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Controlled release colon targeted drug delivery systems of non-steroidal anti-inflammatory drug, indomethacin

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This study aimed to investigate the efficacy of both xanthan gum (XG) and guar gum (GG) controlling the release rate of the poorly water soluble drug, indomethacin (IDM) from colon target drug delivery systems. Binary mixtures of the drug and the hydrophilic carrier (XG) in the ratios of 1:1 and 1:2 and tertiary mixture in the ratio of 1:1:1 IDM: XG: GG were prepared using three different approaches namely, physical mixture, co-grinding and solid dispersion. The prepared binary and tertiary systems were compressed into core tablets. The core tablets were evaluated for their drug content, weight variation, hardness, friability and in-vitro dissolution rate study. The dissolution profiles in pH 6.8 buffer solutions revealed that increasing the gum content in the core tablet resulted in a decrease in the IDM release rate. The core tablets were then coated with two different type of coat (inner and outer). The inner coat consisted of guar gum solutions of different concentrations (0.2, 0.4, 0.6, 0.8% w/v) to prevent the drug release in pH 7.4. Tablets were then coated with an enteric coat by dipping in 5% Eudragit (ER L100) ethanolic solution to inhibit the drug release in pH 1.2. The coated tablets were then dried using hot air. The prepared coated tablets were subjected to release rate study which indicated that the release of the drug was inhibited in pH 1.2 whereas; a low percentage of the drug was released in pH 7.4. In pH 6.8, the release profiles showed a sustained release of the drug over 18 h. The coated tablets that showed the promising sustained release profiles were further evaluated in pH 6.8 buffer solution containing rat cecal content to study the effect of bacterial degradation on the polysaccharide gums.

Key words: Indomethacin, xanthan gum, guar gum, co-grinding, solid dispersion, dissolution rate, colon target drug delivery systems.

INTRODUCTION

The traditional non-steroidal anti-inflammatory drugs (NSAIDs) and selective cyclooxygenase-2 (Cox-2) inhibitors potentially inhibited polyp development and tumour incidence (Gupta and DuBois, 2001). Cox-2 over expression is thought to play an important role in colon carcinogenesis, as it has been found to be elevated in 40% of colonic adenomas and up to 90% of sporadic CRC (Eberhart et al., 1994; Fujita et al., 1998). Its pharmacological inhibition by NSAIDs is the central event in the chemoprevention of colon cancer (Gupta, 2000;
El-Kamel et al., 2008; Asghar and Chandran 2008; Krishnaiah et al., 2002a). Indomethacin (IDM) is a nonsteroidal anti-inflammatory agent with antipyretic and analgesic properties. It is a nonselective inhibitor of COX 1 and 2, enzymes that participate in prostaglandin synthesis from arachidonic acid. It has been used in the symptomatic management of painful and inflammatory conditions. Therefore, many researches intended to formulate IDM as a colon drug delivery for the treatment of colorectal diseases as well as for the management of osteoarthritis (Ravi et al., 2008; Amrutkar and Gattani, 2009).

The aim of this study was to formulate controlled release colon drug delivery systems of IDM using hydrophilic carriers; namely xanthan and guar gums in different ratios. Binary mixtures of the drug and the hydrophilic carrier (XG) in the ratios of 1:1 and 1:2 and tertiary mixture in the ratio of 1:1:1 IDM: XG: GG were prepared using three different approaches namely, physical mixture, co-grinding and solid dispersion. The possible interaction between the drug and the gums in both the liquid and solid states was detected by the phase solubility study and the differential scanning calorimetry (DSC), respectively. The prepared binary and tertiary systems were further evaluated for their flow properties and dissolution rates. These mixtures were then compressed into core tablets to study the effect of the included gums being hydrogels upon IDM release from the compressed matrix tablets. Tablets for colon delivery were prepared by coating the core tablets with an inner coat consisting of guar gum solutions of different concentrations (0.2, 0.4, 0.6, 0.8% w/v) to prevent the drug release in pH 7.4 and with an enteric coat by dipping in 5% Eudragit (ER L100) ethanolic solution to inhibit the drug release in pH 1.2. IDM release studies from coated and uncoated tablets were performed in both pH 6.8 buffer solution with and without rat cecal content to show the effect of bacterial degradation on the drug release.

Evaluation of the prepared powdered mixtures

Drug content

To estimate the drug content, an amount of the prepared powders equivalent to 10 mg of IDM were weighed accurately and transferred into 100 ml volumetric flask containing 25 ml ethanol. The flask was shaken vigorously and left to stand for about 6 h, then complete to volume with phosphate buffer (PB) pH 6.8 and left for 24 h. The concentration of IDM was assayed spectrophotometrically at 320 nm against ethanol-buffer mixture (1:3) as a blank.

Differential scanning calorimetry (DSC)

DSC thermograms of pure materials, PMs, CGs and SDs were recorded using Shimadzu differential scanning calorimeter (DSC-60, Shimadzu, Japan). The samples 2-5 mg of the pure drug or the above mentioned samples were weighed carefully and were hermetically sealed in aluminum pans and heated at a constant rate of 10°C/min, over a temperature range of 25 to 250°C. Thermograms of the samples were obtained using differential scanning calorimeter. Thermal analysis data were recorded using a TA 501 PC system with Shimadzu software programs. Indium standard was used to calibrate the DSC temperature and enthalpy scale. N₂ was used as purging gas at the rate of 40 ml/min. The heat of fusion of crystallized drug in a LD was calculated from the peak area of the melting endotherm. The heat of fusion of pure crystalline drug was determined in a separate experiment. The ratio of these fusion energies was used to calculate the percent crystallinity of drug in the LDs and PMs using the following equation:

\[
\text{Percentage} \, (\%) \, \text{crystallinity} \, = \, \frac{\Delta H_s}{\Delta H_c} \times C \times 100
\]

Where, \( \Delta H_s \) and \( \Delta H_c \) are enthalpies of fusion of the sample and pure drug, respectively, and C is the weight fraction of drug in the mixture assuming that the pure drug was 100% crystalline (Rawlinson et al., 1997).

