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Amini Mahabadi J., Khodayari, M., Hassani Bafrani H.*, Nikzad, H., Taherian A.
Wound healing activity of an herbal ointment containing the leaf extract of *Ziziphus Mauritiana* Lam.

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Accepted 19 November, 2012

A large number of plants are used by folklore traditions in India for treatment of cuts, wounds and burns. Particularly, the leaves of *Ziziphus mauritiana* Lam. (Family: Rhamnaceae) have been practiced on wounds for healing. The aim of the present investigation was to assess the *in vivo* wound healing efficacy of prepared topical formulation of 5% w/w ethanolic extract ointment and 5% w/w of aqueous extract ointment and nitrofurazone ointment (0.2% w/w) on excision wound model in Wistar albino rats. The effect produced by ointment, in terms of wound contracting ability, wound closure, decrease in surface area of wound, tissue regeneration at the wound site in treated Wistar albino rats shows that proliferation of epithelial tissue promotes angiogenesis, multiplication of fibrous connective tissue due to treatment with *Z. mauritiana*. Acute toxicity studies revealed the non-toxic nature of *Z. mauritiana*. Ethanolic extract ointment (5% w/w) of *Z. mauritiana* manifested 99% wound contraction on the 16th day. These results were also comparable to those of a standard drug, nitrofurazone. Topical formulation with 5% w/w ethanolic extract promoted wound contraction and reduced the wound closure time, so increase in tensile strength and wound contraction shows the wound healing potential of *Z. mauritiana*. Thus, the present study supports the scientific rationale for the traditional use of this plant in the management of wounds.

Key words: Herbal ointment, *Ziziphus mauritiana*, wound healing, wistar albino rats.

INTRODUCTION

Wound infection is one of the most common diseases in developing countries because of poor hygienic conditions (Senthil Kumar et al., 2006). Healing of wounds starts from the moment of injury and can continue for varying periods of time, depending on the extent of wound, and the process can be broadly categorized into three stages; inflammatory phase, proliferation phase, and finally the remodeling phase which ultimately controls the strength and appearance of the healed tissue (Sumitra et al., 2005). Wound healing process occurs in several steps which involve coagulation, inflammation, formation of granulation tissue, matrix formation, remodeling of connective tissue, collagenization and acquisition of wound strength (Suresh et al., 2002). Research on wound healing agents is one of the developing areas in modern biomedical sciences and many traditional practitioners across the world, particularly in countries like India and China have valuable information of many lesser known hitherto unknown wild plants for treating wounds and burns (Kumar et al., 2007). Many medicinal plants are claimed to be useful for wound healing in the traditional system of medicine. These plant remedies (both single plant and multitherbal reparations) are used since ancient times even though the mechanism of action and efficacy of only very few of them have been evaluated scientifically (Nagappa et al., 2001). *Z. mauritiana* Lam. (Family: Rhamnaceae) is commonly known as jujube tree or Indian jujube, magarya in Hause and whuya in Kilba
(Nigeria). The leaves of the plant are used in the treatment of diarrhoea, wounds, abscesses, swelling, gonorrhoea, liver diseases, asthma and fever (Morton, 1987; Michel, 2002). The different parts of the plant are used as cuts and ulcers healer, pulmonary ailments, fevers, laxative, sedative, anti-nausea, anti-rheumatic areas, anti-diarrhoeal, wounds and abscesses healer, swelling, gonorrhoea curer (Michel, 2002), and also used as anthelminthic in ethnobotanical medicinal system in Pakistan (Hussain et al., 2008). They are also used to treat pulmonary ailments, dysentery, fevers and skin diseases (Adzu et al., 2001). The antioxidant activity of the aqueous extract of Z. mauritiana leaf has been reported (Dahiru and Obidoa, 2008). The hepatoprotective activity of ethanol extract of Z. mauritiana leaf against CCl₄-induced liver damage in rats and the antidiarrhoeal activity of the methanol root extract have also been reported (Dahiru et al., 2006).

In the present study, an indigenous herbal extract of Z. mauritiana which is being claimed to have the potential in the treatment of wounds was selected for the evaluation of wound healing activity of aqueous and ethanolic extract in excision wound models in wistar albino rats.

**MATERIALS AND METHODS**

**Collection of plant material**

The leaves of Z. mauritiana were collected from the Botanical garden at the Campus of Padmavathi College of Pharmacy, Dhanmapuri. The plant was identified and authenticated by Dr. P. Jayaraman, Botanist, Director of Plant Anatomy Research Centre (PARC), Chennai, India. A voucher specimen (PARC/2009/385) was deposited at the herbarium for future reference. The leaves were powdered by means of a wood-grinder and the powder was passed through the sieve no.60 for powder analysis and the coarse fraction was subjected to phytochemical studies.

**Preparation of aqueous extract**

Crude aqueous extract (CAE) of powdered Z. mauritiana was prepared according to the standard methods (Fenado et al., 1989). Briefly, 100 g of the powdered leaves were mixed with 500 mL of distilled water in a 1 L flask and boiled for 1.5 h. Extract was filtered using Whatman No.1 filter paper after cooling it to 40°C. The filtrate was concentrated in a rotary evaporator under vacuum (40°C) and the concentrated extract was stored at 4°C until use. The yield was found to be aqueous extract 8.16% w/w.

**Preparation of ethanolic extract**

Crude ethanolic extract of powdered plants was prepared according to the standard methods (Giliani et al., 2004). Briefly, 1 kg of ground plant material was soaked in sufficient quantity of 70% aqueous ethanol by cold maceration at room temperature for three days after which the filtrate was collected through a piece of muslin cloth and then filter paper and the plant material was resoaked twice. The filtrate was concentrated in a rotary evaporator at 40°C under reduced pressure to yield crude extract. This extract was stored at 4°C until use. The yield was found to be ethanolic extract 8.4 % w/w.

**Formulation of the Herbal ointment**

After preparation of extract and phytochemical studies, the next step was to formulate a polyherbal preparation. A simple ointment base as per Indian pharmacopoeia was prepared by fusion method. It was of first choice due to their ease of preparation, rapid absorption into the skin and also eases of cleaning after application. The weighed quantities of the extract was weighed, added to the molten ointment base and then homogenized in tile by trituration. Four batches of the simple ointment (Group I to III) were prepared and used in the study. Group I received no drug or extract (control). Group II received 5% ethanolic extract of the ointment base. Group III received 5% aqueous extract of the ointment base. Group IV received nitrofurazone ointment (0.2% w/w) (Standard).

**Animals used**

Wistar male albino rats (150 to 180 g) were selected for evaluation of wound healing activity. Six rats were taken for each group. The rats were used after acclimatization under controlled conditions of temperature of 23 ± 2°C, humidity of 50 ± 5% and 10 to 14 h of light and dark cycles, respectively for 7 days. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment and had free access to sterile food (animal chow) and water ad libitum. The animal experimental study was conducted after obtaining the approval of Institutional Animal Ethics Committee, Padmavathi College of Pharmacy. Animal experiments were performed in accordance with the principles of good laboratory practices and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of the Government of India.

**Study design**

The animals were randomly allocated into four groups of six animals each for the Excision wound experimental model. Group I received 0.5 g of Simple ointment (Control); Group II received 5% ethanolic extract in simple ointment (w/w); Group III received 5% aqueous extract in simple ointment (w/w); Group IV received nitrofurazone ointment (0.2% w/w) (Standard).

**Excision wound model**

An excision wound model was used for studying wound healing activity in male albino rats as described by Nagappa et al. (2001), with some modification. Animals were anesthetized prior to and during creation of the wounds with 1 ml of intravenous ketamine hydrochloride (10 mg/kg body wt). Hair was removed by shaving the nape of the back of all the rats. Ethanol (70%) was used as antiseptic for the shaved region before making the wound. A full thickness of the excision wound of uniform 2.5 cm diameter circular area was created along the markings using toothed forceps, scalpel and pointed scissors. The wound was left undressed to the open environment and no local or systemic anti-microbial agents were used. The rats were distributed in groups randomly and each rat was placed in an individual cage. The wistar albino rats were divided into different groups and the test samples (Z. mauritiana extracts) were formulated as an ointment in simple ointment base. 0.5 g of the formulated ointment was applied on the wound once daily for 16 days starting from the day of wounding. The observation of percentage wound closure were made on 2nd, 4th, 8th and 16th day post wounding days. Wound area was measured by retracing the wound on a millimeter scale graph paper.
Table 1. Qualitative preliminary phytochemical screening of ethanolic and aqueous extracts of powdered leaves of Ziziphus mauritiana, Lam.

<table>
<thead>
<tr>
<th>S/No</th>
<th>Phytoconstituents</th>
<th>Ethanolic extract</th>
<th>Aqueous extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Saponins</td>
<td>(-)</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Glycosides</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>4</td>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Tannins and Phenolic compounds</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Fixed oils and fats</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>7</td>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Phytosterols</td>
<td>(-)</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Proteins and amino acids</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Terpenoids</td>
<td>+</td>
<td>(-)</td>
</tr>
<tr>
<td>11</td>
<td>Gums and Mucilages</td>
<td>(-)</td>
<td>(-)</td>
</tr>
<tr>
<td>12</td>
<td>Lignin</td>
<td>(-)</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) = Present; (-) = Absent.

Statistical data analysis

Results were expressed as mean ± standard error of mean (SEM). Statistical comparison were made by using one-way Analysis of variance (ANOVA), followed by Student and Dunnet multiple ‘t’ comparison test using GraphPad Prism software (GraphPad software Inc., Version 4.0.0.255) and difference were considered statistically significant when P-values were < 0.05.

RESULTS AND DISCUSSION

Phytochemical investigation of ethanolic and aqueous extracts of the test plant showed the presence of alkaloids, flavonoid, phenolic compounds and tannins in Z. mauritiana Lam. Also, no sample showed the presence of glycosides. The details of qualitative chemical tests and phytoconstituents present in the extracts are shown in Table 1. The mean wound contraction in the control group was 53.50% (from 506.5 ± 4.61 to 235.5 ± 4.1%) on 16th day. The measurements of the progress of the wound contraction induced by the simple ointment (control), ethanolic extract in simple ointment (5.0% w/w), aqueous extract in simple Ointment (5% w/w), the nitrofurazone ointment (0.2% w/w, Standard) are shown in Table 2. It is observed that the wound contracting ability of the test formulation was significantly greater than that of the control and reference standard (p < 0.05) (Table 2, Figures 1, 2, and 3).

Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other. The process of wound healing occurs in four phases (i) coagulation, which prevents blood loss; (ii) inflammation and debridement of wound; (iii) repair, including cellular proliferation and; (iv) tissue remodeling and collagen deposition. Any phytochemical which accelerates the above process is a promoter of wound healing. Plant products have been shown to possess good therapeutic potential as anti-inflammatory agents and promoter of wound healing due to the presence of active terpenes, alkaloids and flavonoids (Dahiru et al., 2005; Shafaghat et al., 2010). The wound healing property of Z. Mauritiana Lam. appears to be due to the presence of its active principles which accelerates the healing process and confers breaking strength to the healed wound. Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast (Gabbaiani et al., 1976). Since Z. Mauritiana extract enhanced wound contraction, it would have either enhanced contractile property of myofibroblasts or increased the number of myofibroblasts recruited into the wound area.

The results in this study are in support that the wound healing and repair is accelerated by applying Z.
Table 2. Effect of topically applied *Ziziphus Mauritiana Lam* leaves extracts on excision wound model in rats.

