell number increased along with the growth of the concentrations of leptin from 5 to 200 ng/ml, showing that leptin dose-dependently promoted cell proliferation. However, even when the concentration of leptin increased to 200 ng/ml, cell number was still lower than that in the positive control group treated with EGF.

Detection of cellular proliferation by MTT assay

To further determine the effect of leptin on HT-29 cell growth, MTT assay was performed to further determine cell growth. The results (Tables 1 and 2) were similar to that of cell counting method, that was leptin dose-
and time-dependently promoted HT-29 cell growth and proliferation in 72 h.

**DISCUSSION**

In the present study, leptin was observed to affect cell cycle, promote cell growth and proliferation, but had no obvious effect on apoptosis in colorectal cancer cell line HT-29.

The pathogenesis of colorectal cancer is a complex, multi-step and multi-stage process. In addition to genetic factors, certain promoting factors also play an important role (Morikawa et al., 2011; Liu et al., 2011). Several studies suggested that leptin participated in the developing of certain tumor (Chen et al., 2007; Guo and Gonzalez-Perez, 2011; Zhou et al., 2011; Liu et al., 2011), but the mechanism has not been clearly elucidated. In this study, the effect of leptin on HT-29 was observed through growth curve method.
Figure 2. The effect of leptin on cell cycle of HT-29 by flow cytometry. (A) Cells were treated with 0 ng/ml leptin; (B) cells were treated with 5 ng/ml leptin; (C) cells were treated with 50 ng/ml leptin; (D) cells were treated with 100 ng/ml leptin; (E) cells were treated with 200 ng/ml leptin.
curves method, MTT assay and FCM. Growth curve method showed that with the increasing concentration of leptin ranging from 5 to 200 ng/ml, compared to the negative control group, leptin promoted HT-29 cell proliferation in the dose- and time-dependent manner. Moreover, the proliferative effect was the highest as cells were treated with 200 ng/ml leptin.

The relationship between EGF and a variety of tumor development has been investigated. On one hand, EGF stimulates cellular growth in a single layer conversing to multi-layer, that is the growth characteristics of certain tumor cells. On the other hand, EGF receptor shares a common amino acid sequence segment with the product of oncogene c-erb-B. Our results show that 100 ng/ml EGF promoted HT-29 cell proliferation in positive control group more than 200 ng/ml leptin, and the difference was statistically significant. The proliferative effect of leptin on HT-29 was obvious, but was still lower than that of EGF.

The cell density in our MTT assay was 5 × 10^5/L, serum concentration in culture medium was 10%, and negative control was also set up. MTT method is mainly used to detect cell viability firstly. Later, it is widely applied in the detection of proliferation of cancer cells (Kang et al., 2002). It is simple, fast and accurate, needing less cell number, and without radiation pollution. The results show that leptin had a strong promoting effect on HT-29 cells in a dose- and time-dependent manner. The lowest growth rate of HT-29 cells was 5.1%, and the highest rate was 67.9%. FCM is applied in the detection of cell cycle and apoptosis, with the advantages of quick, comprehensive analysis, accuracy and reliability. Cell cycle can be divided into interphase and mitosis (M phase). Interphase includes G1, S, and G2 phase, among which S phase can reflect the activity of cell proliferation, as in this period DNA synthesis exists strongly. Flow cytometry was used in our experiment to detect the effect of leptin on cell cycle of HT-29 cells.

The results were presented that with the increase of the concentration of leptin, the rates of G0/G1 phase cells gradually declined, and the rates of S and G2/M phase cells gradually increased, suggesting that leptin has obvious growth promoting effect in a dose-dependent manner. DNA fragmentation is one of the main biochemical changes in apoptotic cells, because restriction endonuclease reaction is an assigned procedure.

Detection the exits of sub-G1 phase could indirectly reflect the exist and the extent of apoptosis (Wu et al., 2010). FCM can commendably reflect the status of cell apoptosis according to DNA content. Our data showed that with the increase of concentration, leptin has no obvious promoting or inhibiting effect on HT-29 cells, which was similar with the previous results in esophageal cancer cell lines (Somasundar et al., 2003).

In conclusion, in the same period, with the increase of leptin concentration, the amount of HT-29 cells increased, the growth rates increased, the rates of S and G2/M phase cells also increased, suggesting that leptin promotes the proliferation of colorectal cancer cells in a dose- and time-dependent manner in vitro, and there is no relationship between leptin and apoptosis of colorectal cancer cells.

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REFERENCES


Full Length Research Paper

Toxicological evaluation of zerumbone on antitumor effects in mice

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Zerumbone (ZER), a bioactive compound isolated from Zingiber zerumbet Smith, was examined for single and repeated-dose toxicity at dosages with antitumor effects on imprinting control regions (ICR) mice and mice of either sex for repeated dose, respectively. For the single dose toxicity study, ZER was administrated to ICR mice at a dose of 500 mg/kg via intraperitoneal injection, while for the repeated dose toxicity study, mice of either sex were studied at dosages of 5, 25 and 50 mg/kg for a period of 28 days. The effects on body and organ weight, food and water consumption, hematology, serum biochemistry as well as histology, were evaluated. No mortality or significant changes in the clinical signs were produced at the single dose toxicity. There were no significant differences in the general condition, growth, organ weights, hematology, serum biochemistry, or histopathological analysis in the repeated dose toxicity as well. These results suggest that ZER is safe in a toxicity study for the cancer treatment in mice regardless of whether male or female mice.