Scanning electron microscopy (SEM)

The surface morphology of IDM, XG, GG, PMs, CGs and SDs were examined under scanning electron microscope (Jeol, JSM-6360LV scanning microscope, Tokyo, Japan). Before microscopy, the dried samples were mounted at carbon tape and were sputter-coated using gold (Jeol, JFC-1100 fine coat ion sputter, Tokyo, Japan). The photomicroographies were taken at an acceleration voltage of 10 kV.

Flow properties

In order to ensure good flow properties of the binary and tertiary

MATERIALS AND METHODS

Indomethacin (γ- polymorphic form) (IDM) was kindly supplied by Pharco Pharmaceuticals, Alexandria, Egypt. Guar gum, (Premcem gums Ltd, India); Xanthan gum (Ultrasine, India); microcrystalline cellulose (Avicel pH 102, FMC Co., USA); Eudragit (ER L100); polyethylene glycol 400 (European Co. For Pharmaceutical Industries, Egypt); hydrochloric acid 10 N (Prolab, Adwic, Elnasr Pharmaceutical Chemicals Co., Egypt); sodium tribasic phosphate (Chemajet, Alexandria, Egypt). All other chemicals and buffers were of analytical reagent grades.

Preparation of binary and tertiary systems

IDM-XG binary systems were prepared using varying drug concentration of 50 and 33.33% w/w equivalent to drug: polymer ratios of 1:1 and 1:2, respectively. The binary systems were prepared using different methods. Physical mixtures (PMs) of drug and polymer were obtained by simply blending with spatula, co-ground (CG) by co-grinding of drug and XG for 30 minutes in a ceramic mortar and solid dispersion (SD) by solvent evaporation technique were prepared. Xanthan gum or mixture of xanthan and guar gums was dispersed in a minimum amount of ethyl alcohol. Solution of IDM in minimum amount of ethyl alcohol was prepared and added to gum dispersion. The rota-vapor (IKA RV10, IKA, Germany) was used to evaporate the solvent and the solid powder was collected. The obtained powder was then sieved, and average particle size of 125 μm was used for further evaluation. The different systems were stored in desiccators till used.

In order to ensure good flow properties of the binary and tertiary
systems, angle of repose measurements (fixed height cone method), Carr's index and Hausner's ratio were adopted (Luner et al., 2001). The procedure was done in triplicates and the average angle of repose was calculated for each powder. In the bulk density measurements, fixed weight of each of the powder formulations prepared were placed in graduated cylinder and the volume ($V_f$) occupied was measured and the initial bulk density ($D_0$) was calculated. The graduated cylinder was then tapped at a constant velocity till a constant volume is obtained when the powder is considered to reach the most stable arrangement; the volume of the powder was then recorded as the final bulk volume ($V_t$), then the final bulk density ($D_t$) was calculated. Carr's compressibility index was then calculated according to the following equation (Luner et al., 2001):

$$\text{Carr's index} = \frac{D_t - D_0}{D_t} \times 100 \quad (2)$$

In addition, Hausner's ratio was calculated from the following equation:

$$\text{Hausner's ratio} = \frac{D_t}{D_0} \quad (3)$$

The experiments were done in triplicate. Carr's compressibility index and Hausner's ratio with the corresponding standard deviations for each of the prepared formulations were then calculated.

**In-vitro dissolution study**

IDM dissolution study was evaluated using the USP XXIV dissolution rate apparatus II (Pharmatest, Germany) at a stirring rate of 100 ± 2 rpm. Powders samples containing 75 mg of pure drug or its equivalent amount of PM, CG and SD were placed in 900 ml 0.1 N HCl (pH 1.2) at 37 ± 0.5°C for 2 h then the medium was rendered alkaline to pH 6.8 by the addition of the calculated amount of sodium tribasic phosphate. At predetermined time intervals, 5 ml samples were withdrawn and immediately replaced with an equal volume of pre-heated (37 ± 0.5°C) dissolution medium. All samples were run in triplicate, filtered through 0.45 µm membrane filter and the amount of dissolved IDM was analyzed by spectrophotometer at 320 nm (guar gum did not interfere in the spectrophotometric reading of the drug at 320 nm). The percentage cumulative amount of the drug dissolved was plotted against time.

**Phase solubility studies**

In order to detect any possible interaction between IDM and XG in solution and explain the results of in-vitro dissolution studies, phase solubility experiments were performed. Aqueous solubility of IDM in the presence of xanthan gum was carried out according to the method described by Higuchi and Connors (1965). An excess amount of IDM was added to 10 ml of aqueous solutions containing an increasing concentration of XG (0, 2, 4, 6, 8 and 10 mM) in screw-capped vials. The suspensions were shaking in a thermostatically controlled water bath (type 1083, GFL GmbH, Burgwedel, German) at 37 ± 0.5°C for 48 h. After equilibrium has been attained (2 days), aliquots were withdrawn, filtered through 0.45 µm membrane filters, suitably diluted and analyzed for IDM using UV spectrophotometer at 320 nm (xanthan gum had no effect on the spectrophotometric reading at 320 nm). The apparent stability constant ($K_s$) of 1:1 complexes was calculated from the linear phase solubility diagram obtained by plotting the molar concentration of IDM in the solution vs. XG molar concentration according to the equation:

$$K_s = \frac{\text{Slope}}{S_0} \cdot \text{(1-slope)} \quad (4)$$

Where, $S_0$ is the intrinsic solubility of the drug in absence of XG. The solubilization efficiency of XG was calculated as the ratio of IDM aqueous solubility at the highest XG concentration used and IDM intrinsic solubility in pure water.