<table>
<thead>
<tr>
<th>Animal treatment groups*</th>
<th>Wound contraction (mm²) on day ± SE and percentage of wound contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
</tr>
<tr>
<td>Group 1: Simple ointment (control)</td>
<td>506.5±4.61 (0.0)</td>
</tr>
<tr>
<td>Group 2: 5% w/w Ethanol extract in simple ointment</td>
<td>512.6±3.5 (0.0)</td>
</tr>
<tr>
<td>Group 3: 5% w/w Aqueous extract in simple Ointment</td>
<td>507.4±4.7 (0.0)</td>
</tr>
<tr>
<td>Group 4: Nitro furazone ointment (0.2% w/w) (Standard)</td>
<td>510.5±5.1 (0.0)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (n = 6) statically significant difference in comparison with control group: P < 0.05. *Once a day, for 16 day; control, no treatment.

*Figure 1.* Excision wound healing effect of aqueous extract (5% w/w) in simple ointment treated rat on 8th (A) and 16 (B) days observation.

*Mauritiana Lam.*, which was highlighted by the full thickness coverage of the wound area by an organized epidermis in the presence of mature scar tissue in the dermis (Table 2, Figures 1 and 3). The enhanced capacity of wound healing with *Z. Mauritiana Lam.* could be explained on the basis of the anti-inflammatory effects of the plants that are well documented in the literature (Radhika et al., 2012). On the basis of the results obtained in the present investigation, it is possible to conclude that the ointment of the ethanolic extract of *Z. Mauritiana Lam.* has significant wound healing activity (Table 2 and Figure 2). The antioxidant and anti-inflammatory activities of flavonoids in ethanolic extract and aqueous
Figure 2. Excision wound healing effect of ethanolic extract (5% w/w) in simple ointment treated rat on 8th (A) and 16 (B) days observation.

Figure 3. Excision wound healing effect of Nitrofurazone ointment (0.2% w/w) treated rat on 8th (A) and 16 (B) days observation.

extract were believed to be one of the important mechanisms in wound healing (Marwah et al., 2007), and in the presence of tannin it improved the regeneration and organization of the new tissue and hastened the wound healing process (Leite et al., 2002).

Several phytoconstituents like alkaloids and saponins are known to promote wound healing process due to their antioxidant and antimicrobial activities. The active principal agent responsible for the wound healing activity of leaves of *Z. mauritiana* and their mechanisms of action have not so far been elucidated. However, phytochemical screening of the plant extract revealed the probable presence of tannins and flavonoids which are reported to have good wound healing property (Shafaghat et al., 2010; Mughal et al., 2011; Kamal et al., 2009). The results of wound contraction studies indicate that both the formulations enhance wound healing in open wounds due to antioxidant and anti-inflammatory properties.

**Conclusion**

In this study, topical application of the ethanolic extract of *Z. Mauritiana* Lam. incorporated into an ointment base on the excision wound in rats caused a significantly (*P* < 0.05) higher rate of wound healing and reduced the epithelialization period. The study reveals that both ethanolic extract and aqueous extract of *Z. Mauritiana* Lam. possesses good wound healing properties which may be attributed to the individual or combined action of phytoconstituents like alkaloids, and terpenoids present in the extract. Further investigations are necessary to determine the bioactive constituents present in the extracts to prove its potential in clinical studies.

**REFERENCES**


Full Length Research Paper

Positive inotropic activity exerted by the montelukast-androsterone derivative in a model of congestive heart failure (CHF)


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Accepted 21 November, 2012

Several studies indicate that some steroid derivatives have inotropic activity; nevertheless, there is scarce information about the effects of the androsterone derivatives at cardiovascular level. Therefore, in this study, the inotropic activity of montelukast-androsterone was evaluated using an isolated rat heart model. In the first stage, the Langendorff technique was used to measure perfusion pressure and coronary resistance changes in isolated rat heart in absence or presence of androsterone and its derivative. In second stage, the inotropic activity of montelukast-androsterone was evaluated by measuring left ventricular pressure (LVP) in absence or presence of the following compounds; flutamide, prazosin, metoprolol, nifedipine and carbachol. The results showed that the montelukast-androsterone derivative significantly increase the perfusion pressure and coronary resistance in comparison with androsterone and control conditions. In addition, other data indicate that montelukast-androsterone derivative increase LVP in a dose-dependent manner [1 × 10^-9 to 1 × 10^-4 mmol]; nevertheless, this phenomenon was significantly inhibited by flutamide at a dose of 1 × 10^-6 mmol. Finally, other data indicate that the effect exerted by androsterone derivative [1 × 10^-7 to 1 × 10^-4 mmol] on LVP was blocked in the presence of carbachol [1 × 10^-3 mmol]. All these data suggest that the montelukast-androsterone derivative induces positive inotropic activity through activation of the androgen receptor. In addition, this phenomenon may involve changes on the cAMP concentration.

Key words: Montelukast-androsterone derivative, Langendorff, inotropic activity.

INTRODUCTION

Congestive heart failure (CHF) is a main cause of death in patients with heart disease (Braunwald and Bristow, 2000). Several positive inotropic agents have been used for the treatment of CHF (Feldman, 1993). For example, the use of digoxin (ATP-ase inhibitor) for CHF; unfortunately, the use of this agent is limited by their narrow therapeutic window and their propensity to cause life-threatening arrhythmias (Kersten et al., 2000; Silverberg et al., 2000). Another drug used for treatment of CHF is dobutamine (β1-adrenergic agonist); however, its poor oral bioavailability, involves that it is administered intravenously; in addition, the continuous administration for 24 to 72 h leads to progressive loss of efficacy (Mayes et al., 1995). Additionally, there are studies which compare the effect of dobutamine with levosimendan (Ca2+-sensitizing), the results indicate that treatment with levosimendan in patients with CHF showed an improvement in hemodynamic function in comparison with the group treated with dobutamine; it is important to

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mention that molecular mechanism of levosimendan involved Ca²⁺-sensitization through binding to troponin C in the myocardium, and the opening of ATP-sensitive K⁺ channels in vascular smooth muscle. In general, these mechanisms evoke positive inotropic activity and vasodilation (Papp et al., 2005).

On the other hand, another type of positive inotropic agents such as milrinone have been used for treatment of CHF. In this sense, there are studies which indicate that administration of milrinone at dose of 0.75 µg/kg/min to patients with CHF exerts benefic effects and induce higher cardiac output and decrease systemic vascular resistance; nevertheless, also it can induce ischemic cardiopathy and ventricular arrhythmia in a dose-dependent manner (Hoffman et al., 2003); therefore, the therapeutic management of this drug conditioned its use to large scale. Other pharmacological alternatives have been used for treatment of CHF; for example, the use of inhibitors of angiotensin convertase enzyme (IACE) such as captopril or enalapril. It is important to mention that the use of these agents for CHF may prevent development of patchy myocardial fibrosis and its inherent arrhythmias and thus reduce the incidence of sudden death (Struthers, 1995). There are clinic data which have shown that addition of spironolactone as an adjuvant in patient treated with IACE can induce natriuresis and magnesium retention, increase myocardial noradrenalin uptake, reduce the incidence of arrhythmias and decrease ischemic cardiopathy (Tamargoa and López-Sendón, 2004). Nevertheless, some reports indicate that excessive use of spironolactone was associated with higher number of hospitalizations of patients with hyperkalemia and increase morti-mortality (Cruz et al., 2003). All these data indicate that there are several drugs with inotropic activity for the treatment of CHF; however, the various effects generated by its administration in patients with this clinic pathology may be due to the different doses or different routes of administration or the molecular mechanism involved in the effect induced by each drug used for treatment of CHF. Therefore, in the search of therapeutic alternatives, there has been a resurgence of interest in cardiotoxic steroids derivatives, it is important to mention that these molecules exert a large number of effects in cardiac tissue (Wier and Hess, 1984). For example, the strophanthinid (steroid derivative) increase the force of contraction by changes in the calcium levels (Clark, 1914). In addition, there are studies that show the synthesis of a steroid derivative (F90927) which exerts a positive inotropic activity in cardiac muscle via activation of the L-type Ca²⁺ channel (Pignier et al., 2006). Additionally, a series of steroid derivatives (Gobbini et al., 2001) were synthesized, which showed a positive inotropic effect mainly by inhibition of Na⁺, K⁺-ATPase. Other reports indicate that 14β-hydroxyl progesterone (Templeton et al., 1987) increases the contractility of isolated cardiac tissue via glycoside receptor. Additionally, it is important to mention that recently a furosemide-pregnenolone derivative was synthesized which induce positive inotropic activity in cardiac muscle via activation of the L-type Ca²⁺ channel (Figueroa-Valverde et al., 2011). All these data show that several steroid derivatives induce inotropic effects in the cardiovascular system; nevertheless, the cellular site and molecular mechanism involved in its inotropic activity are quite confusing, perhaps this phenomenon is due to differences in the chemical structure of the steroid derivatives. Therefore, data information is needed to characterize the activity induced by steroid derivatives at cardiovascular level. To provide this information, the present study was designed to investigate the effects of an androsterone derivative on perfusion pressure and vascular resistance in isolated rat hearts using the Langendorff technique. In addition, to evaluate the molecular mechanism involved in the inotropic activity induced by the montelukast-androsterone derivative on left ventricular pressure (LVP), the following compounds were used as pharmacological tools: flutamide [antagonist of androgen receptor] (Simard et al., 1986), prazosin [α₁ adrenoceptor antagonist] (Graham et al., 1977), metoprorolol [selective β₁ receptor blocker] (Bengtsson et al., 1975), nifedipine [antagonist of calcium-channel] (Henry, 1980), and carbachol [non-selective agonist cholinergic] (Billman, 1990).

MATERIALS AND METHODS

Montelukast-androsterone derivative (compound 1; Figure 1) was prepared according to a previously reported method by Figueroa-Valverde (2012) and the other compounds evaluated in this study were purchased from Sigma-Aldrich Co., Ltd. All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of Universidad Autonoma de Campeche (UAC) and were in accordance with the Guide for the Care and Use of Laboratory Animals (Bayne, 1996). Male rats (Wistar; weighing 200 to 250 g) were obtained from UAC.

Reagents

All drugs were dissolved in methanol and different dilutions were obtained using Krebs-Henseleit solution (≤ 0.01%, v/v)

Langendorff technique

Briefly, the male rat (200 to 250 g) was anesthetized by injecting them with pentobarbital at a dose rate of 50 mg/kg body weight. Then, the chest was opened, and a loose ligature passed through the ascending aorta. The heart was then rapidly removed and immersed in ice cold physiologic saline solution. The heart was trimmed of non-cardiac tissue and retrograde perfused via a noncirculating perfusion system at a constant flow rate. It is important to mention that perfusion medium was the Krebs-Henseleit solution (pH 7.4, 37°C) composed of (mmol); 117.8 NaCl; 6 KCl; 1.75 CaCl₂; 1.2 Na₂HPO₄; 1.2 MgSO₄; 24.2 NaHCO₃; 5 glucose and 5 sodium pyruvate. The solution was actually bubbled with a mixture of O₂:CO₂ (95:5). The coronary flow was adjusted with a variable speed peristaltic pump. An initial perfusion rate of 15
ml/min for 5 min was followed by a 25 min equilibration period at a perfusion rate of 10 ml/min. All experimental measurements were done after this equilibration period.

**Induction of CHF**

CHF was developed mainly following a method previously reported (Mondragón et al., 2003). In this process, the pentobarbital (100 mg/kg) was administered through a cannula inserted into the aorta to induce CHF.

**Perfusion pressure**

Evaluation of measurements of perfusion pressure changes induced by drugs administration in this study were assessed using a pressure transducer connected to the chamber where the hearts were mounted and the results entered into a computerized data capture system (Biopac).