Key words: Zerumbone, toxicity, antitumor effect, single and repeated dose, safety.

INTRODUCTION

Natural products obtained from plants have a long history of beneficial use by mankind for the treatment of diseases (Lucas et al., 2010). With developments of new bioassay methods, isolation of bioactive components from natural sources (plants and animals) and identification of their molecular mechanism of action in the living system has become important pharmacological research area (Aggarwal et al., 2009; Harvey, 2008; Shanmugam et al., 2011). Several promising natural products and natural product-inspired compounds are currently in clinical and pre-clinical developmental stages for cancer treatment (Prasannan et al., 2012). The rationale for the utilization of medicinal plants has rested largely on the long-term clinical experience with little or no scientific data on their efficacy and safety.

Zerumbone (ZER; 2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one) is a sesquiterpenoid found in large amounts in the rhizome of Zingiber zerumbet Smith, a plant traditionally used in Southeast Asian counties as an anti-inflammatory and anti-rheumatic agent and as a condiment. ZER has been reported to have chemopreventive (Ohnishi et al., 2009), anti-inflammatory (Abdelwahab et al., 2010; Murakami et al., 2004) and free radical (hydroxyl free radicals, nitric oxide, singlet oxygen, etc) scavenging activities (Murakami et al., 2002) in several in vitro studies. Intrinsc antioxidant properties of ZER are believed to contribute to its antitumor action (Conti, 2006). ZER induces apoptosis in glioblastoma multiforme, breast cancer, pancreatic cancer, ovarian and cervical cancer cell lines (Abdelwahab et al., 2012; Sehrawat et al., 2012; Weng et al., 2012). In addition, cytotoxic effect of ZER has been reported to be selective toward cancer cells as compared to normal cells (Abdul et al., 2009). ZER also has been reported to inhibit tumor growth in various animal models of inflammation and cancer (Huang et al., 2005; Kim et al., 2009; Prasannan et al., 2011).
et al., 2012; Sulaiman et al., 2010). ZER has been shown to suppress cervical intraepithelial neoplasia by diethylstilbestrol (DES) in female Balb/c mice, at an efficacy close to that of the anti-tumor drug cisplatin (Abdelwahab et al., 2010). Taha et al. (2010) reported that ZER protects rat livers from the carcinogenic effect of diethylnitrosamine (DEN) (single injected dose) and dietary 2- acetylaminoﬂuorene (AAF). ZER has been suggested to modulate many inﬂammation-related molecular targets, and it has also been investigated as a chemopreventive agent. ZER has been used against colorectal and lung cancers, which are known to have chronic inﬂammation as a high risk factor for carcinogenesis (Azad et al., 2008; Xie and Itzkowitz, 2008). However, toxicity has not been reported in ZER for preclinical use.

In a previous study, we investigated that ZER mitigates radiation resistance by heat shock protein 27 (HSP27). Overexpression of HSP27 in tumor cells increases tumor-igenicity and protects against cell death triggered by a number of stimuli (Lelj-Garolla and Mauk, 2005). ZER induced cross-linking of HSP27 protein by its insertion between the disulfide bond, which resulted in a sensitiizing effect to tumors. ZER-mediated altered cross-linking of HSP27 that modiﬁed normal HSP27 dimerization. ZER may be a novel strategy for inhibition of HSP27-mediated radio- and chemo-resistance (Choi et al., 2011). In vivo data using nude mice after grafting of human lung cancer cells indicated that ZER showed synergic tumor regression effects with radiation via radiation-sensitize effect.

Therefore, ZER would be beneﬁcial for cancer therapy as a single and/or combination treatment, especially in tumor types with high HSP27 protein expression. As toxicity tests are important for clinical use, we have assessed the safety of ZER by employing single and repeated-dose toxicity studies in imprinting control regions ICR mice.

**MATERIALS AND METHODS**

**Test and control reagents**

ZER were isolated and puriﬁed from the dried rhizomes of Z. zerumbet Smith (Figure 1). The purities of all compounds (>97%) were identiﬁed and analyzed by high-performance liquid chromatography and nuclear magnetic resonance (Choi et al., 2011). The vehicle control was 1% dimethyl sulfoxide (DMSO) dissolved in phosphate buffer saline (PBS).

**Experimental animals**

All ICR mice (Japan SLC Inc.), aged 7 weeks and weighing 24 to 26 g in females and 27 to 29 g in males, were used after 1 week adaptation in this study. The animals were housed in autoclaved polycarbonate cages on hardwood bedding throughout the study.

![Figure 1. Structure of ZER (2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one).](https://example.com/fig1.png)

The room was maintained at a controlled temperature (22 ± 1°C) and humidity (50 ± 5%) under a time-controlled system with 12 h light/12 h dark cycle. Food and water were supplied ad libitum. Studies were conducted according to the guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS).