**Preparation of core tablets**

The drug binary and tertiary mixtures (125 µm) were prepared according to Table 1, and were compressed into core tablets using single punch tablet machine with punch of 9 mm in diameter to a tablet hardness of 7 Kg/cm².

**Drug content of core tablets**

To estimate the drug content, 10 tablets of each IDM formulation were weighed accurately, triturated and transferred into 500 ml volumetric flask containing 250 ml ethanol. The flask was shaken vigorously and left to stand for about 6 h, then complete to volume with phosphate buffer pH 6.8 and left for 24 h. The concentration of IDM was assayed spectrophotometrically at 320 nm against buffer as a blank.

**Preparation of coated tablets**

The prepared core tablets were coated with two different coats; a primary or an inner coat and an outer coat. The inner coat consisted of guar gum solutions of different concentrations (0.2, 0.4, 0.6, 0.8%) prepared by dissolving the appropriate weight of guar gum in a plasticized 2% cellulose acetate solution in acetone: methanol solvent system. PEG 400 was used as a plasticizer. The outer coat was an enteric coat using Eudragit ER L100 polymer. Tablets were coated by dipping in 5% ER L100 ethanolic solution and then dried using hot air (10 coats of each coating solution were applied which were equivalent to 10% increase in tablet weight).

**In-vitro release study of core tablets**

Dissolution rate study of IDM core tablets was carried out using the USP XXIV dissolution rate apparatus II (Pharmatest, Germany) at a stirring rate of 100 ± 2 rpm. Core tablets were placed in 900 ml 0.1 N HCl (pH 1.2) at 37 ± 0.5°C for 2 h then the medium was rendered alkaline to pH 6.8 by the addition of the calculated amount of sodium tribasic phosphate. At predetermined time intervals, 5 ml samples were withdrawn and immediately replaced with an equal volume of pre-heated dissolution medium at 37± 0.5°C. All samples were run in triplicate, filtered through 0.45 µm membrane filter and the amount of dissolved IDM was analyzed by spectrophotometer at 320 nm. The percentage cumulative amount of the drug released was plotted against time.

**In-vitro dissolution of coated tablets**

The same procedure mentioned above was followed. Coated tablets were placed in 900 ml 0.1 N HCl (pH 1.2) at 37± 0.5°C for 2 h then the medium was rendered alkaline to pH 7.4 by the addition of the calculated amount of sodium tribasic phosphate and the release study was continued for another 3 h. The tablets were then placed in pH 6.8 for 24 h. At predetermined time intervals, 5 ml samples were withdrawn and immediately replaced with an equal
The enthalpy of drug fusion was plotted of rat caecal content were noticed indicating partial, 2
displaced was plotted indicating that the present methods for the preparation of

RESULTS AND DISCUSSION

Drug content of the prepared mixtures

The drug content of the prepared binary systems were found to be in the range 97.23 ± 0.35 to 99.96 ± 0.29% indicating that the present methods for the preparation of solid systems can be applied with a high content uniformity.

Differential scanning calorimetry (DSC)

The DSC thermograms of IDM (Figure 1a) exhibited sharp endothermic peak at 161.17°C corresponding to its melting point, such sharp endothermic peaks signify that IDM used was in pure crystalline state (Mahmoud et al., 2009). XG and GG being amorphous anhydrous carriers did not show any characteristic peaks except broad band (Figure 1b). All the binary mixtures prepared by different methods revealed the presence of the drug peak with slight shift in the melting temperature of the drug along with significant decrease in the endothermic peak intensity (Figure 1 and Table 1). The intensity of the drug peak is reduced in the drug; XG ratio of 1:1 further increase in the gum content (1:2 drug: gum ratio) showed slight decrease in the intensity of the drug peak. No complete disappearance was noticed indicating partial loss of drug crystallinity. SD binary mixtures showed the highest reduction in the intensity of the drug fusion peak. This modification of the DSC profile of the drug may be related to a chemical or physical interaction between the drug and XG or the possible formation of an amorphous system. In case of tertiary systems PM3, CG3 and SD3, the incorporation of GG potentiated the amorphization characteristic of XG. The order of reducing the intensity of the drug peak was as follow SD3>CG3>PM3>IDM. The drug peak was still detectable in the SD3 system but with a significant reduction of drug crystallinity assuming a partial dispersion at a molecular level in the solid product, but did not seem to be indicative of a true complex formation. The enthalpy of drug fusion decreased sharply with the incorporation of the carrier indicating that the drug lost an appreciable percentage of its crystallinity (Table 1). For example, the heat of fusion of the pure IDM was significantly reduced from -380.81 to -194.83, -159.61 and -148.03 in case of PM1, CG1 and

### Table 1. Thermoanalysis data of IDM and its binary and tertiary systems.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Peak °C</th>
<th>Heat (mJ)</th>
<th>Crystallinity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDM</td>
<td>161.17</td>
<td>-380.81</td>
<td>100</td>
</tr>
<tr>
<td>IDM: XG (1:1)</td>
<td>159.63</td>
<td>-194.83</td>
<td>51.19</td>
</tr>
<tr>
<td>IDM: XG (1:2)</td>
<td>159.72</td>
<td>-184.42</td>
<td>48.43</td>
</tr>
<tr>
<td>IDM: XG: GG (1:1:1)</td>
<td>159.43</td>
<td>-145.67</td>
<td>38.25</td>
</tr>
<tr>
<td>IDM: XG (1:1)</td>
<td>159.32</td>
<td>-159.61</td>
<td>41.91</td>
</tr>
<tr>
<td>IDM: XG (1:2)</td>
<td>158.97</td>
<td>-138.36</td>
<td>36.33</td>
</tr>
<tr>
<td>IDM: XG: GG (1:1:1)</td>
<td>159.30</td>
<td>-133.13</td>
<td>34.96</td>
</tr>
<tr>
<td>IDM: XG (1:1)</td>
<td>159.20</td>
<td>-148.03</td>
<td>38.87</td>
</tr>
<tr>
<td>IDM: XG (1:2)</td>
<td>159.23</td>
<td>-88.76</td>
<td>23.31</td>
</tr>
<tr>
<td>IDM: XG (1:1)</td>
<td>159.78</td>
<td>-87.78</td>
<td>23.05</td>
</tr>
</tbody>
</table>