**Inotropic activity**

Contractile function was assessed by measuring left ventricular developed pressure (LVdP), using a saline-filled latex balloon (0.01 mm, diameter) inserted into the left ventricle via the left atrium. It is important to mention that latex balloon was bound to cannula which was linked to pressure transducer that was connected with the MP100 data acquisition system.

**Biological evaluation**

**First stage**

Effect induced by the montelukast-androsterone derivative on perfusion pressure: Changes in perfusion pressure as a consequence of increases in time (3 to 15 min) in the absence (control) or presence of androsterone and androsterone-derivative at a concentration of $1 \times 10^{-9}$ mmol were determined. The effects were obtained in isolated hearts perfused at a constant-flow rate of 10 ml/min.

Evaluation of effects exerted by the androsterone derivative on coronary resistance: The coronary resistance in the absence (control) or presence of androsterone and androsterone-derivative at a concentration of $1 \times 10^{-9}$ mmol was evaluated. The effects were obtained in isolated hearts perfused at a constant flow rate of 10 ml/min. Since a constant flow was used, changes in coronary pressure reflect the changes in coronary resistance.

**Second stage**

Effects induced by the montelukast-androsterone derivative on LVP through androgen receptor: Intracoronary boluses (50 µl) of the androsterone derivative ($1 \times 10^{-9}$ to $1 \times 10^{-4}$ mmol) were administered and the corresponding effect on the LVP was determined. The dose-response curve (control) was repeated in the presence of flutamide at a concentration of $1 \times 10^{-6}$ mmol (duration of preincubation with flutamide was by a 10 min equilibration period).

Effect exerted by the androsterone derivative on LVP in the presence of $\alpha_1$ adrenergic blocker: The boluses (50 µl) of the androsterone derivative [$1 \times 10^{-6}$ to $1 \times 10^{-4}$ mmol] were administered and the corresponding effect on the LVP was evaluated. It is important to mention that the bolus injection administered was done at the point of cannulation. The dose-response curve (control) was repeated in the presence of prazosin at a concentration of $1 \times 10^{-6}$ mmol (duration of preincubation with prazosin was by a 10 min equilibration period).

Effects induced by the montelukast-androsterone derivative on LVP in the presence of $\beta_1$ adrenergic blocker: The boluses (50 µl) of androsterone derivative [$1 \times 10^{-9}$ to $1 \times 10^{-4}$ mmol] were administered and the corresponding effect on the LVP was evaluated. The dose-response curve (control) was repeated in the presence of metoprolol at concentration of $1 \times 10^{-6}$ mmol (duration
of preincubation with metoprolol was by a 10 min equilibration period).

Effects of the androsterone derivative on LVP through the calcium channel: Intracoronary boluses (50 µl) of androsterone derivative \([1 \times 10^{-9} \text{ to } 1 \times 10^{-4} \text{ mmol}]\) were administered and the corresponding effect on the LVP was evaluated. The dose-response curve (control) was repeated in the presence of nifedipine at a concentration of \(1 \times 10^{-9} \text{ mmol}\) (duration of preincubation with nifedipine was by a 10 min equilibration period).

Effects induced by the montelukast-androsterone derivative on LVP through non-selective agonist cholinergic: Intracoronary boluses (50 µl) of the androsterone derivative \((1 \times 10^{-9} \text{ to } 1 \times 10^{-4} \text{ mmol})\) were administered and the corresponding effect on the LVP was determined. The dose-response curve (control) was repeated in the presence of carbachol at a concentration of \(1 \times 10^{-3} \text{ mmol}\) (duration of preincubation with carbachol was by a 10 min equilibration period).

Statistical analysis
The obtained values are expressed as average ± standard error (SE) using each heart as its own control. The data obtained were put under an analysis of variance (ANOVA) using the Bonferroni correction factor (Hocht et al., 1999). The differences were considered significant when \(P\) was equal or smaller than 0.05.

RESULTS

First stage

In this study, the activity induced by the montelukast-androsterone derivative on perfusion pressure and coronary resistance in the isolated rat heart was evaluated.

The results obtained from changes in perfusion pressure as a consequence of increases in the time (3 to 15 min) in absence (control) or in presence of androsterone or the androsterone derivative (Figure 2), showed that montelukast-androsterone \([1 \times 10^{-9} \text{ mmol}]\) significantly increases the perfusion pressure \((P = 0.006)\) in comparison with the control conditions and androsterone. Each bar represents the mean ± standard error (SE) of 8 experiments.
Figure 3. Activity exerted by the montelukast-androsterone derivative on coronary resistance. The results show that coronary resistance was higher \( (P = 0.005) \) in the presence of androsterone derivative in comparison with the control conditions and androsterone. Each bar represents the mean ± standard error (SE) of 8 experiments.

Discussion

Several inotropic agents have been used to treat heart failure resulting from myocardial ischemia. Nevertheless, there is scarce information about the effects of the steroid hormones on this clinical pathology. Therefore, in this study the inotropic activity of a new steroid was evaluated using several strategies.

First stage

The activity induced by an androsterone derivative on the perfusion pressure and coronary resistance in isolated rat heart (Langendorff technique) was evaluated. The results obtained showed that the androsterone derivative significantly increased the perfusion pressure in comparison with the control conditions and androsterone. These experimental data indicate that the androsterone derivative exerts effects on perfusion pressure, which could consequently bring modifications in coronary resistance as happening in other type of steroid derivatives (Figueria et al., 2009; Figueroa-Valverde et al., 2010). In order to verify this hypothesis, the effects
Figure 4. Effects induced by the montelukast-androsterone derivative on left ventricular pressure (LVP) through androgen receptors. Intracoronary boluses (50 μl) of androsterone derivative [1 × 10^{-9} to 1 × 10^{-4} mmol] were administered and the corresponding effect on the LVP was determined. The results showed that androsterone derivative increase the LVP in a dependent dose manner and this effect was inhibited significantly (P = 0.005) in presence of flutamide [1 × 10^{-6} mmol]. Each bar represents the mean ± standard error (SE) of 8 experiments.

Figure 5. Effect of the montelukast-androsterone derivative (1 × 10^{-9} mmol) on LVP in an isolated rat heart model. In the graphic, (A) observed androsterone derivative increase the LVP through of time; however, this effect is inhibited in presence of flutamide (B) at a dose of 1 × 10^{-6} mmol.

induced by androsterone and its derivative on coronary resistance were evaluated. The results indicate that coronary resistance in the presence of androsterone derivative was higher in comparison with control conditions.
and androsterone. All this data suggest that androsterone derivative may induce a positive inotropic activity in the isolated rat heart.

**Second stage**

In order to characterize the molecular mechanism of positive inotropic effect from androsterone derivative and analyzing previous reports (Simard et al., 1986) which indicate that some androsterone derivatives exert their effect by activation of androgen receptor in the vascular smooth muscle. For this reason, we used flutamide an androgen receptor blocker to determine if the inotropic activity of androsterone derivative on LVP was via the androgen receptor which may be a key requirement for the biological activity as in the case of other androgen derivatives (Figueroa-Valverde et al., 2010). Our results showed that the effect of androsterone derivative was inhibited by flutamide, suggesting that the molecular mechanism involved in the positive inotropic activity is via the androgen-receptor. Analyzing these data and the reports of Charalampopoulos et al. (2005), which indicate that some steroids derivatives may induce an inotropic activity by exerting indirect effect by the release of adrenal catecholamines concentration. In this study, the effect exerted by the androsterone derivative on LVP was evaluated in the absence or presence of prazosin and metoprolol. The results showed that the effect induced by the androsterone-derivative was not inhibited in the presence of these compounds. These data indicate that the molecular mechanism involved in the effects of this
Figure 7. Activity exerted by the montelukast-androsterone derivative on LVP through L-type calcium channel. Intracoronary boluses (50 μl) of androsterone derivative [1 × 10^{-9} to 1 × 10^{-4} mmol] were administered in absence and presence of nifedipine [1 × 10^{-6} mmol]. The results showed that, the effect induced by androsterone derivative on perfusion pressure in presence of nifedipine was not inhibited. Each bar represents the mean ± standard error (SE) of 8 experiments.

steroid-derivative on LVP is not through adrenergic activity. Therefore, analyzing these results and other reports which suggest that activity induced by an androsterone derivative on blood pressure involved a molecular mechanism via calcium-channels (Figueroa et al., 2009). In addition, there are reports which have shown that positive cardiotonic agents (Smith et al., 1984) act by an increase in intracellular Ca^{2+} and consequently induce an increase in the sensitivity of contractile proteins to Ca^{2+} ions or by combinations of the two mechanisms (Blinks and Endoh, 1986). Therefore, in this study, the activity induced by the androsterone derivative on LVP was evaluated in absence or presence of nifedipine. The results showed that effect exerted by androsterone derivative was not inhibited in the presence of nifedipine. These results suggest that the effect of the androsterone derivative on LVP is not through activation of the L-type calcium channel. Analyzing this data and some reports which indicate that some positive inotropic agents induce higher intracellular Ca^{2+} levels by inhibition of PDE and regulate cAMP concentration (Endoh et al., 1986). Therefore, it was interesting to evaluate whether cAMP could be involved in positive inotropic activity of the montelukast-androsterone derivative, using carbachol as pharmacological tool. It is important to mention that there are reports which indicate that carbachol inhibits selectively the cAMP-dependent positive inotropic effect of cardiotonic agents in mammalian ventricular myocardium (Endoh, 1987). The results showed that positive inotropic effect of the androsterone derivative was inhibited partially by carbachol.

The inhibition induced by carbachol was observed at higher concentrations [1 × 10^{-7} to 1 × 10^{-4} mmol] of montelukast-androsterone derivative; these results suggest that positive inotropic effect of montelukast-androsterone derivative can affect the cAMP levels and consequently bring increase to the LVP in a dose-dependent manner. This phenomenon is similar to inotropic positivity activity exerted by other drugs on ventricular muscle, which involves changes in cAMP concentration that results from the inhibition of cyclic AMP phosphodiesterase (Endoh et al., 1986) which plays an important role in the regulation of LVP. Analyzing these data, the montelukast-androsterone derivative is a particularly interesting drug because the positive inotropic activity induced by this steroid derivative involves a molecular mechanism different in comparison with other inotropic drugs (Akera and Brody,
Figure 8. Activity induced by the montelukast-androsterone derivative on LVP through cAMP changes. Intracoronary boluses (50 μl) of estradiol derivative [1 × 10^-9 to 1 × 10^-4 mmol] were administered and the corresponding effect on the perfusion pressure was evaluated in absence and presence of carbachol (1 × 10^-3 mmol). The results showed that the activity induced by androsterone derivative on LVP was inhibited in presence of carbachol only at higher concentrations [1 × 10^-6 to 1 × 10^-4 mmol] of montelukast-androsterone. Each bar represents the mean ± standard error (SE) of 8 experiments.

Figure 9. Activity exerted by the montelukast-androsterone derivative on LVP in presence of carbachol in an isolated rat heart model. In C, it was observed that the effect induced by androsterone derivative at a dose of 1 × 10^-9 mmol on LVP in presence of carbachol [1 × 10^-3 mmol] was not inhibited. However, with increasing doses of the androsterone derivative (1 × 10^-6 mmol), the inotropic activity was partially blocked by carbachol at the same dose (D).
1977). This phenomenon may result in a decrease in adverse effects such as cardiac arrhythmia and ischaemia induced by several cardiotoxic agents such as cardiac glycosides and sympathomimetic amines (Figueroa et al., 2009).

Conclusions

All these experimental data suggest that positive inotropic activity induced by the montelukast-androsterone derivative on the LVP may involve activation of androgen-receptor and induce indirectly changes in the cAMP concentration.