**Single-dose toxicity study**

Six male and six female ICR mice were given a single dose intraperitoneal (i.p.) injection of zerumbone at 500 mg/kg. Since Ibrahim et al. (2010) reported that a 500 mg/kg single dose of ZER via oral administration produced severe liver and renal damage in female Sprague Dawley rats, we choose 500 mg/kg as a single injected dose. The control mice were given equal volumes of 1% DMSO in saline. When we compared 1% DMSO in PBS, there was no alteration related toxic effect to mice (Data not shown). All animals were examined for mortality and clinical signs twice a day. Body weights were measured on 0, 2, 4, 6, 8, 12 and 14 days. All of the animals were sacrificed on 14 days and examined by macroscopic lesions. Organs, including the brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus were collected from all animals in control (0 mg/kg) and maximum dose (intraperitoneal injection of 500 mg/kg) mice and were routinely processed, embedded in parafﬁn, sectioned, and stained with hematoxylin and eosin for microscopic evaluation.

**Repeated-dose toxicity study**

After a one-week adaptation, the male or female mice were randomly assigned to each group: vehicle control, ZER at 5 mg/kg (low dose), 25 mg/kg (medium dose), or 50 mg/kg (high dose). The dosage levels of ZER were determined based on the findings in a tumor regression study with NCI460 and NCI1299 human lung cancer cells, in which a decrease in tumor growth was observed at doses of 5 mg/kg (Data not shown). Test drug and control articles.
were administered as an intraperitoneal injection (0.1 ml/mouse) on every odd numbered day for 28 days. Animals were observed each day for signs of toxicity at 30 min, and 1, 3, and 6 h after injection. Daily food consumption was measured and estimated as gram of food eaten over 24-h period. Water was available to animals at all times. Body weights were recorded on all animals prior to the start of dosing (D0) and then at weekly intervals (D7, D14, and D21) until the end of the study period (D28). Measurements of body weight were carried out prior to dose injection.

Blood samples were collected from abdominal vein for hematological and biochemical analysis. Hematology parameters were measured using Hemavet950 Multispecies Hematology Analyzer (DREW Scientific Inc. Oxford, CT, USA) and included: white blood cell count (WBC), neutrophiles (NE), lymphocytes (LY), monocytes (MO), eosinophiles (EO), basophiles (BA), red blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean cell hemoglobin (MCH), and platelets (PLT). Serum biochemical analyses were performed using Dri-Chem4000i (Fujifilm, Japan) for glucose (GLU), total cholesterol (TCH), creatinine (CRE), blood urea nitrogen (BUN), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP).

During the autopsy, the brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus, were grossly evaluated, and were fixed in fixative solution for histopathological analysis. Fixed tissues were embedded in paraffin, sectioned at 3 µm, and stained with hematoxylin and eosin. Any abnormalities in other organs were noted and the organs were collected for histopathological evaluation.

Data analysis

Data are presented as the mean ± standard deviation (SD). Statistical comparisons were conducted using the statistics software SPSS, version 14.0 (SPSS, Inc., Chicago, IL, USA). In the case of body weight, one-factor (time) analysis of variance (ANOVA) with post hoc testing was used for determining group differences. The residuals were analyzed for homogeneity of variance and normality of distributions. Statistical comparisons were made using the Student’s t-test (independent group) and Dunnett’s test. Statistical significance was determined whenever a P value of 0.05 or less was observed.

RESULTS

General condition, symptoms and mortality

All tested animals survived the duration of the experiment. No adverse effects were observed in mice given ZER when compared to the control group. All animals did not show any clinical signs of toxicity. Body weight and food consumption were measured 1 to 3 times a week. Body weight and food consumption were similar in all groups.

Single-dose toxicity study

A single intraperitoneal injection of ZER at 500 mg/kg did not induce any clinical sign of toxicity. ZER in animals showed no differences in body weight, organ weight, and histological examinations when compared with the control during the 14 days study. Based on these data, we conducted a repeated-dose toxicity test in mice.

Repeated-dose toxicity study

ZER was injected at 0, 5, 25, and 50 mg/kg in 14 dosages over 28 days. During the treatment period, no mortality or clinical signs of general toxicity were observed at all the selected doses. Estimated food and water consumption did not differ in all ZER treated groups as compared to the control group (Data not shown). Body weights were measured weekly and no dose-related changes were found in males or females among ZER treated groups as compared to the control group (Figure 2). The organ weights (absolute and relative) did not show significant differences between the control and treated groups of either sex at the end of the treatment period (Table 1).

Hematological parameters

The hematology data indicated that ZER did not induced significant changes in hematological parameters such as erythrocyte, platelets and white blood cells including differential counts (Table 2). All parameters obtained from ZER treated and control groups were in the normal range. Serum biochemistry indicates toxicity of a test material. The results of the effect of the repeated administration of ZER on serum chemistry are summarized in Table 3. ZER did not exhibit any effect on the level of serum chemistry at all groups.