Preparation to mimic enzymatic media of the colon (rat cecal matter, RCM)

A group of 5 rats each weighing (150-200 g) and maintained on normal diet (soaked grain) were used to induce enzymes specifically acting on guar gum. The rats were treated with 1 ml of 2% w/v guar gum dispersion using oral needle for 7 days. The rats were then killed using CO₂ asphyxiation, 45 min before the study. The abdomen were opened, the cecai were traced, ligated at both the ends, dissected and immediately transferred into phosphate buffer of pH 6.8, previously bubbled with CO₂. The ceacal bags were opened, their content individually weighed, pooled and then suspended in pH 6.8 to give 4% w/v dilution. As the ceacum is naturally anaerobic, all operations were carried out under CO₂ (Rama et al., 1998).

In vitro drug release in rat cecal matter

Drug release studies in the presence of rat caecal content were also carried out using USP dissolution test apparatus II, but with slight modification. After completing test in Ph 1.2 for 2 h and pH 7.4 for 3 h, baskets containing tablets were immersed in 250 ml beaker containing phosphate buffer solution (pH 6.8) and rat caecal content maintained in the jars of the dissolution apparatus for up to 24 h. Samples of 5ml each were withdrawn at different time intervals (6, 7, 8, 9, 24 h), filtered using filter paper and assayed spectrophotometrically for IDM at 320 nm. The same volume of fresh medium bubbled with CO₂ was added after each withdrawn sample (Krishnaiah et al., 2002b).
Figure 1. A, DSC thermogram of indomethacin (IDM), xanthan gum (XG) and their physical and co-grinding mixtures in different ratios. A: IDM, B: XG, C: IDM: XG 1:1 physical mixtures (PM), D: IDM: XG 1:2 PM, E: IDM: XG 1:1 CG, F: IDM: XG 1:2 CG. B, DSC thermogram of indomethacin (IDM), xanthan gum (XG), guar gum and their physical mixture (PM), co-grinding (CG) and solid dispersion (SD) in ratio, 1:1:1. A: IDM, B: XG, C: guar gum, D: PM, E: CG, F: SD, C, DSC thermogram of indomethacin (IDM), xanthan gum (XG) and their solid dispersion (SD) in different ratios. A: indomethacin, B: xanthan gum, C: 1:1 SD, D: 1:2 SD.
Scanning electron microscopy (SEM)

Micrographs of Indomethacin powder are shown in (Figure 2a-g). IDM was formed of plate crystals with smooth borders, but irregularly shaped. Both XG and GG showed irregular shaped particles. PM of 1:2 IDM: XG micrograph demonstrated the plate crystals of IDM in combination with the irregular shaped XG particles. Co-ground mixture of 1:2 IDM: XG ratio showed large particles formed of aggregated small particles. Upon grinding, particle size reduction occurred which resulted in the formation of charged particles. These charged particles were aggregated through electrostatic attraction. The micrograph showed a conversion from the crystalline to the amorphous state. In case of 1:2 IDM: XG solid dispersion, the particle shape becomes completely different from the original shape of the drug particles.

Flow properties

Angle of repose (θ) is a characteristic of the internal friction or cohesion of the particles. Its value will be high if the powder is cohesive and low if the powder is non-cohesive. Table 2 represents flowability parameters of IDM and prepared powdered mixtures using different techniques in terms of angle of repose, Carr index and Hausner’s ratio. PM3, CG2, CG3, SD1, SD2 and SD3 formulations demonstrated (θ) values in the range of 29.15 to 39.42 and Carr’s index up to 21, indicating that these formulations were systems with acceptable flowability. The prepared mixtures were found to have significantly lower angle of repose (P= 0.05) in comparison to the raw crystals of IDM, which could be due to the irregular shaped crystals of IDM, which hindered the uniform flow of crystals. The reason for the excellent flowability of prepared mixtures was due to significant reduction in interparticle friction because of their shape modification from crystal state to amorphous state or fragmented particles (Table 2). In addition, Hausner found that the ratio D1 /D0 was related to the inter particle friction, so, he showed that powders with low interparticle friction, had ratios of approximately 1.250 indicating good flow (Fahmy and Kassem, 2008). SD3 showed the lowest angle of repose, Carr’s index and Hausner’s ratio. Good flow properties were in the order of SD3 > SD2 > SD1 > CG3 > CG2 > PM3.

In-vitro dissolution and phase solubility studies

The effect of varying drug: gum ratios and method of preparation of drug: polymer mixtures on IDM dissolution rate in both pH 1.2 and 6.8 is shown in (Figures 3, 4 and 5). In 0.1 N HCl (pH 1.2), IDM exhibited a tendency to form large aggregates and floated on the surface of dissolution medium, due to its hydrophobic nature. The aggregation caused reduction in effective surface area of drug particles available for dissolution which resulted in 9.14% drug release after 2 h of dissolution test.

In general, XG is present predominantly in a unionized state at low pH, whereas XG is ionized under dilute acidic and alkaline conditions. This difference in the ionization state of xanthan gum in the dissolution media affected hydrogel formation and consequently, the retardation of drug release. When XG was present in a unionized state, an intramolecular hydrogelation was prevented due to the absence of ionic bonds, resulting in a considerable release of IDM in 0.1 N HCl (Ramani et al., 2010).