REFERENCES


Full Length Research Paper

Ultrasound and microwave assisted extraction of luteolin from *Eclipta prostrata*

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Accepted 3 December, 2012

Luteolin (LU) is considered as one of the most important flavonoids with many beneficial functions to human health. In our present study, an efficient, convenient and reproducible extraction method of LU extracted from *Eclipta prostrata* by ultrasound assisted microwave extraction (UAME) was established. The extraction process of LU using UAME was optimized according to single factor test and orthogonal design, and the optimum parameters were obtained as follows: the ratio of 80% ethanol, hydrochloric acid and plant material: 50:0.3:1 (v/v/w), microwave power: 40 W, extracting time: 3 × 3 min. The extraction yield of LU was 0.690 mg/g under the optimum conditions, compared with heat reflux extraction (HRE) and reflux acid extraction (RAE) in terms of extraction yield, time consumption and labor intensiveness, indicating that the UAME was more efficient than RE and RAE. In conclusion, UAME could markedly reduce extraction time and simplify the extraction process of LU from *E. prostrata*.

Key words: Ultrasound assisted microwave acid extraction, luteolin, *Eclipta prostrata* L.

INTRODUCTION

*Eclipta prostrata* L. (Asteraceae) is one of the oldest tonic herbs in traditional Chinese medicine. Its aerial parts have been extensively used in China, Korea, India and Thailand for tonifying the liver and kidney, promoting the growth of hair, resisting hyperlipidemia, improving antioxidant and treating snake venom poisoning (Pithayanukul et al., 2004; Kumari et al., 2006; Roy et al., 2007; Kim et al., 2008; PCCn, 2010). Chemical constituent investigation indicates that *E. prostrata* is rich in flavonoids accounting for the beneficial functions on the human health (Zhang et al., 1997; Wang et al., 2009).

Among the flavonoids in *E. prostrata*, luteolin (LU, Chemical structure shown in Figure 1a) is the main flavones possessing a wide spectrum of pharmacological properties including anti-amnesic (Liu et al., 2009), anti-tumorigenic (Lee et al., 2006; Samy et al., 2006; Du et al., 2008), anti-hepatotoxic (Domitrović et al., 2009), anti-osteoporotic (Choi, 2007), anti-proliferative (Hou et al., 2009), anti-inflammatory (Choi, 2007; Leemans et al., 2010) and antioxidant activities (Horváthová et al., 2005; Ashokkumar et al., 2008). Thus, it prompted us to investigate the extraction technology of LU from *E. prostrata* previously.

Plant cell walls consist of cellulose, hemi-cellulose and pectin, which represent the barrier for the release of intracellular substances (Dong, 2010a). It was reported that the method of heat reflux extraction (HRE) has been used to extract LU from *Herba Ecliptae* associated with such undesirable traits as low yield, time consumption and labor intensiveness (Yang et al., 2008). Recently, Ultrasound assisted microwave extraction (UAME) has been used as an effective method to extract chemical constituents from plant materials (Zhang et al., 2008), which could accelerate the extracting process and improve the release of bioactive compounds (Martino et al., 2006; Fang et al., 2008; Golmakani and Rezaei, 2008; Huang et al., 2009). On the other hand, ultrasound can facilitate the solvation of plant materials by causing cell swelling and enlarging pores of the cell wall. Better swelling could
improve the rate of mass transfer, resulting in the increased extraction efficiency and reduced extraction time (Dong, 2010b). Also, microwave extraction can heat the extracts quickly and accelerate the extraction process for adsorption and desorption of the targeted compounds from matrix but nevertheless, with the concomitant disadvantage of inhomogeneous heating (Bonrath, 2004).

This deficiency can be complemented by coupling microwave extraction with ultrasound (Zhang et al., 2008). Moreover, flavonoids are always occurring in combination with glucoses as glucosides with glucosidic linkages. When the glucosidic linkages were broken, free flavonoids were released (Fu et al., 2008). In order to obtain more free flavonoids, acids such as hydrochloric acid, sulfurous acid and perchloric acid are usually used to break the glucosidic linkages (Dong, 2010c).

The objective of the present study was to investigate the effect of ultrasound assisted microwave on the extraction of LU from *E. prostrata*. Several parameters affecting UAME of LU, that is, types of solvent, ratio of hydrochloric acid to solvent, ratio of solvent to material, microwave power and extraction time were optimized according to single factor test and orthogonal design. Ultimately, to better understand the advantages of UAME, we also compared it with heat reflux extraction (HRE) and reflux acid extraction (RAE) methods.

**MATERIALS AND METHODS**

**Plant**

The herb of *E. prostrata* was purchased from Tianren Pharmaceutical Company (Fujian, China) and identified as the aerial parts of *E. prostrata* by Cheng-Zi Yang, Department of Pharmacy, Fujian University of Traditional Chinese Medicine, Fuzhou, China. Then, voucher specimen was deposited in the herbarium of Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou, China. Samples were dried in vacuum oven at 40°C, then ground and sieved for homogenization (40 mesh). The ground samples were kept in a dry place prior to use.

**Chemicals and reagents**

Luteolin (LU, 3’,4’,5,7-tetrahydroxyflavone) standard was purchased from the Chengdu Mansite Pharmaceutical Co. Ltd. (Sichuan, China) with a purity > 98%. The standard was dissolved in methanol to obtain the stock solution at concentration of 0.2 mg/mL for LU. Methanol of High-performance liquid chromatography (HPLC) grade was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ethanol, methanol, sodium hydroxide and hydrochloric acid (12 mol/L) of analytical grade were obtained from Fuchen Chemicals Reagent Factory (Tianjin, China). Deionized water was purified by a Milli-Q water-purification system from Millipore (Bedford, MA, USA).

**Instrument and analytical conditions**

UAME experiment was performed with an ultrasonic and microwave extracting apparatus (CW-2000, Shanghai Xintuo Analytical Instruments Co., LTD). An open microwave with maximal power of 800 W at a frequency of 2450 MHz, and an ultrasonic transducer with a fixed power of 50 W at a frequency of 40 KHz were the conditions of extraction. The determination of LU was carried out on a Waters liquid chromatographic system (Waters Company, USA) consisting of Masslynx 4.0 system software, Model Waters Delta 600 pump and Model Waters 2996 Photodiode Array Detector (PAD). Chromatographic separation was carried out by Ultrasphere ODS C18 reversed-phase column (4.6 × 150 mm, BECKMAN Corporation, USA) packed with 5 μm diameter particles. LU was quantified by a PAD at 352 nm following Reversed phase-high performance liquid chromatography (RP-HPLC) separation. The mobile phase was methanol 0.4% phosphoric acid (45:55, v/v), which was filtered through a 0.45 μm membrane filter and then deaerated ultrasonically prior to use. The flow rate was 1 mL/min, the injection volume was 10 μL, the column temperature was maintained at 30°C and the retention time for LU was 11.5 min. Chromatographic peaks of LU was confirmed by comparing their retention time and UV spectrum with the reference compound. The working calibration curve based on reference compound of LU showed good linearity over the range of 10 to 200 μg/mL. The regression line was Y = 14630X - 630.14 (R² = 0.9998, n = 7), where Y is the peak area of analysis, and X is the concentration of reference compound (μg/mL).

**Different extraction methods**

**Optimums of the extraction parameters of UAME**

According to LU yield of the extraction from *E. prostrata*, the optimum extraction parameters of UAME were investigated. The ultrasonic and microwave extracting apparatus was used to extract LU from *E. prostrata*: the sample (about 2 g) was transferred into the flask, proper volume (assigned according to the experiment planning) of the extraction solvent added and then the flask was transferred into the chamber of the apparatus connected with a condensing tube. Finally, the door of chamber was closed and the program of the parameters (microwave power and extraction time) was set according to the experimental design. After the process was finished, the flask was removed from apparatus. The mixture was filtered through filter paper immediately and evaporated to dryness by removing the solvent in a rotary evaporator (RE-52, Shanghai splendor and biochemical instrument Co., China) at 40°C under reduced pressure.

If the solvent containing hydrochloric acid was used to extract, the extracts were neutralized by sodium hydroxide solution (5 mol/L) before the solvent evaporation. After cooling, the extract dissolved in methanol was transferred to a 10 mL volumetric flask and diluted with methanol to volume. The obtained sample solution was then passed through a 0.45 μm Millipore membrane prior to HPLC analysis.

Single factor and orthogonal experiments were performed to optimize the extraction condition of LU from *E. prostrata* using UMAE. Firstly, the effects of solvent type (water/methanol/ethanol), ethanol concentration (20 to 100% v/v), ratio of hydrochloric acid (12 mol/L) to solvent (0.006 to 0.024 v/v), ratio of solvent to material (20:1 to 50:1 v/w), microwave power (10 to 50 W) and extraction time (120 to 360 s) on the extraction yield of LU from *E. prostrata* were investigated, respectively. Secondly, an orthogonal test was designed to optimize the extraction parameters depending on the results of the single factor experiments. The factors and levels tested in this study were presented in Table 1. The orthogonal test design consisted of sixteen separate experiments in Table 2. The sequence of the experiments was randomized to ensure the validity of the test results. The statistical software Statistical Package for the
Table 1. Independent factors and levels of orthogonal test by UAME.

<table>
<thead>
<tr>
<th>Level</th>
<th>Ethanol concentration (%)</th>
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<th>Extraction power (w)</th>
<th>Extraction time (s)</th>
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<td>20</td>
<td>10</td>
<td>180</td>
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<td>3</td>
<td>60</td>
<td>40</td>
<td>30</td>
<td>300</td>
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<tr>
<td>4</td>
<td>80</td>
<td>50</td>
<td>40</td>
<td>360</td>
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Table 2. Orthogonal test design and results by UAME.

<table>
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<th>Test no.</th>
<th>Ethanol concentration (%)</th>
<th>Ratio of solvent to material (v/w)</th>
<th>Extraction power</th>
<th>Extraction time</th>
<th>Extraction yield of LU (mg/g)</th>
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</thead>
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<td>1</td>
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<td>1</td>
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<td>3</td>
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<td>4</td>
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<tr>
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<td>1.905</td>
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<td>$K_4$</td>
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<td>$R^2$</td>
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<td>0.228</td>
<td>0.158</td>
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</table>

*Extreme difference (range).*

Social Sciences (SPSS) 18.0 was used to calculate the result of variance analysis (Table 3).

Comparison of luteolin yields extracted from *E. prostrata* by UAME, HRE and RAE

The HRE method was widely used in the field of medicine, food and light industry. However, HRE, which is due to using a large volume of a solvent and spending a lot of time, may gradually be substituted by some modern extraction methods, such as ultrasonic and microwave, supercritical fluid extraction, and so on. In our paper, UAME, HRE, and RAE were used to extract LU from *E. prostrata*, respectively. Each test was two repeats and the result of LU yield was an average of two times. To enhance LU yield, hydrochloric acid was added to the solvent of extraction. HRE was done such that the extracts mixed with hydrochloric acid after the extraction of LU from *E. prostrata*. Both UAME and RAE were done such that the...
Table 3. Analysis of variance (ANOVA) table for the orthogonal experiment.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>Sum of square</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol concentration</td>
<td>0.255</td>
<td>3</td>
<td>0.085</td>
<td>13.180</td>
<td>*</td>
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<tr>
<td>Ratio of solvent to material</td>
<td>0.127</td>
<td>3</td>
<td>0.042</td>
<td>6.551</td>
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<tr>
<td>Extraction power</td>
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<tr>
<td>Extraction time</td>
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<td>0.004</td>
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<td>Error</td>
<td>0.019</td>
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<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.434</td>
<td>15</td>
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</table>

*Significant (p < 0.05), df = difference

Table 4. Comparison of the results among UAME, HRE and RAE (n = 2).