Histopathological evaluations

We analyzed hematoxylin and eosin stained tissue sections from brain, heart, lung, kidney, intestine, liver, reproductive organs, lymph nodes, salivary glands and thymus for general toxicity such as inflammation, hemorrhage, hyperplasia, necrotic cell death, etc. Some of the animals in the ZER treated and control groups showed mild inflammation in the lung, however, there was no statistical difference among the groups. No treatment related gross lesions or histopathological alteration under microscope were observed in all organs of ZER and control groups. The observations in this study imply that mice tolerated well in the single dose and repeated dose toxicity evaluations.

DISCUSSION

The present study was conducted to determine the single
Figure 2. Effect of ZER on body weight changes in male and female mice in the 28-day repeated dose toxicity study. Data represented as mean and SD (n = 6). No statistical differences.

and repeated-dose general toxicity of ZER. In a previous study, we found that the combination of ZER and radiation showed synergistic antitumor effects as compared to ZER or radiation single treatment in vivo and in vitro. ZER produced cross-linking of HSP27, which is dependent on inhibition of the monomeric form of HSP27. ZER directly inserts between the disulfide bond in the HSP27 dimer and modifies normal HSP27 dimerization. Pretreatment with ZER before radiation inhibited the binding affinity between HSP27 and apoptotic molecules, such as cytochrome c and protein kinase C (PKC)δ, and induced sensitization in vitro and in an in vivo xenografted nude mouse system (Choi et al., 2011). It is well-known that overexpression of HSP27 in various tumors including breast, colorectal, ovarian, prostate cancers (Arrigo et al., 2007) increases malignancy and metastasis, and protects against cell death triggered by a number of stimuli (Lei-Garolla and Mauk, 2005). However, in the case of HSP27, no strategies such as developing small molecules for HSP27 inhibitors in application to cancer therapy has been attempted, even though functional HSP27 inhibition may be a good strategy for combination therapy with chemotherapy agents or radiation. So, if it confirmed that ZER is safe as a HSP27 sensitizer with a therapeutic combination of chemotherapy and radiation, it would be a novel strategy for cancer therapy.

Our results showed no toxic effects on all organs including liver and kidney in both single and repeated dose of ZER. There are few reports which investigate the toxic effects of ZER in vivo, and are controversial. Ibrahim et al. (2010) showed that ZER causes severe renal and hepatic damage at the single dose of 500 mg/kg in Sprague Dawley rats, but not at the dose of 100 to 200 mg/kg. However, other group reported that several doses of ZER (15, 30, and 60 mg/kg for 11 weeks) protected rat livers in various indicators such as serum enzymes, proliferation and anti-apoptotic index of liver in the liver tumor model (Taha et al., 2010). And Fakurazi et al. (2008, 2009) reported that low concentration of ZER (0.05 to 0.5%) effectively mitigates liver damage induced by chemicals such as paracetamol and ethanol. Currently, it is also reported that ZER attenuates acute pancreatitis and pancreatitis-induced hepatic injury (Wenhong et al., 2012). We used ICR mice in our toxicity study and did not find toxicity in all organs including liver.
No statistical difference. The unit of organ weight: g; ( ): relative organ weights were presented as the mg organ weight per 25 mg/kg body weight.

Table 1. Absolute and relative major organs weights of mice in 28-day repeated dose toxicity of ZER.

<table>
<thead>
<tr>
<th></th>
<th>Male (N=6)</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0.47±0.02 (12.4±0.6) 0.21±0.03 (5.4±0.7) 0.31±0.04 (8.2±1.1) 2.26±0.05 (59.4±1.32) 0.81±0.11 (21.3±1.3) 0.14±0.02 (3.6±0.5)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>5 mg/kg</strong></td>
<td>0.50±0.02 (13.0±0.6) 0.22±0.02 (5.7±0.5) 0.31±0.03 (8.1±0.7) 2.29±0.32 (59.8±8.3) 0.74±0.09 (19.3±2.2) 0.14±0.02 (3.7±0.5)</td>
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</tr>
<tr>
<td><strong>25 mg/kg</strong></td>
<td>0.48±0.03 (12.4±0.7) 0.22±0.03 (5.7±0.8) 0.33±0.05 (8.3±1.2) 2.23±0.13 (57.0±3.3) 0.81±0.05 (20.7±1.3) 0.15±0.03 (3.8±0.8)</td>
<td></td>
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</tr>
<tr>
<td><strong>50 mg/kg</strong></td>
<td>0.48±0.06 (12.5±1.4) 0.23±0.02 (6.1±0.5) 0.30±0.04 (7.8±0.9) 2.27±0.09 (58.9±2.2) 0.86±0.10 (22.4±2.7) 0.14±0.03 (3.7±0.8)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Female (N=6)</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0.48±0.03 (14.3±0.9) 0.21±0.03 (6.3±0.9) 0.31±0.08 (9.2±2.3) 2.05±0.37 (61.4±11.0) 0.48±0.02 (14.3±0.6) 0.13±0.04 (4.2±1.2)</td>
<td></td>
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</tr>
<tr>
<td><strong>5 mg/kg</strong></td>
<td>0.49 ± 0.06 (14.8±1.0) 0.21±0.04 (6.1±1.2) 0.30±0.04 (8.6±1.0) 2.20±0.14 (63.3±3.9) 0.50±0.04 (14.3±1.1) 0.16±0.03 (4.3±0.9)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>25 mg/kg</strong></td>
<td>0.48±0.05 (14.8±1.7) 0.21±0.05 (6.4±1.7) 0.29±0.04 (8.9±1.3) 2.01±0.31 (62.1±9.5) 0.46±0.06 (14.3±1.9) 0.14±0.03 (4.3±0.9)</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>50 mg/kg</strong></td>
<td>0.50±0.05 (14.9±1.4) 0.20±0.03 (6.0±1.0) 0.30±0.05 (8.9±1.4) 2.04±0.23 (61.4±7.0) 0.50±0.02 (14.9±0.6) 0.15±0.03 (4.4±1.1)</td>
<td></td>
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</tr>
</tbody>
</table>