It was observed that drug dissolution progressively improved with increasing the polymer proportion in the mixture and reached the highest values at the 1:2 drug: XG ratio. This result is evidenced by the phase solubility study. The phase solubility profiles for the IDM-XG systems are presented in Figure 6. The plot indicates a typical A2-type (positively deviating isotherms) solubility curve as classified by Higuchi and Connors (1965). The diagram shows an increase in IDM solubility occurred as the amount of the complexing agent, XG increased. This is due to soluble complex formation which might be through hydrogen bonding between the hydroxyl (OH) groups XG and the carboxylic (COOH) group of IDM, thereby increasing the total amount of the IDM in the solution. Furthermore, A2-type phase solubility diagram is formed when more than one molecule of the complexing agent is found in the complex (Luner et al., 2001). Positive deviation from linearity (A2-type systems) are thought to indicate formation of complexes that are first order with respect to the drug but second or higher order with respect to the complexing agent (Karsten, 2009). The A2-type phase solubility curve revealed the formation of a complex of IDM in 1.2 stoichiometric ratios with XG. The stability constants (Kd) for the complex at 37 °C ± 0.5, assuming a 1:2 stoichiometric ratio, calculated from the slope of preliminary straight line portion of the phase solubility curve (333.733 mM⁻¹), demonstrated the relative affinity of the drug for the polymer and good complexation ability (Najib and Suleiman, 1989). This also suggests that there is an increase in the dissolution profile which would certainly increase bioavailability of IDM.

The slight increase in drug dissolution shown by simple physical mixtures could be due to a reduction of the interfacial tension between the hydrophobic drug particles and the dissolution medium, owing to the presence of the hydrophilic polymer, thus increased the wettability of the drug (Najib and Suleiman, 1989). It could also be due to the increased surface area available for dissolution being carried on by the hydrophilic amorphous XG. The high
Figure 2. Scanning electron micrographs. A, Indomethacin; B, xanthan gum; C, guar gum; D, 1:1 PM of IDM: XG; E, 1:2 PM of IDM: XG; F, 1:2 CG of IDM: XG; G, 1:2 SD of IDM: XG
rate of drug dissolution shown by the solid dispersion could be attributed to the intimate physical contact between IDM and hydrophilic carrier. It might also be due to the better dispersion of the drug on the surface of XG. Whereas in case of co-ground mixtures, the increase in drug dissolution rate could be due to particle size reduction brought about by the mechanical treatment and to a decrease in drug crystallinity during co-grinding with the amorphous carrier (Ramanji et al., 2010). These finding were in agreement with the results of the thermoanalysis studies, confirming that the best dissolution performance of SD products is mainly attributable to the almost higher degree of drug amorphization achieved in these systems (Figure 1). The incorporation of GG in the different formulations PM3, CG3 and SD3 increased the drug dissolution rate. The improvement of dissolution may be due to the swelling nature of the guar gum which resulted in increasing the
extensive surface of the gum during dissolution, and the dissolution rate of deposited drug was markedly enhanced. In addition, the hydrophilic and amorphous nature of the GG may be attributed to the increase in drug dissolution rate (Suchetha et al., 2011; Shah et al., 2010).

In phosphate buffer pH 6.8, it was expected that the drug dissolution rates from the various formulations could be reduced as a result of the intramolecular hydrogelation of XG being in the ionized state in alkaline medium (Ramanji et al., 2010). The drug dissolution rate increased as the acidic pH of medium was shifted to alkaline pH. This result can be attributed to the solubility of the acidic drug IDM in alkaline medium although the carrier in its ionized state.

The results of the dissolution rate studies revealed that the drug dissolution increased as the XG content increased in the different formulations. Physical mixture showed a slight increase in drug dissolution rate that might be due to the presence of the drug particle individually dispersed in the dissolution medium leading to an increase in its surface area. Consequently, this increase in IDM surface area increased its dissolution rate (Elkhodairy et al., 2011). Whereas, in the co-ground
Table 2. Flowability parameters of IDM and the different binary and tertiary mixtures.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Angle of repose (θ)°</th>
<th>Carr’s Index</th>
<th>Hausner’s ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug</td>
<td>49.32±0.004</td>
<td>40.32±0.001</td>
<td>1.99±0.011</td>
</tr>
<tr>
<td>Xanthan gum</td>
<td>32.45±0.006</td>
<td>20.00±0.003</td>
<td>1.37±0.012</td>
</tr>
<tr>
<td>PM1</td>
<td>43.98±0.010</td>
<td>29.14±0.004</td>
<td>1.62±0.021</td>
</tr>
<tr>
<td>PM2</td>
<td>40.62±0.021</td>
<td>28.32±0.002</td>
<td>1.60±0.012</td>
</tr>
<tr>
<td>PM3</td>
<td>39.42±0.031</td>
<td>21.32±0.003</td>
<td>1.23±0.031</td>
</tr>
<tr>
<td>CG1</td>
<td>42.08±0.024</td>
<td>27.98±0.012</td>
<td>1.67±0.014</td>
</tr>
<tr>
<td>CG2</td>
<td>36.94±0.030</td>
<td>18.32±0.024</td>
<td>1.15±0.012</td>
</tr>
<tr>
<td>CG3</td>
<td>36.33±0.006</td>
<td>18.67±0.012</td>
<td>1.15±0.025</td>
</tr>
<tr>
<td>SD1</td>
<td>34.61±0.016</td>
<td>16.73±0.021</td>
<td>1.11±0.015</td>
</tr>
<tr>
<td>SD2</td>
<td>32.31±0.023</td>
<td>15.69±0.026</td>
<td>1.12±0.013</td>
</tr>
<tr>
<td>SD3</td>
<td>32.15±0.024</td>
<td>15.32±0.014</td>
<td>1.11±0.012</td>
</tr>
</tbody>
</table>