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>UAME</th>
<th>HRE</th>
<th>RAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of extraction cycles</td>
<td>1</td>
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<td>3</td>
</tr>
<tr>
<td>Total extraction time (min)</td>
<td>3</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Total solvent amount (mL)</td>
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<td>200</td>
<td>300</td>
</tr>
<tr>
<td>Extraction yield (mg/g)</td>
<td>0.632</td>
<td>0.676</td>
<td>0.690</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Chromatographic results

Identification of the LU was carried out by comparing its retention time and on-line UV spectrum. As shown in Figure 1a, the chromatograms of standard showed the standard substance with retention time of 11.5 min for LU. In Figure 1b, the chromatograms of E. prostrata extracts by UAME (Figure 1b1), HRE (Figure 1b2) and RAE (Figure 1b3) were presented. These results indicated that different extraction methods possessed similar chromatographic characteristic and contained the standard substance of LU.

Effects of extraction variables on extraction yield of LU

Selection of solvent

The extraction follows the principle of “like dissolves like”. Low polarity solvent yields more lipophilic components, while alcoholic solvent gives a larger spectrum of apolar and polar compounds (Stecher et al., 2003). LU possesses a planar structure and has the molecular close packing, so its solution in water is little (Dong, 2010d). However, LU has four phenol hydroxyls, it shows certain polar degree. Therefore, water, methanol and ethanol were used to extract LU from E. prostrata in this study. Figure 2 shows the effects of different solvents on the
extraction yield of LU from the powder of *E. prostrata*. The extraction conditions were as follows: ratio of solvent to material: 20:1, ratio of hydrochloric acid to solvent: 0.006, microwave power: 30 W, and the extraction time: 180 s. Under the same extraction conditions, five different extraction solvents that is, water, methanol, 80% methanol, ethanol and 80% ethanol exhibited different efficiency on the extraction yield of the objective constituent as shown in Figure 2, which might be mainly due to their different polarity. According to the result, the yield of LU extracted by different extraction solvents followed this order: 80% ethanol > 80% methanol ≈ methanol > ethanol > water. 80% ethanol was found to be the best extraction solvent with the highest yield of 0.53 mg/g. Therefore, it was chosen as the extraction solvent.

In order to gain the optimized extraction aqueous ethanol solvent, the effects of different concentrations of ethanol on the extraction yield of LU was investigated. As shown in Figure 3, when the ethanol content is less than 80%, the yield of LU increased with increased ethanol content. However, absolute ethanol did not show the highest yield and 80% ethanol was verified to be the optimized extraction solvent with the highest extraction yield of LU (0.53 mg/g). This is probably due to the solvent-solute affinity and the effective swelling of the plant material by the solvent, which can increase the

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**Figure 1.** Chromatograms of standard substance and samples by HPLC–UV detected at 352 nm: (a) standard substance and structure; (b) *Eclipta prostrata* extracts: (b1) UAME, (b2) HRE, (b3) RAE.
Figure 2. Effects of different solvents on the extraction yield of LU from *E. prostrata*. ratio of solvent to material (v/w): 20:1; ratio of hydrochloric acid to solvent (v/v): 0.006; microwave power: 30 W; extraction time: 180 s.

Surface area for solute-solvent contact (Li et al., 2004).

**Ratio of hydrochloric acid to aqueous ethanol**

Hydrochloric acid can facilitate the release of flavonoids by breaking the hydrogen bonding interactions and glucosidic linkages (Dong, 2010c). Thus, the effect of ratio of hydrochloric acid (12 mol/L) to solvent (v/v) on the extraction yield of LU was investigated in our present study. As shown in Figure 4, the extraction yield increased when ratio of hydrochloric acid to solvent ranged from 0 to 0.006 (v/v), then became fairly constant when ratio of hydrochloric acid to solvent was above 0.006 (v/v). Hence, the ratio of hydrochloric acid to solvent was set at 0.006 (v/v).

**Ratio of aqueous ethanol to material**

Generally, a larger solvent volume can dissolve target components more effectively leading to an improvement of the extraction yield. However, this will lead to excess work in the concentration process, resulting in the unnecessary waste of solvent and time. To obtain the optimum volume of extraction solvent, we observed the effect of ratio of solvent to material (v/w) on the extraction yield of LU. As presented in Figure 5, the result indicated that ratio of solvent to material (50:1) should be suitable for the extraction of LU from *E. prostrata*.

**Extraction time**

Extraction time is another important factor in extraction procedures. Before the establishment of the equilibrium between the objective constituents in and out the plant cells, the yield of extraction increases with time. However, it will not increase with time after the establishment of the equilibrium (Fang et al., 2008). Figure 6 shows the effect of extraction time on the extraction efficiency of LU. The extraction yield of LU slightly increased when the extraction time was extended from 120 to 300 s, and it decreased along with time when the extraction time was longer than 300 s. This may be due to ionization, hydrolysis and oxidation during extraction with increasing the extraction time (Li et al., 2005). Therefore, longer time of extraction is unnecessary after the maximum extraction yield is achieved.

**Microwave power**

The samples of *E. prostrata* were extracted for 180 s with different extraction power, respectively. Figure 7 shows the effects of microwave power on the extraction
Figure 3. Effects of different concentrations of aqueous ethanol on the extraction yield of LU from *Eclipta prostrata*. Ratio of solvent to material (v/w): 20:1; ratio of hydrochloric acid to solvent (v/v): 0.006; microwave power: 30 W; extraction time: 180 s.

Figure 4. Effect of ratio of hydrochloric acid to solvent (v/v) on the extraction yield of LU from *Eclipta prostrata*. Ethanol concentration (v/v): 80%; ratio of solvent to material (v/w): 20:1; microwave power: 30 W; extraction time: 180 s.
The extraction yield increased with increased microwave power from 10 to 40 W. This may be ascribed with the acoustic cavitation bubble size increase with increasing acoustic power (Adam et al., 2009). However, when the extraction power was set above 40 W, the extraction yield starts to decrease. This suggests that a relatively stronger acoustic power was crucial to the chemical structures of components, leading to a lower extraction yield because the cavitation causes a great deal of noise and damage to components (Durmus et al., 2008).

Orthogonal experiment

Based on the above results from single factor experiments, an orthogonal experiment (L₁₆(4⁵)) of UAME of LU was designed in order to optimize the combination of parameters. Four factors, ethanol concentration, ratio of solvent to material, microwave power and extraction time were selected for optimization (Table 1). For four factors at four levels each, the orthogonal test design required only 16 experiments while the traditional full factorial design would require 256 experiments. The results of orthogonal experiment and extreme difference analysis were presented in Table 2, and the result of variance analysis calculated by the statistical software SPSS 18.0 is listed in Table 3.

The analysis of extreme difference indicated that the influential order of the four factors on the extraction yield of LU was ethanol concentration > ratio of solvent to material > microwave power > extraction time (Table 2). The order is in agreement with the order based on the values of $F$ in variance analysis (Table 3). According to variance analysis, the contribution of ethanol concentration for the extraction yield of LU is significant ($P < 0.05$), whereas ratio of solvent to material, microwave power and extraction time are not significant factors. In the orthogonal test, the higher extraction yield (0.629 mg/g) in the fifteenth experiment provided evidence that the deduced extraction condition might be the optimum one. To reconfirm this deduced optimum condition, UAME under this condition was carried out, and the extraction yield of luteolin reached 0.632 mg/g. So, this deduced condition was rationally confirmed to be the best combination of different parameters.

Comparison of luteolin yields extracted from E. prostrata by UAME, HRE and RAE

In Table 4, the LU yields extracted from E. prostrata by UAME increased with the number of extraction cycles. The LU yields of one cycle, two cycles and three cycles were 0.632, 0.676, 0.690 mg/g, respectively. It was evident that most of LU was extracted from E. prostrata in
Figure 6. Effect of the extraction time on the extraction yield of LU from *Eclipta prostrata*. Ethanol concentration (v/v): 80%; ratio of hydrochloric acid to solvent (v/v): 0.006; ratio of solvent to material (v/w): 50:1; microwave power: 40 W.

Figure 7. Effect of the microwave power on the extraction yield of LU from *Eclipta prostrata*. Ethanol concentration (v/v): 80%; ratio of hydrochloric acid to solvent (v/v): 0.006; ratio of solvent to material (v/w): 50:1; extraction time: 180s.
one cycle by UAME. Based on these results, it should be weighed to ascertain whether further extraction after the first cycle is to be continued or not, because of the solvent consumption increasing with the extraction times. To evaluate the advantages of UAME, the comparison of LU yields extracted by UAME, HRE and RAE were carried out under the same solvent consumption (300 mL). After 9 min extraction (three extraction cycles), UAME could produce the similar extraction yield (0.690 mg/g) as HRE after 240 min (two extraction cycles) (0.676 mg/g) and RAE after 60 min (0.689 mg/g).

The results showed that the extraction time of UAME were significantly shorter than that of HRE and RAE. From the above, it was evident that UAME is a more attractive extracting method when compared with HRE and RAE in terms of extraction yield, time consumption and labor intensiveness.

CONCLUSION

The extraction process of LU from E. prostrata by UAME was optimized using single factor test and orthogonal design. The optimal extraction condition of UAME was gained as follows: the ratio of 80% ethanol, hydrochloric acid and plant material: 50: 0.3: 1 (v/v/w), microwave power: 40 W and extraction time: 3 × 3 min. Moreover, ethanol content affected significantly on the extraction yield of LU (P < 0.05). The extraction yield of LU was 0.690 mg/g under the optimum conditions, compared with HRE and RAE in terms of extraction yield, time consumption and labor intensiveness, indicating that the UAME was more efficient than HRE and RAE.

Conclusion

UAME could markedly reduce extraction time, simplify the extraction process and gain higher LU yield from E. prostrata

ACKNOWLEDGEMENTS

This work was conducted in Class III Laboratory of Traditional Chinese Medicine on Pharmacognosy of State Administration of Traditional Chinese Medicine of People’s Republic of China and was financially supported by Key Project of Fujian Provincial Universities for Haixi Development (No. 5), the Research Foundation of Education Bureau of Fujian Province of China (No. JA10181), the Research Foundation of Health Bureau of Fujian Province of China (No. 2011-1-39) and the Natural Science Foundation of Fujian province of China (No. 2011J01214).

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Histopathological study on susceptible and resistant *Bulinus truncatus* snails to infection with *Schistosoma haematobium*

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Accepted 4 October, 2012

In this study, the distribution pattern of *Schistosoma haematobium* miracidia level to host susceptibility/resistance and the basic cellular responses during the parasite development was investigated. Several snail stocks showed a wide spectrum of host reaction to the parasite. A vigorous "resistant-type" cellular response to invading miracidia was seen in the histological sections of non-susceptible snails. In this respect, they were classified as "resistant snails". *Bulinus truncatus* infected with *S. haematobium* exhibited a wide range of histopathological change, suggesting the presence of endogenous factors preventing the immune system of susceptible snails from destroying the developed parasite larvae. Therefore, the mechanism underlying the susceptibility of the snails should be investigated by studying the host-parasite interactions by light and electron microscopy.

Key words: *Bulinus truncatus*, *Schistosoma haematobium*, snails, susceptible, resistance, light microscopy, electron microscopy.