Table 2. Hematological values of mice in 28-day repeated dose toxicity of ZER.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male (N=6)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5 mg/kg</td>
<td>25 mg/kg</td>
<td>50 mg/kg</td>
<td></td>
</tr>
<tr>
<td>RBC (10^6/μl)</td>
<td>7.05±1.33</td>
<td>7.21±2.14</td>
<td>7.63±0.63</td>
<td>7.19±1.00</td>
<td></td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>13.1±2.0</td>
<td>14.2±3.2</td>
<td>13.5±1.3</td>
<td>13.3±1.7</td>
<td></td>
</tr>
<tr>
<td>HCT (%)</td>
<td>44.4 ± 7.9</td>
<td>41.6±3.0</td>
<td>47.9±4.4</td>
<td>43.6±6.0</td>
<td></td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>60.1±0.6</td>
<td>59.1±0.6</td>
<td>58.4±0.6</td>
<td>58.2±0.7</td>
<td></td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.2 ± 0.3</td>
<td>15.8±0.1</td>
<td>24.2±0.3</td>
<td>24.5±0.5</td>
<td></td>
</tr>
<tr>
<td>WBC (10^3/μl)</td>
<td>8.02±1.76</td>
<td>7.16±0.74</td>
<td>7.51±0.52</td>
<td>7.50±1.23</td>
<td></td>
</tr>
<tr>
<td>NE (%)</td>
<td>20.4 ± 2.7</td>
<td>21.9±1.6</td>
<td>20.57±0.6</td>
<td>21.5±7.5</td>
<td></td>
</tr>
<tr>
<td>LY (%)</td>
<td>73.2±3.4</td>
<td>7.25±1.49</td>
<td>74.56±0.8</td>
<td>73.0±9.6</td>
<td></td>
</tr>
<tr>
<td>MO (%)</td>
<td>5.59±1.26</td>
<td>4.32±2.15</td>
<td>4.25±0.23</td>
<td>5.24±0.74</td>
<td></td>
</tr>
<tr>
<td>EO (%)</td>
<td>0.39±0.23</td>
<td>0.84±1.08</td>
<td>0.51±1.03</td>
<td>0.18±0.08</td>
<td></td>
</tr>
<tr>
<td>BA (%)</td>
<td>0.43 ± 0.10</td>
<td>0.39±0.5</td>
<td>0.11±0.17</td>
<td>0.07±0.05</td>
<td></td>
</tr>
<tr>
<td>PLT (10^3/μl)</td>
<td>1226±341</td>
<td>982±308</td>
<td>1054±193</td>
<td>1300±272</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Female (N=6)</th>
<th>Heart</th>
<th>Lung</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>9.59±0.54</td>
<td>9.84±1.44</td>
<td>9.17±1.26</td>
<td>9.72±1.60</td>
<td></td>
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</tr>
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</table>

No statistical difference. The unit of organ weight: g; ( ): relative organ weights were presented as the mg organ weight per 1 g body weight.
Table 2. Contd.

<table>
<thead>
<tr>
<th>General parameter</th>
<th>Renal function parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dl)</td>
<td>14.6±0.7 15.6±2.4 16.9±2.1 16.1±2.3</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>38.1±3.0 40.1±9.1 37.6±7.9 38.6±9.6</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>60.5±0.6 61.0±0.6 60.5±0.5 59.9±0.8</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.2±0.3 15.8±0.3 15.1±0.3 15.0±0.3</td>
</tr>
<tr>
<td>WBC (10^3/μl)</td>
<td>6.11±2.10 6.48±1.78 6.49±0.70 6.74±1.69</td>
</tr>
<tr>
<td>NE (%)</td>
<td>26.4±2.9 26.1±5.4 21.2±1.9 24.3±8.4</td>
</tr>
<tr>
<td>LY (%)</td>
<td>69.0±3.3 68.4±7.4 73.6±3.0 70.4±10.7</td>
</tr>
<tr>
<td>MO (%)</td>
<td>2.13±0.88 3.54±1.74 3.43±1.57 3.46±1.99</td>
</tr>
<tr>
<td>EO (%)</td>
<td>1.15±0.60 1.53±1.10 0.98±0.61 1.47±0.91</td>
</tr>
<tr>
<td>BA (%)</td>
<td>0.26±0.20 0.39±0.34 0.72±0.37 0.35±0.26</td>
</tr>
<tr>
<td>PLT (10^3/μl)</td>
<td>1301±284 836±405 1121±367 1153±543</td>
</tr>
</tbody>
</table>

No statistical difference.