Table 3. Composition of the different core tablets.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Ingredients in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IDM</td>
</tr>
<tr>
<td>Control Table (CT)</td>
<td>75</td>
</tr>
<tr>
<td>1:1 PM (PM1)</td>
<td>75</td>
</tr>
<tr>
<td>1:2 PM (PM2)</td>
<td>75</td>
</tr>
<tr>
<td>1:1:1 PM (PM3)</td>
<td>75</td>
</tr>
<tr>
<td>1:2 PM (PMG)*</td>
<td>75</td>
</tr>
<tr>
<td>1:1 CG (CG1)</td>
<td>75</td>
</tr>
<tr>
<td>1:2 CG (CG2)</td>
<td>75</td>
</tr>
<tr>
<td>1:1:1 CG (CG3)</td>
<td>75</td>
</tr>
<tr>
<td>1:1 SD (SD1)</td>
<td>75</td>
</tr>
<tr>
<td>1:2 SD (SD2)</td>
<td>75</td>
</tr>
<tr>
<td>1:1:1 SD (SD3)</td>
<td>75</td>
</tr>
</tbody>
</table>

PMG*: 1:2 IDM: GG physical mixture. Total tablet weight: 380 mg.

mixture, the drug crystals were reduced in size during grinding leading to the increased dissolution rate compared to IDM original crystals. In case of SDs, in the dissolution medium, the drug particles were present in the form of amorphous powder of increased solubility and dissolution rate as compared to its original crystalline state. Formulations of SD showed an improved release behavior of IDM in comparison with the PM and CG mixtures. This could be explained by reducing the particle size and consequent increase in surface area, decreased crystallinity occurring during the formulation. Moreover, presence of the drug in a molecular dispersion in hydrophilic carrier matrix increased the wettability and the surface available to dissolution by reducing the interfacial tension between the hydrophobic drug and dissolution medium (Mortada, 2006).

The obtained results could be explained on the basis that in alkaline medium ionization of the acidic groups of both IDM and XG took place leading to the formation of anionic species. The absence of ionic interaction between the anionic species resulted in the presence of the drug in a free state in the dissolution medium. Thus, the high affinity of the anionic drug to the cationic medium resulting in liberation of the drug from the hydrogel formed, thus overcome the drug release retardation effect of the carrier. The replacement of half the amount of XG with GG reduced the hydrogel capacity of XG and the presence of GG which resulted in increasing the swelling capacity of the gum mixture over its hydrogelation power. This is the cause for the increased drug release in case of 1:1:1 IDM: XG: GG mixtures compared to 1:2 IDM: XG mixtures.

Evaluation of IDM tablets

Drug content of core tablets

The drug content of the prepared binary systems were found to be in the range of 95.73 ± 0.15 to 99.86 ± 0.19%. 

The obtained results could be explained on the basis that in alkaline medium ionization of the acidic groups of both IDM and XG took place leading to the formation of anionic species. The absence of ionic interaction between the anionic species resulted in the presence of the drug in a free state in the dissolution medium. Thus, the high affinity of the anionic drug to the cationic medium resulting in liberation of the drug from the hydrogel formed, thus overcome the drug release retardation effect of the carrier. The replacement of half the amount of XG with GG reduced the hydrogel capacity of XG and the presence of GG which resulted in increasing the swelling capacity of the gum mixture over its hydrogelation power. This is the cause for the increased drug release in case of 1:1:1 IDM: XG: GG mixtures compared to 1:2 IDM: XG mixtures.
In-vitro release study of core tablets

Figures 7 and 8 illustrate the effect of varying drug/gum ratios and method of preparation of drug powder on the amount of IDM released from tablets prepared from either PM, CG and SD mixtures by direct compression technique in 0.1N HCl for 2 h then in phosphate buffer (pH 6.8) for 24 h at 37°C ± 0.5. It is clear that the drug release rate from the control IDM tablet in pH 1.2 was very low because of the acidic nature of the drug. Increasing the pH of the medium from pH 1.2 to pH 6.8, the amount of drug released increased from 1.3 to 47 mg% (Table 4). The figures showed high retardation of IDM release from gum containing tablets compared to IDM control tablets. It is obvious that the release of IDM from tablets prepared of the SD was higher than that of tablets prepared by direct compression of the co-ground and physical mixtures. PM has higher retardant release rate than CG and SD, this was evident by the increase in $T_{25\%}$ of PM1 (3-4 h) compared to that of the correspond-
ing CG1 (2-3 h) and SD1 (1-2 h) (Table 4). This may be explained on the basis that tablets prepared by CG presented the drug particles in the reduced form and those prepared by SD showed higher wettability increasing front erosion and hence increased drug release. It was also observed that an inverse relationship was found between amount of gum and release rate of IDM. Increasing the gum concentration from 1:1 to 1:2 caused more retardation of drug release, as evidenced by lower dissolution efficiency (DE%) values and higher T25\% values (Table 4). This result was not in agreement with that obtained in case of powder mixtures. The obtained results could be explained as the gum concentration increased, the thickness of the gel layer increased and the diffusion path length increased leading to delay in drug release. Hydration of individual xanthan gum particles causes swelling of each particle. The swollen particles coalesced together, resulting in a continuous viscoelastic matrix that fills the interstices, maintaining the integrity of the tablet, and retarding further penetration of the dissolution medium (Yeole et al., 2006).