INTRODUCTION

Schistosomiasis is a chronic debilitating disease in tropical regions of Africa, Americas and Asia. The relationships between schistosomiasis and its intermediate hosts, mollusks of the genus *Biomphalaria* and *Bulinus* have been a concern for decades. It is known that the vector mollusk shows different susceptibility against parasite infection whose occurrence depends on the interaction between the forms of trematode larvae and the host defense cell (Oliveira et al., 2010). The genetics of susceptibility of the different strains of snails to *Schistosoma haematobium* infection is complex and likely involves the interaction of several snail and parasite genes (Stothard et al., 2001; Mubila and Rollinson, 2002).

As a vector of *S. haematobium*, the gastropod *Bulinus truncatus* represents the primary model used to investigate the molluscan internal defense system. Hemocytes are the primary effectors of the snail defense system and its resistance lies in the ability of circulating hemocytes to recognize and bind the parasite surface and then undergo a cytotoxic activation, resulting in the effective killing of the parasite (Matricon-Gondran and Letocart, 1999; Bahgat et al., 2002; Martins-Souza et al., 2006). Some gene combinations allow the parasite to develop and proliferate because the snail fails to recognize it as foreign. In this combination, the parasite is recognized and phagocytized in one to a few days (Lie et al., 1987), or the parasite fails to develop because the host constitutes an unsuitable medium (Lie et al., 1983).

The fate of sporocysts of *S. haematobium* in non-susceptible *B. truncatus* has been studied by numerous investigators. Early study by Lie et al. (1977) reported that manifestations of non-susceptibility range from rapid, hemocyte-mediated destruction of sporocysts, a phenomenon termed resistance, and one that is believed to represent an immunological response (Adema et al., 1997) to arrested or delayed development (Lewis et al., 1993)
Laboratory-reared, adults *B. truncatus* snails (6 -10 mm shell height; age, 6 - 8 weeks) and miracidia of *S. haematobium* were supplied by the Schistosome Biological Supply Center (SBSC, Theodor Bilharz Research Institute [TBRI], Giza, Egypt). Snails were maintained as described by Liang et al. (1987). *B. truncatus* snails were exposed individually for 3 h in 1 ml dechlorinated water to 5 - 10 freshly hatched *S. haematobium* miracidia. Exposed snails were examined daily for cercarial shedding by exposing them to fluorescent light for 2 h from the first week up to 6 weeks. Snails were classified into susceptible and resistant according to Lewis et al. (1993).

### Light microscopy

To study parasite development, at each time of testing, 3 - 5 snails from susceptible and resistant strains were fixed in Bouin’s fixative for at least 24 h and then placed in gradually increasing concentrations of ethanol. Hematoxylin-eosin-stained 5-µM sections were examined microscopically for the histological condition of larval trematodes, as categorized by Borges et al. (1998). Histological analysis was performed on about 38 and 45 susceptible and resistant snails, respectively.

### RESULTS

A total number of 150 snails from both susceptible (75) and resistant (75) groups, respectively, were used. Survival rate of snails after exposure to *S. haematobium* miracidia was found to be quite similar in both groups (80 - 81.3%). The data depicted in Figure 1 indicated that normal infection was observed in 53.3 and 9.3% in the susceptible and resistant snail groups, respectively, at 1 - 2 weeks post exposure (WPE). Moreover, 1.8 and 3.3% of susceptible and resistant snails, respectively, developed such retarded infection signs with the development of foot-sporocyst before the 3 WPE. Meanwhile, retarded infection signs were observed in 0.78 and 9.7% of susceptible and resistant snails, respectively, but with the development of foot-sporocyst in more than 3 - 5 WPE. The data also shows that 46.7 and 90.7% of the susceptible and resistant snails, respectively, did not show any sign of infection at 7 WPE.

Moreover, cellular reaction to the sporocysts varied with sporocysts location and the length of infection. At 1 WPE, most of the sporocysts contained one or more germinial cells with nucleoli and, therefore, were judged to be viable. Approximately 8 - 12% of the sporocysts were elongated, showing transverse constrictions and were categorized as normal (Figure 2A); those that showed no elongation or folding were categorized as retarded. Some sporocysts were surrounded by several layers of flattened hemocytes (Figure 2B), but many others were free of encapsulation. All remaining sporocysts at 3 - 4 WPE were categorized as dead, that is degenerated (all germinial cells lacking nucleoli) or destroyed (disintegrated and undergoing phagocytosis) (Figure 2C). At 5 WPE, all sporocysts were dead. Most degenerated sporocysts were compact, round, and contained scattered pyknotic nuclei in a vacuolated eosinophilic matrix (Figure 2D). In addition, other bodies lacking nuclei were seen at these two subsequent time periods. These were usually round to oval, homogenously eosinophilic except for vacuolated areas, and were surrounded by a clear space (Figure 2E).

The histological study showed that the final site of infection, developmental and growth dynamics were similar with previous reports. Although encapsulation of sporocysts never occurred in susceptible snails, hemocyte aggregations could sometimes be observed in the proximity of well developed sporocysts (Figure 3A). Figure 3B shows...
Figure 2. Histological sections of *B. truncatus* exposed to *S. haematobium*. (A) Normal sporocyst in head-foot at 1 week post exposure (WPE); note absence of hemocytic response. (B) Sporocysts (arrow) surrounded by hemocytes (arrow head) at 4 WPE in the tentacles. (C) Degenerating sporocyst (arrow) and large capsule formation (arrow head) in the tentacles at 4 WPE. (D) Destroyed sporocysts in the tentacles of susceptible *B. truncatus* exposed to *S. haematobium* at 5 WPE; note eosinophilic fragments (arrows) undergoing phagocytosis by hemocytes (arrow head). (E) Amorphous sporocyst in the pericardial cavity of the heart at 5 WPE. Sporocysts consisted of vacuolated eosinophilic matrix (arrow) (x160).

Figure 3. (A) Degenerating (collapsed) sporocyst in the mantle of susceptible *B. truncatus* exposed to *S. haematobium* at 3 WPE; note narrow, darkly stained germinal cells and surrounded by several layers of hemocyte. (B) Degenerating (rounded) sporocyst in the kidney of susceptible *B. truncatus* exposed to *S. haematobium* at 4 WPE; note eosinophilic masses (arrow), pyknotic nuclei surrounded by flattened hemocytes. (C) A numerous multiplying sporocysts (arrow) appeared within of the heart of resistant *B. truncatus* exposed to *S. haematobium* at 2 WPE; note the absence of any tissue reaction. (D) An encapsulated focal reaction (arrow) in the mantle collar of resistant *B. truncatus* exposed to *S. haematobium* at 3 WPE. (E) A sporocyst (arrows) in the anterior cephalopodal sinus of resistant *B. truncatus* exposed to *S. haematobium* at 4 WPE; note individual hemocytes (arrow head) adhering to the parasite surface. (F) Destroyed sporocysts (arrow) in the head-foot of susceptible *B. truncatus* exposed to *S. haematobium* at 6 WPE (x160).
normal developing sporocysts in susceptible snails at 4 WPE for comparison with sporocysts in resistant snails. In the resistant snails, miracidia were able to transform into sporocysts, to migrate and reach the heart area by the end of the 2 WPE (Figure 3C). Although the migration pattern was overall similar to that of susceptible snails, differences could be observed regarding sporocysts final site of infection (Figure 3C). This cellular response continued to increase and, after 3 - 4 WPE, resulted in the encapsulation of the sporocysts settled in the ventricle and aorta. Sporocysts tegumental destruction was observed and hemocytes were infiltrating the sporocyst tissues. In some cases, a clear space was still observed between the sporocyst tegument and the capsule (Figure 3D), although individual hemocytes were present at the surface of the parasites (Figure 3E).

Interestingly, this encapsulation process was not observed for sporocysts settled in the pericardial cavity. Despite this absence of encapsulation, the development of the sporocysts settled in the digestive gland was not normal as compared with the development of sporocysts in susceptible snails. After 4 WPE, sporocysts settled in the kidney, mantle collar, ventricle and the aorta were clearly degraded. In addition to the large number of hemocytes encapsulating sporocysts, numerous hemocyte aggregations were observed in the heart area, such as the pericardial cavity. After 5 WPE, sporocysts were fully degraded and hardly identifiable within capsules. In some cases, degenerate sporocyst materials were observed (Figure 3F). The capsule was characterized by numerous dead or degenerating hemocytes in the center next to the dead sporocysts surrounded by live hemocytes. The external surface of the sporocysts is increased by irregular knobs and short ramified projections. The 1 to 4 µM thick tegument may have deep fissures, or numerous deeply channels. The tegument is rich in vesicles and vacuoles which vary in size and shape and have more or less electron-dense contents. Mitochondria, myelin-like bodies and electron dense spherical bodies are common in the tegument. The spherical bodies are formed in the tegumented cell bodies that occur below the basal lamina and are in common with the syncytial tegument through thin cytoplasmic bridges (Figure 4A). Poorly developed and sparsely described muscle fibers occur below the basal lamina (Figure 4B). The sub-tegumental layer also contains different types of parenchymatous cells, some of which are rich in lipid droplets and glycogen patches (Figure 4C). Many sporocysts occur close to tubules of the digestive gland. The tegument of the sporocyst and the basal lamina of the hemocyte-producing organ (HPO) are separated by connective tissue cells or muscle fibers (Figure 4D).

Aggregating hemocytes form a compact capsule around the damaged sporocysts. The peripheral flattened hemocytes are very similar to the flattened cells that surround living sporocysts. Some cells contain small spherical, lysosome-like vesicles and high concentrations of supposed glycogen particles (Figure 4E). The innermost cells of the capsule act as phagocytes. Phagocytosis of parasite tissue is followed by formation of residual bodies of indigestible material in cells in the center of the capsule (Figure 4F). As some of these cells die a dense core of residual bodies is formed. It is likely that some of the peripheral cells of the capsule become amoebocyte and migrate to other areas of the snails (Figure 4G). The cells containing the residual bodies may migrate from the capsule center to the peripheral layer. However, most cells in the capsule remain and a homogenous tissue replaces the removed parasite (Figure 4H). This granuloma formation is composed of large rounded cells containing lysosome-like vesicles and small spherical vesicles (Figure 4I).

DISCUSSION

In the present study, we preferred to select laboratory-reared snails from different stocks to investigate resistance in B. truncatus to infection with S. haematobium. This selection process allows adult snails to self-fertilize, exposes the juvenile progeny to infection and ensures the isolation of the susceptible/resistant (positive/negative) snails. Through different analysis, some snail stocks showed normal developing infection, although some snails of each stock showed no signs of infection and/or delayed infection with or without foot sporocysts development according to the classification of Kristensen and Christensen (1989) and Sesen and Yildirim (1993). In snails that develop foot-sporocysts, it seems that the genetics of this phenotype probably involve multiple factors expressed in variable quantitative doses in the snail (Özcel et al., 1996). Although retarded schistosome infections have been discussed by other researchers, Sesen (2004) proposed that an additional category of snail/parasite interaction must be added to the four general classification described in details by Mukaratirwa et al. (1998).