Table 3. Biochemical parameters in mice after 28-day repeated administration of different dose of ZER.

<table>
<thead>
<tr>
<th>Group</th>
<th>General parameter</th>
<th>Renal function parameter</th>
<th>Hepatic function parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLU P value</td>
<td>TCH P value</td>
<td>CRE P value</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>309±62</td>
<td>-</td>
<td>158±26</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>311±82</td>
<td>0.970</td>
<td>126±20</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>332±53</td>
<td>0.609</td>
<td>144±31</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>297±45</td>
<td>0.777</td>
<td>138±19</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>206±14</td>
<td>-</td>
<td>122±20</td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>219±51</td>
<td>0.561</td>
<td>107±24</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>202±17</td>
<td>0.652</td>
<td>107±14</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>208±35</td>
<td>0.927</td>
<td>108±40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Male</th>
<th>TP p value</th>
<th>AST p value</th>
<th>ALT p value</th>
<th>ALP p value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.52±0.48</td>
<td>49.6±10.0</td>
<td>30.2±7.0</td>
<td>235±36</td>
<td>-</td>
</tr>
</tbody>
</table>


and kidney. Most antitumor studies using ZER in mice induced tumors with the exception of the liver (Abdelwahab et al., 2010; Kim et al., 2009; Murakami et al., 2004). Mouse might have more tolerance to drug toxicity than rats (Finch et al., 1998; McKenna et al., 1997). We also investigated toxicity in ZER treated mice bearing human lung cancer cells at the autopsy and noticed no drug-related toxicity in preliminary study (Data not shown). Therefore, our results suggested that ZER at the dose of 500 mg/kg as a single treatment and 700 mg/kg as total repeated doses did not produce any toxicity to mice. Further studies are needed to clarify ZER toxicity to rat at the effective dosage which can inhibit tumor growth.

ACKNOWLEDGEMENTS
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REFERENCES


Full Length Research Paper

Antidiarrheal activity of methanol extract and major essential oil contents of *Saussurea lappa* Clarke

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Earlier folklore claims reveal that *Saussurea lappa* Clarke (family Asteraceae) is used in the treatment of abdominal pain, dysentery and chronic skin diseases. This species has a good economic potential as its essential oil is commercially valuable in the flavour and fragrance industry. The major bioactive components of *S. lappa* are costunolide and dehydrocostustost lactones. However, other sesquiterpene lactones are also active constituents. The present study reports antidiarrheal activity and major constituents of *S. lappa*. Five groups of Wistar rats (210 to 230 g), each group consisting of 5 animals were taken for the study. Group I was kept as control, providing only saline while group II, III and IV were considered as test group, and the plant extracts (100, 300 and 500 mg/kg body weight) were administrated orally. The fifth group received the standard drug loperamide (5 mg/kg body weight). Qualitative and quantitative analysis of extracted essential oil of *S. lappa* was performed on Perkin-Elmer Gas Chromatography equipped with Perkin-Elmer-Clarus-500 Mass Spectrometry (GC-MS). The individual constituents were identified by comparing their mass spectra to National Institute of Standards and Technology (NIST) and Wiley mass spectral libraries. We reported that application of three different doses of 100, 300 and 500 mg/kg inhibited diarrhea by 26.33, 32.28 and 66.77%, respectively. GC-MS analysis of extracted essential oil of *S. lappa* showed presence of sesquiterpenes, among these, δ-castor oil, 3-elemene were found as major components. The methanol (MeOH) extract significantly exhibited antidiarrheal activity in dose dependent manner. The study supports the traditional claims of *S. lappa* as an antidiarrheal agent.

Key words: Qualitative and quantitative analysis, gas chromatography-mass spectrometry (GC-MS), castor oil, loperamide, wistar rats.

INTRODUCTION

*Saussurea lappa* Clarke [Synonym: *Saussurea costus* (Falc.) Lipschitz] (family Asteraceae) is a well known medicinal plant growing in the Himalayan region between 2500 to 3000 m above sea level. In view of increasing national and international market demand of *S. lappa*, it is also cultivated in a few states of India, including Uttarakhand and Himachal Pradesh. Sesquiterpene lactones such as costunolide and dehydrocostustost lactone, are major components of the roots, and have been reported to possess various biological activities such as antifungal (Barrero et al., 2000), anthelmintic (Seki et al., 1991), antidiabetic (Upadhyay et al., 1996), antitumor (Ko et al., 2005), antimicrobial (Khalid et al., 2011), immunostimulant (Kulkarni and Desai, 2001), antiulcer (Sutar et al., 2011), antiinflammatory (Yashvanth et al., 2010) and antihepatotoxic (Yaeesh et al., 2010). The medicinal importance of *S. lappa* is well reviewed (Pandey et al., 2011).

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Diarrhea disease is a leading cause of mortality and morbidity among children in many developing countries (Das et al., 1999). The majority of the people in developing countries rely on herbal medicine for the treatment of diarrhea. *S. lappa* is used traditionally for the treatment of abdominal pain, dysentery and chronic skin diseases (Kala and Manjrekar, 1999).