It was reported that Xanthan gum has the highest water uptake compared to HPMC and guar gum (Sinha et al., 2004). Based on this consideration, 3 mechanisms were suggested namely; swelling, erosion and then diffusion, which synchronized together forming gel layer of constant thickness. Accordingly, a zero-order release could be expected (Sinha et al., 2004). The higher water uptake but lower erosion of the tablets may describe the lower release rate of IDM (Lu et al., 1991). Incorporation of guar gum caused less retardation of drug release than pure xanthan gum. DE% values increased with decreasing T25\% values as shown in Table 2. A combination of the anionic xanthan gum and the non ionic guar gum seems to produce a synergistic increase in viscosity. This may be attributed to the stronger hydrogen bonding between the carboxyl groups of the xanthan and the hydroxyl groups of guar gum, leading to stronger physical cross-linking between the polymers. Interaction between nonionic and ionic polymers has been reported to be greater than between molecules of the same species (Cerdeira et al., 1998).

**In-vitro drug release of coated tablets**

Core tablets that showed a promising results in sustaining the drug release namely PM 2 XG, PM 2 GG, PM 3, CG 2 and SD 2, were coated and subjected to dissolution rate studies. The obtained results are presented in Figures 9a and 9b. It was found that coated tablets prepared from the physical mixtures showed the highest retardation rate compared to those prepared from CG and SD mixtures. Therefore, tablets prepared from PM mixtures were selected for studying the release in the presence of rat cecal content, because bacteria accelerate the degradation of gums resulted in increase in drug release rate. Figure 10 shows coated tablets of PM2 and PM3 mixtures release profiles in pH 6.8 compared to that in the rat cecal content. It is obvious that the coating prevented the release in pH 1.2 and a slight release was observed in pH 7.4 then the presence of the rat cecal content (pH 6.8) increased the drug release rate to about three folds. The release profiles of IDM from the coated tablets indicated that these formulations could be used as extended colon targeted drug delivery systems.

**Conclusion**

It can be concluded that mixtures of xanthan and guar gum with different ratios could be used to modulate the

| Table 4. Comparison of the release rate of IDM from different core tablets. |
|-----------------------------|-------------------|-------------------|-------------------|
| Formula                     | Parameter of comparison |               |
|                             | 2 h          | 3 h          | DE% (6 h) | T25\%(h) | 24 h          |
| Control Table (CT)          | 1.3      | 47          | 43.50     | 1.596     | 98.00         |
| 1:1 PM (PM1)                | 1.3      | 15.5        | 25.54     | 3.226     | 62.70         |
| 1:2 PM (PM2)                | 1.0      | 8.8         | 17.13     | 5.112     | 46.20         |
| 1:1:1 PM (PM3)              | 0.8      | 11.2        | 19.31     | 3.751     | 50.70         |
| 1:2 PM (PMG)*               | 1.0      | 29.6        | 31.65     | 2.534     | 69.50         |
| 1:1 CG (CG1)                | 1.1      | 37          | 35.76     | 2.027     | 75.40         |
| 1:2 CG (CG2)                | 1.0      | 10.8        | 21.81     | 4.255     | 50.33         |
| 1:1:1 CG (CG3)              | 0.9      | 14.7        | 23.67     | 3.778     | 56.34         |
| 1:1 SD (SD1)                | 1.7      | 39.2        | 36.82     | 1.932     | 79.30         |
| 1:2 SD (SD2)                | 1.3      | 16.8        | 22.37     | 4.032     | 53.87         |
| 1:1:1 SD (SD3)              | 1.4      | 18.7        | 24.26     | 3.345     | 58.34         |

PMG*: 1:2 IDM (Indomethacin): GG (guar gum) physical mixture; DE%, dissolution efficiency.
Figure 9. Release profiles of IDM from uncoated and coated tablets prepared from A, physical mixtures; B, CG and SD mixtures.

Drug release rate from either powder mixtures or from compressed tablets. Xanthan gum showed higher drug release retardation from tablets compared to guar gum. Physical mixture approach demonstrated the slowest drug release compared to co-ground and solid dispersion techniques. Core tablets prepared from 1:2 drug: xanthan gum and 1:1:1 drug: xanthan gum; guar gum showed the highest drug release retardation among the other formulations under investigation. In addition, release profiles of IDM from coated tablets showed that tablets prepared from physical mixtures had the highest release retardation effect. Therefore, they are selected for formulating colon target drug delivery systems since the drug release in the colon will be increased in the presence of the rat cecal content. Tablets of PM2 and PM 3 could be used as once a day colon target drug delivery systems.
ACKNOWLEDGEMENT

This research project was supported by a grant from the Research Center of the Center of Female Scientific and Medical Colleges in King Saud University.

ABBREVIATIONS

NSAIDs, Non-steroidal anti-inflammatory drugs; Cox, cyclooxygenase; IDM, indomethacin; XG, xanthan gum; GG, guar gum; DSC, differential scanning calorimetry; PMs, physical mixtures; CG, co-ground; SD, solid dispersion; SEM, scanning electron microscopy; DE%, dissolution efficiency.

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Full Length Research Paper

Comprehensive study of the intestinal absorption of four phenolic compounds after oral administration of Ananas comosus leaf extract in vivo and in vitro

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The extract of Ananas comosus leaf (EAL) was proved to be antihyperlipidemia and antihyperglycemia consisting of rich phenolic acids. Pharmacokinetic study showed p-coumaric acid was a rich compound detected in the mouse plasma after EAL oral administration. The aim of this study was to explore the alteration of the absorption of four principle phenolic compounds through the intestines by oral administration. Liquid chromatography-mass spectrometry (LC-MS-MS) was used to detect the phenolic compounds in EAL. The assay for the absorption of the small intestines of mice was employed in vivo and in vitro. According to our results, 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol would be converted into p-coumaric acid and caffeic acid by passing though the intestines. Caffeic acid might be transformed into a new compound with relative molecular mass of 359 in liver. The phenolic components of EAL were absorbed mainly in the form of p-coumaric acid while caffeic acid were absorbed through the intestines. p-Coumaric acid and caffeic acid are considered the active components of EAL in the body.