The presence of cytoplasmic prolongations from numerous cells appears under the light microscope as containing fibers, sometimes mimicking the process of fibrosis seen in vertebrates. Fibers with staining characteristics of collagen or elastin have been demonstrated in normal snail tissues (Lemos, 1999; Borges and Andrade, 2003). However, the presence of elements from the extracellular matrix in the granulomatous lesions of Biomphalaria glabrata against Schistosoma mansoni has been a controversial issue. Although Yoshino (1976) and Krupa et al. (1977) noted that the presence of extracellular fibrils contributed to the formation of the encapsulating lesions, Harris (1975) did not find extracellular elements associated with the molluscan cellular reactive responses. One probable cause of this divergence could be the presence of true collagen and orcein-positive elastic fibers only at the periphery of the lesions as noted recently by Borges and Andrade (2003). Both at light and electron microscopy, collagen-like fibers are noted at the proximity of hemocytes accumulations. Since no active connective-cell was visualized and no real accumulation of such fibers
Figure 4. (A) An electron micrograph of a section through the tegument of sporocyst from *B. truncatus* exposed to *S. haematobium*. Note the large nucleus (N) with its prominent nucleolus, large secretory granules (arrow), Golgi complex (G), mitochondria (m) and granular endoplasmic reticulum (ER). (B) The tegument is moderately electron dense and contains mitochondria (m) and membrane-bound granules. Adjacent cells (arrow) are ventricular smooth muscle. (C) Intercellular space (arrow heads) between newly aggregated cells; sporocyst tegument is swollen with loss of mitochondria (m) and erosion of membrane-bound granules (arrow). (D) Hemocytes extending homogenous pseudopodia (arrow) toward lipid-containing tegument of sporocyst. (E) Debris-laden hemocytes surrounding degenerating sporocysts (arrow). (F) The outer part consists of lightly packed flattened hemocytes (arrows); remains of dead sporocysts are (arrow head) seen in the center of the capsule. (G) Note portion of hemocytes in cell aggregation in the ventricle. Immature features include abundant free ribosomes (R). (H) Cells containing residual bodies (arrow) from the center of the capsule. (I) Rounded cells from the center of the granuloma formation (x1200).

could be documented in the present study, they are probably pre-existing normal component of the molluscan tissues as suggested by Imbert-Establet et al. (1992), Vuong et al. (1996) and Botros et al. (2008).

Encapsulation and subsequent destruction of helminths by molluscan hemocytes is well known (Mubila and Rollinson, 2002; Remy and Arouna, 2005). The speed and severity of encapsulation responses against trematode larvae have been hypothesized to reflect the degree of host resistance, with rapid encapsulation and destruction being interpreted as evidence of strong resistance (Kirinoki et al., 2000; Sasaki et al., 2003; Azevedo et al., 2006). Accordingly, resistance in *B. truncatus* has not been deemed strong because the hemocytic reaction is unimpressive and dead parasites persist for some time. Since the parasites seem to be recognized and
encapsulated but not readily phagocytized, these snails may be useful in determining if encapsulation and phagocytosis require specific genetic activators as reviewed by Richards (1975). This fact must be considered in discussing the biological control of *S. haematobium* by the introduction of refractory snails into endemic areas.

Among non-susceptible stocks of *B. truncatus* to *S. haematobium* in the present study, there is a range of host tissue response and parasite deterioration at several intervals following infection. Meanwhile, we do not know whether the range of the deterioration of *S. haematobium* sporocysts observed is the result of a differential host response to a homogenous parasite population or whether it reflects the range in parasite diversity as suggested by Sullivan et al. (2004). In the present study, a snail stock was categorized as non-susceptible. Although a few snails in this stock became infected, a vigorous “resistant-type” cellular response to invading miracidia was seen in histological section as that of non-susceptible snails. In this respect, they resemble the other resistant snails stocks (Lie et al., 1987; Lewis et al., 1993; Cooper et al., 1994; Cousin et al., 1995), and are classified in our study as “resistant snails” notwithstanding the low percentage of susceptible reactions in this stock.

The present light microscopic observations of the interaction between sporocysts of *S. haematobium* and *B. truncatus* snail tissue generally agree with similar studies of sporocysts in natural or susceptible hosts (Sminia and Barendsen, 1980; Arfaa et al., 1989; Lemos, 1999; Borges and Andrade, 2003). Electron microscope studies of the relation between sporocysts and snail cells are limited and possibly rare. The general morphology of the body wall of the sporocysts of *S. haematobium* does not differ essentially from that of most sporocysts or radiae described in the ultrastructural level (Kirkinoi et al., 2000; Sasaki et al., 2003; Remy and Arouna, 2005). However, some sporocysts have been described as possessing an external nucleated layer outside the tegument. This external layer is often mentioned as the “Paletot”. Such two-layered sporocysts have been described at the ultrastructural level (Kirkinoi et al., 2000; Sasaki et al., 2003; Remy and Arouna, 2005). However, the electron micrographs clearly showed that the outer nucleated layer is of host origin. It must be emphasized that a nucleated cell layer of parasite origin never occurs outside the syncytial tegument of any sporocysts or redia when these occur in the molluscan tissue. The cell layer (primitive epithelium) which surrounds developing daughter sporocysts disappears before these leave the mother sporocysts (Meuleman et al., 1980; Southgate et al., 1989; Joubert et al., 1991; Souza et al., 1995; Lemos, 1999).

Host reactions resulting in the formation of few layered or loosely packed host capsule around daughter sporocysts in susceptible host have been described at the ultrastructural level by Krupa et al. (1977). These host responses may, similarly to the layers of flattened hemocytes around the sporocyst of *B. truncatus*, be regarded as an attempt by the host to wall off the parasite. The tegument of the sporocyst is the sole organ for uptake of nutrients. Absorption by the external surface of sporocysts may be enhanced by the presence of structural modification such as microvilli, folds, ridges and invaginations (Zdarska and Soboleva, 1982) that amplify the surface area of the tegument. The extensive amplification in surface area of trematode may be correlated with their capacity to actively transport among other things (Üglem and Lee, 1985).

**Conclusion**

The cellular response observed in *S. haematobium* resistant stock of *B. truncatus* support the notion that a rapid destruction of the mother sporocysts depends on hemocyte capability to adhere to and encapsulate the parasite. However, observations of a related development and degeneration of un-encapsulated or partially encapsulated sporocysts raise the question of the possible role of humoral factor in the resistance to *S. haematobium*. Therefore, further studies are needed to determine the impact *S. haematobium* infection on morphological and functional properties of *B. truncatus* hemocytes.

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Effect of diet containing sesame seed on epididymal histology of adult rat

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Accepted 8 November, 2012

Sesamin is a major lignan constituent of sesame seed and considered as a key factor in a number of beneficial effects on human health. The intake of sesame leaves has been shown to improve and increase epididymal spermatocytes reserve in adult male Sprague Dawley rats. The aim of the study was to determine the effect of a diet containing sesame seeds on epididymis histological structure of the adult wistar rat. Thirty adult male rats were divided into two groups consisting of 15 rats each. The regime group received a diet containing 30% sesame seeds, while the control group received a standard diet for 12 weeks. The right epididymis was removed and minced into several pieces in a specimen bottle containing normal saline for a few minutes to allow the sperm to swim out. Sperm parameters, sperm count and motility was determined. The left epididymis was divided into three sections and fixed into bouin’s solution for histological evaluations. Serum Follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentrations were estimated by Enzyme-linked immunosorbent assay (ELISA) techniques while testosterone concentration was determined using a Chemo-luminence method. The body weight gain during the treatment period did not differ significantly between the two groups. The mean epididymal sperm motility and count of the experimental group was significantly higher (p < 0.05) when compared to the control group. LH levels were significantly increased in the experimental group compared to control group. No significant changes in FSH and testosterone levels were reported. The mean epididymal diameter of the tubular, lumen and epithelium in three parts were not significantly different (p > 0.05) when the regime group was compared to the control group. From our study, we can conclude that the consumption of sesame seeds improves sperm parameters (motility and count) and increases LH levels. Sesame seeds had no effect on epididymal tissue and body weight of the rats.

Key words: Diet, epididymis, sesame seed, sperm, hormone

INTRODUCTION

The function of the epididymis, including production of the epididymal specific micro-environment is necessary for the maturation, storage, and survival of spermatozoa which is regulated by hormones and testicular growth factors (Swider-Al-Amawi et al., 2010). More recently, it has been hypothesized that both testicular cancers and sub-fertility may be caused by the exposure of the developing male embryo to agents that disrupt normal hormonal balance (Sharpe and Skakkebaek, 1993; Sharpe, 2003; Izegbu et al., 2005). Sesame (Sesamum indicum) is an important oil seed crop due to its high nutritional and therapeutic values which is cultivated widely in tropical, subtropical, and southern temperate regions (Chakraborty et al., 2008).

Sesamin is a major lignan constituent of sesame seeds and considered as a key factor in a number of beneficial effects on human health. These benefits include anti-cancer properties (Yokota et al., 2007), anti-hypertensive properties (Miyawaki et al., 2009), anti-inflammatory properties (Jeng et al., 2005), anti-oxidative properties (Nakano et al., 2003), cholesterol-lowering activity (Chen et al., 2005), enhancement of hepatic fatty acid oxidation and alcohol metabolism (Tsuruoka et al., 2003).
2005), neuroprotection (Khan et al., 2010) and promotion of angiogenesis (Chung et al., 2010).

In addition, sesame plant is one of the richest food sources of phytoestrogenic lignans, a valuable phytochemical known to man since the dawn of civilization (Thompson et al., 1991). This plant source is now increasingly being incorporated into the human diet worldwide because of their reported health benefits (Shittu et al., 2009). Shittu et al. (2007) reported that sesame leaves intake improve and increase epididymal spermatocytes reserve in adult male Sprague Dawley rats. Sesame lignan, such as: sesamin, sesamolin, sesaminol, pinorsinol, sesamol and gammatocopherol are isolated from S. indicum and S. radiatum seeds and have more anti-tumorigenic, estrogenic or anti-estrogenic and antioxidant features compared with other plant species (Jeng and Hou, 2005).

In terms of phytochemicals, this plant has phenolic compounds (phenols, sterols, flavonoids and lignans), non-protein amino acids, cyanogenic glucoside, alkaloids, unsaturated fats and lipids with multiple double bonds, glazes, phospholipids and E, B1 and B2 vitamins (Koran et al., 2008). Minerals or trace elements such as calcium, iron, magnesium, zinc, copper and phosphorus exist in this plant (Shittu et al., 2006). Approximate analysis of sesame seed has made it clear that the seeds contain 50 to 60% oil, 8% protein, 5.8% water, 3.2% crude fiber, 18% carbohydrate and 5.7% ash (Obiajunwa et al., 2005). It has been reported by Shittu and Shittu (2012) that phytochemicals in sesame leaves had an effect on improving fertility potential and antimicrobial activities, as well as significantly increasing the mean epididymal diameter and volume density of the tubular lumen in low dose sesame group. When compared to the control group. In addition, the consumption of sesame leaves extract enhanced the quality of the spermatozoa produced with improvement in the storage capacity of the epididymis for these spermatozoa in a dose related manner (Shittu and Shittu, 2012).

The influence of lead and zinc on the rat male reproductive system at the biochemical and histopathological levels has been evaluated (Obiajunwa et al., 2005). These authors reported that zinc (an element of sesame seed) supplementation to lead-exposed rats had a protective effect. Zinc could compete significantly and effectively reduce the availability of lead binding sites (Batra et al., 2001). The aim of our study was to determine the effect of a sesame seed rich diet on the structure of the epididymis using histomorphometric methods as well as on hormone (LH, FSH and testosterone) levels.

**MATERIALS AND METHODS**

**Animal experiment**

Thirty mature and healthy adult male wistar rats weighing between 190 to 210 g were procured from Kashan University, College of Medicine and Kashan-Iran from the period of 2010 to 2011. They were housed in well ventilated wire-wooden cages in the departmental animal house. They were maintained under controlled light schedule (12 h light and 12 h darkness) at room temperature (28°C) and with constant humidity (40 to 50%). The animals were allowed to acclimatize for a period of 7 days before treatment during the experiments. During the next 12 week period, they were fed with standard rat chows supplied, and water was available ad libitum (Shittu et al., 2007, 2008). All of the clinical trials conducted on animals were approved by Animal Care Committee of Kashan University of Medical Sciences.