Essential oils are valuable natural products used as raw materials in perfumes, cosmetics, aromatherapy, phototherapy, spices and nutrition (Buchbauer, 2000). Earlier studies on essential oil from roots of *S. lappa* showed the presence of (−)-α-selinene, (+)-selina-4, 11-diene, (−)-α-trans-bergamotene, (−)-α-costol, (+)-γ-costol, (−)-elema-1,3,11(13)-triene-12-ol, (−)-α-costal, (+)-γ-costal, (−)-elema-1,3,11(13)-triene-12-al, (−)-(E)-trans-bergamota-2,12-dien-14-al, (−)-ar-curcumene, (−)-caryophyllene oxide (Maurer and Grieder, 1997) and 12-methoxydihydrodehydrocostuslactone (Dhillon et al., 1987). α-Piene, p-cymene, δ-elemene, β-selinene, α-selinene and caryophyllene oxide were also reported from *Artemisia arborescens*, *Michelia macclurei* and *Atractylodis rhizomes* (Younes et al., 2012; Zhou et al., 2012; Wang et al., 2012).

In recent times, the species has been introduced in farmers’ field and successfully cultivated. Except antidiarrheal activity, *S. lappa* has been investigated for several types of biological activities. There is little published data on essential oil contents of *S. lappa*. Therefore, the present study is aimed at evaluating the antidiarrheal activity of *S. lappa* methanol extract and its major essential oil contents.

**MATERIALS AND METHODS**

Collection of plant material and extraction of essential oil

Roots of *S. lappa* (cultivated) were collected from the Herbal Garden of Herbal Research and Development Institute, Gopeshwar, India. The plant was authenticated by Dr. C. S. Rana, State Medicinal Plant Board, Dehradun, Uttarakhand, through existing literature (herbarium No. GUH 19278). The dried roots were pulverized and extracted with MeOH using Soxhlet apparatus. Thereafter, solvent was removed in a rotary evaporator. The methanol extract of *S. lappa* at 100, 300 and 500 mg/kg (body weight) and the standard drug loperamide 5 mg/kg (body weight) were prepared. For extraction of essential oil, the fresh roots of *S. lappa* were cut into small pieces and subjected to hydro-distillation in a Clevenger apparatus (5 h). The pale yellow colored essential oil was collected, dried over anhydrous sodium sulphate and stored in a sealed glass vial at low temperature (0 to 4°C) prior to analysis. All the chemicals and reagents were of Guaranteed Reagent (GR) quality of Merck.

**Animals**

Experiment was carried out on Wistar rats (210 to 230 g) housed in standard cages at room temperature (23 ± 1°C). Food and water ad libitum were provided to the animals and allowed seven days acclimatization period prior to commencement of the experiment. All the experiments were carried out according to the recommendation of International Association for the Study Pain (IASP) committee for research and ethical issues guidelines.

**Acute toxicity test**

The acute toxicity study was carried out on the basis of the method described by Lorke (1983). The MeOH extract of *S. lappa* was administered orally in doses of 2000, 3000 and 5000 mg/kg body weight to the animals, because lower doses were not found toxic. The animals were observed for signs and symptoms of toxicity including mortality for 48 h. The final lethal dosage (LD₅₀) was calculated as the square root of the product of the lowest lethal and highest non lethal dose (that is, geometric mean of consecutive doses for which 0 and 100% survival of rats were recorded). The extract had an LD₅₀ of 2215.8 mg/kg body weight.

**Castor oil induced diarrhea**

Five groups of Wistar rats (210 to 230 g), each group consisting of 5 animals, were used for the study. Group I was kept as control, providing only saline while group II, III and IV were considered as test groups, and the plant extracts (100, 300 and 500 mg/kg body weight) were administered orally using gavage. These doses were prepared in saline as solutions and administered to respective rats groups. The fifth group received the standard drug loperamide (5 mg/kg body weight). After one hour, each experimental animal received castor oil (1 ml/kg body weight basis) orally by gavage. For an early protection of diarrhea, *S. lappa* was administered 1 h before castor oil administration. The animals were placed in individual transparent plastic container over clean non-wetting paper. Diarrhea was determined by the presence of fluid material in the stool, which stained the absorbent paper placed beneath at the cage. Stools were collected on non-wetting paper sheets of uniform weight, up to 24 h after administration of the castor oil. The urine was drained off every 15 min during the first 8 h by gravity, and the net stools weights were recorded. The time between castor oil administration and the occurrence of first diarrheal output was considered as diarrhea free period, while the period between the first and the last diarrheal output of the 8 h observation was considered as acute diarrheal phase. The stools occurring between 8 and 24 h after castor oil administration are considered as late diarrheal excretion (Pazhani et al., 2001). The total score of diarrheal stool for control group was considered as 100%, and results were expressed as percentage of inhibition of diarrhea.