**Experimental design**

An experimental study was designed in that, two types of rats (A and B) were randomly selected and were assigned into either the experimental (n = 15) or the control groups (n = 15). Group A served as the control while B constituted the treated group. Group A animals received normal diet while group B received normal diet (70%) plus 30% sesame seeds. All the animals consumed diets for a period of 12 weeks.

**Animal sacrifice**

The rats were anaesthetized at the time of sacrifice using a sealed inhalation jar with ether-soaked cotton wool for 3 and 5 min. The weights of the animals were taken weekly and before sacrifice (Shittu et al., 2008).

**Semen collection and semen analyses**

The right epididymis from rats of each of the two groups were removed and minced into several pieces in a specimen bottle containing normal saline. It was allowed to stand for a few minutes to allow the sperm to swim out into the saline medium. The semen was collected with a 1 ml pipette and dropped on a clean slide and covered with slips. The slides were placed under a light microscope and examined for the sperm number and motility (Saalu et al., 2006, 2007a).

**Organ harvest and tissue processing for light microscopy**

The left epididymis was carefully dissected, trimmed of all fat and blotted dry to remove any blood. The epididymis was divided into three sections (head, body and tail). The fixed tissues were transferred into bouin's solution and then processed for 17.5 h in an automated Shandon processor after which the tissues were passed through a mixture of equal concentration of xylene. Following clearance in xylene, the sections were then infiltrated and embedded in molten paraffin wax. Prior to embedding, it was ensured that the mounted sections were cut using a rotary microtome which was orientated perpendicularly to the long axes of the epididymis. These sections were designated “vertical sections”. Ranomic sections of 5 μm thickness were cut (per 5 sections), floated onto clean slides coated with 2% formaldehyde for proper cementing of the sections to the slides and were then stained with Haematoxylin and eosin stains (Shittu et al., 2006, 2007).

**Determination of morphometric parameters**

The diameter (D) of the epididymal tubules with profiles that were round or nearly round for each animal was estimated. A mean diameter “D” was taken as the average of two diameters, D1 and
Table 1. Effects of sesame seeds consumption on body weights, sperm motility and count in epididymis of wistar male rats treated during 12 weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weights (g)</td>
<td>195.88±7.67</td>
<td>194.42±8.04</td>
</tr>
<tr>
<td>Sperm count (×10⁶)</td>
<td>62.14±3.91</td>
<td>74.23±2.52*</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>57.86±3.77</td>
<td>72.31±2.98**</td>
</tr>
</tbody>
</table>

Results expressed in mean ± SEM. *Indicates P < 0.01; **indicates P < 0.006.

Table 2. Effects of the sesame seeds consumption on serum hormone concentrations of wistar male rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (IU/L)</td>
<td>2.7±0.29</td>
<td>3.5±0.22*</td>
</tr>
<tr>
<td>FSH (mIU/L)</td>
<td>3.9±0.44</td>
<td>3.3±0.28</td>
</tr>
<tr>
<td>T (nmol/L)</td>
<td>10.88±2.9</td>
<td>8.45±2.9</td>
</tr>
</tbody>
</table>

Results expressed in mean ± SEM. *Indicates P < 0.05. LH = luteinizing hormone, FSH = follicle stimulating hormone, T = testosterone.

RESULTS

Body weight

The body weight gain during the treatment period did not differ significantly between the two groups. Moreover, no clinical and behavioral changes were observed in the animals treated with the sesame seeds (Table 1).

Epididymal sperm motility and count

The mean epididymal sperm motility and count of the experimental group was significantly higher than control group (Table 1).

Serum hormone levels

LH levels were significantly increased in the experimental group compared to the control group; however no significant changes in FSH and testosterone levels were observed (Table 2).

Determination of morphometric parameters

The results in Table 3 indicate that the mean epididymal diameter of the tubular, lumen and epithelium in three parts were not significantly different (P > 0.05) between the two groups. Measuring the mean diameter of epididymal tubular was done by Zeiss optical microscope with a magnification of ×10.

DISCUSSION

The purpose of the study was to examine the effects of a diet containing sesame seeds on the histological profile of the epididymis of the adult wistar rat. Moreover, there is an increasing role of sesame lignans research because of its contribution to medicine and of its immense economic value to man. However, sesame being rich in trace elements or minerals, vitamins and antioxidant lignans (phytoestrogens) possesses the ability to improve fertility potential in the male (Shittu et al., 2007).

According to our study, epididymal sperm motility and sperm count in experimental group was significantly higher in comparison to control group. It has been reported that antioxidants have the ability to enhance fertility (Ganong, 2003). Most plants rich in antioxidants have been found to increase sperm counts, sperm motility and enhance sperm morphology (Oluyemi et al., 2007). Vitamins C and E (as a sesame seed antioxidant) are free radical scavengers and they protect sperm against lipid peroxidation with an increase in peripheral testosterone levels (Fridovich, 1986). Dalsenter et al. (2004) suggested that the daily sperm production, as well as the number of sperms in the caudal epididymis were unaffected by aqueous crude extract of Achillea millefolium leaves, suggesting absence of adverse effects on the spermatogenic process. Treatment of wistar rats with the highest dose of yarrow aqueous extract altered the sperm morphology (Dalsenter et al., 2004).

Pro-fertility effects of the alcoholic extract of Sesame in male Sprague Dawley rats were investigated. These authors found a significant increase in motility of sperm in the treatment group that received vitamin C (Saalu et al., 2007b). In the absence of vitamin C, decreased sperm motilities were recorded in treatment groups even upon withdrawal from the extract, when compared to the group...
Table 3. Effects of the sesame seeds consumption on morphometric parameters in epididymis of male wistar rats.

<table>
<thead>
<tr>
<th>Parameters (µm)</th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubule diameter in head part</td>
<td>290.23±11.04</td>
<td>316.43±8.5</td>
</tr>
<tr>
<td>Tubule diameter in body part</td>
<td>284.75±10.32</td>
<td>302.39±8.11</td>
</tr>
<tr>
<td>Tubule diameter in tail part</td>
<td>284.44±8.45</td>
<td>304.87±5.74</td>
</tr>
<tr>
<td>Lumen diameter in head part</td>
<td>231.4±7.06</td>
<td>245.31±8.6</td>
</tr>
<tr>
<td>Lumen diameter in body part</td>
<td>223.64±8.76</td>
<td>240.68±8.43</td>
</tr>
<tr>
<td>Lumen diameter in tail part</td>
<td>226.95±9.14</td>
<td>239.3±7.0</td>
</tr>
<tr>
<td>Epithelium diameter in head part</td>
<td>58.81±5.0</td>
<td>64.01±7.72</td>
</tr>
<tr>
<td>Epithelium diameter in body part</td>
<td>61.11±3.65</td>
<td>61.71±2.35</td>
</tr>
<tr>
<td>Epithelium diameter in tail part</td>
<td>60.48±2.4</td>
<td>65.57±4.88</td>
</tr>
</tbody>
</table>

Results expressed in mean ± SEM.

that received vitamin C alongside extract (Ukwenya et al., 2008). Ethanolic extract of sesame was shown to enhance morphology of the spermatozoa in the epididymis at the time of sacrifice of the animals (Saalu et al., 2007b).

Ofusori et al. (2007) evaluated the effects of ethanolic extract of croton zambesicus (as a potent antioxidant and free radical scavenger) on the testes of Swiss albino mice. A significant increase in the sperm concentration, motility, and progressivity in the treated group was observed. *C. zambesicus* ameliorated the increased free radicals generated by the natural and experimental stress (Ngadjui et al., 2002; Okokon et al., 2005). The mechanism of action of the extract for the increased sperm concentration is yet to be elucidated (Ofusori et al., 2007). Spermatozoa continuously proliferate to replenish themselves and differentiate through definite changes of development (Guyton and Hall, 2000). The extract could possibly have an effect on the mitochondria in the body of the tail of the spermatozoon influencing its ability to synthesize energy in the form of adenosine triphosphate (Guyton and Hall, 2000).

The data obtained in our study show that LH levels were significantly increased in the experimental group when compared to control but significant changes in FSH and testosterone levels were not observed in both groups. Dahia and Roa (2006) reported that LH and its receptors (LHR) are necessary for regulation of the morphology of epithelial cells of the epididymis and epididymal steroidogenesis. It has been shown for the first time that the FSH receptor is also expressed in epithelial cells of the caudal epididymis of rat and monkey (Dahia and Rao, 2006).

The epididymis is also richer in androgen receptors, the site for action of the testosterone, dihydrotestosterone and probably estradiol (Oliveira et al., 2003). Shittu et al. (2007) suggested that testosterone levels in the high dose sesame group was significantly higher than the control and the low testosterone level observed, which could be due to the fact that some of the testosterone were aromatized to estradiol and/or converted to dihydrotestosterone by the aromatase and reductase enzymes present within the epididymis. Moreover, Huang et al. (1987) demonstrated that as little as 25% of normal testicular testosterone concentration was sufficient to support all stages of spermatogenesis. Shittu et al. (2007) reported that the low dose sesame will reduce endogenous estradiol with less competition, although there is synergism at this level between the testosterone and estradiol to favour spermatogenesis. However the high dose of sesame could possibly result in more estradiol production with more competition with dihydrotestosterone for aromatization to occur in its favour. The low testosterone observed in this study is not a result of destruction of the Leydig cells, but a reflection of the complex hormonal interplay at the level of the hypothalamic-pituitary-testicular axis.

Shittu et al. (2009) observed the effects of mesterolone (proviron) to induce low sperm quality with a reduction in sex hormone profile of the testis of adult male Sprague Dawley rats. It was concluded that testosterone and FSH levels in the proviron group was significantly lower than the control group. These results are in contradiction to our findings and could be attributed to variations in the type of diet, type of rats and duration of using a particular diet. However, it was found that FSH had a synergistic effect with testosterone hormone and stimulating synthesis of the androgen receptor at the receptor level. According to a study by Shittu et al. (2008), FSH concentration decreased in the group that received the high dose liquid extract of sesame leaves group compared to the control group. The histological features of the caudal epididymis were not affected in treated mice, except there was a non significant decrease in the tubular diameter and tubular epithelial height as compared to controls (Shittu et al., 2008).

The results of the study indicate that no significant changes in body weight gain were evident. These were confirmed in other studies where no significant differences in the animal body weights were observed.
(Awoniyi et al., 1997). Ukwenya et al. (2008) demonstrated the pro-fertility effects of the alcoholic extract of sesame in male Sprague Dawley rats studied. The effects of the ethanolic extract of beniseed (sesame) at 3000 mg/kg body weight, with vitamin C administered as adjuvant, had the potential to increase the mean body weights of rats. This is mostly due to the high fat content of the seeds. Fats are stored in the form of triacylglycerol in adipose tissues in mammals via lipogenic pathways. The increased weight gain is in line with the work of Shittu et al. (2007) where they reported significant weight gain in all the animals.

They also recorded a dose-dependent increase in weight gain upon administration of 14.0 and 28 mg/kg body weight of aqueous extract of sesame to rats for six weeks.

Shittu et al. (2007) have suggested that significant weight gain was observed in all the animals. The weight gain observed in the treated groups was dose dependent such that weight gain in the high dose was more than that in the low dose. However, the low dose weight was significantly lower. However, the results are in disagreement with those of Shittu et al. (2007) where they reported significant weight gain in all the animals.

**Conclusion**

This is the first study which has evaluated the histological effect of sesame seed on epididymis of adult Wistar rat. It can be concluded that sesame seed could improve sperm motility and sperm count, and also it may increase LH. A diet rich in sesame seed did not have any effect on epididymal tissue and body weight of animals.

**REFERENCES**