**Gas chromatography-mass spectrometry (GC-MS) analysis**

Qualitative and quantitative analysis of extracted essential oil of *S. lappa* was performed on Perkin-Elmer-Clarus-500 GC equipped with Clarus-500 MS and capillary column (60 m × 0.25 mm, film thickness 0.25 μm). Injector and detector temperatures were 210 and 280°C, respectively, while helium was used as carrier gas. Oven temperature was held for 5 min at 50°C with 5 min solvent delay, then programmed at 3°C/min up to 220°C/min, and then maintained isothermally at 220°C for 20 min. GC-MS was operated in electron ionization (EI) mode at 70 eV.

**Statistical analysis**

Results were presented as mean ± standard deviation (SD), and the data were analyzed by one way Analysis of variance (ANOVA) at 99% confidence level (p < 0.01).
RESULTS AND DISCUSSION

The data of antidiarrheal activity test of methanol extract of S. lappa roots on Wistar rats are presented in Table 1. It was observed that administration of a dose of 100, 300 and 500 mg/kg body weight showed 26.33, 32.28 and 66.77% inhibition of diarrhea, respectively. The standard drug (loperamide) at the dose of 5 mg/kg body weight showed significant reduction (68.02%) in diarrheal stool. Our results revealed that the dose of 500 mg/kg body weight showed almost similar effect to that of standard drug loperamide in reducing diarrheal stool, while the extract at doses of 100 and 300 mg/kg body weight also exhibited remarkable reduction in diarrhea.

Hemamalini et al. (2011) reported that methanol extracts of Anogessius acuminatus at the dose of 300 mg/kg body weight exhibited 57.05% inhibition of diarrhoea similar to standard drug (5 mg/kg diphenoxylate), while in present study, methanol extract of S. lappa roots exhibited 32.28% inhibition of diarrhea at this dose. The protective role of the extract at 500 mg/kg was comparable to that of the reference drug, loperamide (5 mg/kg). Comparison of our results with these findings clearly shows that the MeOH extract of S. lappa exhibited significant antidiarrheal activity.

Hydro-distillation of S. lappa (roots) yielded pale yellow colored oil (yield 0.23% w/v). The individual constituents were separated by gas chromatography and identified by comparing their MS to those of National Institute of Standards and Technology, U.S. Department of Commerce (NIST) and Wiley (John Wiley & Sons Ltd) mass spectral libraries. Upon GC-MS analysis, the hydro-distilled oil was found to contain 42 constituents eluted between 10 to 65 min. Among these constituents, 12 are found to be major constituents representing 58.18% of the oil, which are mainly comprised of sesquiterpenes.

In this study, the major component of the essential oil resulted to be β-costa1 (13.55%) and δ-elemene (12.69%). Other compounds characterizing from S. lappa essential oil were α-selinene (5.02%), β-selinene (4.47%), α-costol (4.02%), 4-terpinol (3.38%), elemol (3.21%), α-ionone (3.13%), β-elemene (3.00%), (-)γ-elemene (2.08%), p-cymene (1.96%) and 2-β-pinene (1.57%). Maurer and Grieder (1997) had also reported


In another study, Liu et al. (2012) identified 39 components from the essential oil of S. lappa roots. The principal compounds in S. lappa essential oil were dehydrocostus lactone (46.75%), costunolide (9.26%), 8-cedren-13-ol (5.06%) and α-curcumene (4.33%). However, the percentages of all these compounds greatly varied in the other reported studies. Existing variations in essential oil composition of S. lappa may be attributed to factors related to ecotype, chemotype, phenophases and the variations in environment conditions such as temperature, relative humidity, irradiance and photoperiod. Moreover, the genetic background may also affect the chemistry of secondary metabolites of plants (Marotti et al., 1994).

Conclusion

The MeOH extract of S. lappa significantly protected the rats against diarrhea evoked by castor oil in dose dependent manner. β-Costa1 and δ-elemene were found as major components in the extracted essential oil. Based on variations in the composition of the essential oil, it can be concluded that the studied accession seems quite different from those investigated in the past. The results of the present study would be useful in promoting research, aiming at the development of a new agent for diarrhea control based on bioactive chemical compounds from S. lappa. Further research is needed to fractionate the MeOH extract and isolate components responsible for the antidiarrheal activity.

REFERENCES


Table 1. Antidiarrheal activity of methanol extract of S. lappa on castor oil induced diarrhea in rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Weight of stool (g) mean ± SD</th>
<th>% inhibition of diarrhea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control) + castor oil</td>
<td>5 ml</td>
<td>3.19±0.24</td>
<td>-</td>
</tr>
<tr>
<td>Lopeamide (standard) + castor oil</td>
<td>5</td>
<td>1.02±0.72*</td>
<td>68.02</td>
</tr>
<tr>
<td>MeOH extract + castor oil</td>
<td>100</td>
<td>2.35±0.21*</td>
<td>26.33</td>
</tr>
<tr>
<td>MeOH extract + castor oil</td>
<td>300</td>
<td>2.16±0.35*</td>
<td>32.28</td>
</tr>
<tr>
<td>MeOH extract + castor oil</td>
<td>500</td>
<td>1.06±0.14*</td>
<td>66.77</td>
</tr>
</tbody>
</table>

*Statistically significant (p < 0.01) relative to control, SD = standard deviation.


UPCOMING CONFERENCES

International Conference on Pharmacy and Pharmacology, Bangkok, Thailand, 24 Dec 2013


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