Key words: Ananas comosus, phenolic acid, absorption, mouse, metabolism.

INTRODUCTION

Ananas comosus (Linn.) Merr or pineapple, which originated from Brazil, is one of the most popular tropical and subtropical fruits in the world. The current major pineapple-producing countries include Thailand, Philippines, Indonesia, Vietnam, Brazil, South Africa, United States, and southern China. Aside from being a delicious food, pineapple has been used as a folk medicine for digestion and diarrhea (Borrelli et al., 2011). By recent study, the extract of A. comosus leaves (EAL) have been detected with richness of polyphenols, which are known as antimicrobial agents (Ma et al., 2007; Gazzani et al., 2011). Our previous study showed that EAL has comprehensive effects on diabetic-dyslipidemic rats (Xie et al., 2005). It was shown that EAL has significant anti-diabetic, anti-dyslipidemic, and antioxidative effects as well as insulin sensitivity improving in animal models (Xie et al., 2006, 2007). However, studies on the metabolism of polyphenols in EAL by oral administration are few.

Polyphenols, a large group of natural antioxidants, are generally involved in defense against ultraviolet radiation or aggression by pathogens. Epidemiological studies and associated meta-analyses strongly suggested that long term consumption of plants rich in polyphenols offer protection against hypertensive (Rodrigo et al., 2012), oxidants (Attaia et al., 2013), dermatophytes (Tao et al.,...
2013), anxiolytic activity (Malik et al., 2013), development of lung cancer, cardiovascular disease, diabetes and neurodegenerative diseases (Pandey et al., 2009). Here, we present the absorption and biological effects of phenolic compounds after oral administration of EAL in the context of relevance to hypolipidemic and hypoglycemic.

We previously reported that EAL is rich in phenolic acids, including p-coumaric acid, caffeic acid, 1-O-p-coumaroylglycerol, and 1-O-caffeoylglycerol (Figure 1) (Ma et al., 2007; Wang et al., 2006). The most abundant and stable component of EAL is p-coumaric acid, which is the main form of phenolic component presented and excreted in vivo. p-Coumaric acid has been reported to inhibit the growth of Staphylococcus aureus, Shigella, and Escherichia coli (Pereira et al., 2007). Animal experiments have demonstrated that the lipid-lowering effect of p-coumaric acid manifests by protecting low-density lipoprotein cholesterol from oxidation (Morais et al., 2009). Therefore, it is used as a bioactive marker for quality control of EAL (Zang et al., 2000). The kinetic behavior of p-coumaric acid in mouse plasma has been detected after oral administration of EAL. p-Coumaric acid was found with quick absorption ($T_{max}$ 0.062 h) and short half-life time ($t_{1/2}$ 0.023 h) (Meng et al., 2006). Four major ingredients were able to be detected in the plasma, bile and urine (data are not shown). However, the ratio of the ingredients was altered in these samples, p-coumaric acid became more and the other three ingredients became less. It was mentioned that there stayed a metabolism during the ingredients of EAL absorbed just through the intestines. Therefore, the absorption of the four ingredients of EAL after oral administration needs to be studied. In this study, HPLC and LC-MS-MS were used to detect and identify the phenolic compounds of EAL. The absorption by pass through the intestines in vivo and in vitro was used as model. The microsome incubation of live and small intestines was used to confirm the metabolites and bio-transformation of the four compounds.

MATERIALS AND METHODS

Chemicals and reagents

p-Coumaric acid (trans-p-hydroxycinnamic acid) standard (98% purity) was purchased from Sigma (U.S.). 1-O-p-Coumaroylglycerol, caffeic acid, and 1-O-caffeoylglycerol standards were provided by Dr. Wei Wang. EAL with batch number 110801 was produced in our laboratory, containing 0.14% of 1-O-p-coumaroylglycerol, 1.31% of caffeic acid, 0.19% of 1-O-caffeoylglycerol and 1.98% p-coumaric acid by high performance liquid chromatography (HPLC) determination. Methanol and acetonitrile (HPLC grade) were purchased from Xinhua Special Reagent Factory, Tianjin, China.

Animals

Male ICR mice weighing 23-25 g and male Wistar rats weighing 230-250 g were purchased from Vital River Laboratories (Beijing, China). The animals were raised in an environment with 60 ± 5% humidity at 25°C under a 12 h dark/light cycle. The animals were fasted overnight before the experiment. All the animals and the experimental procedures were approved by the Animal Welfare and Ethics Committee of Tsinghua University and the Institutional Animal Care and Use Committee of Tsinghua University.

Chromatographic system and conditions

Phenolic acids were detected using HPLC for quantity and LC-MS-MS for indentify of four compounds in EAL. The HPLC system were used to detect and identify the phenolic compounds of EAL. The absorption by pass through the intestines in vivo and in vitro was used as model. The microsome incubation of live and small intestines was used to confirm the metabolites and bio-transformation of the four compounds.
for data collection. Sample analysis was carried out by applying a 10 μL volume to an ODS-3 C18 reversed-phase column (5 mm, 150 × 4.6 mm) and detected at 310 nm (1-O-p- coumaroylglycerol and p-coumaric acid) and 324 nm (1-O-caffeoylglycerol and caffeic acid). The mobile phase [0.1% phosphate acid, pH 3: water-acetonitrile (88:12, v/v)] filtered through a 0.45 μm millipore filter and degassed prior to use] was utilized at a flow rate of 1 mL/min with a constant temperature (25°C).

A liquid chromatography/ linear ion trap mass spectrometry (LC-MS) system (Agilent 1200/6340). The LC equipment (Agilent 1200) comprised of MS pump (Agilent 6340), an autosampler with a 10 μL loop. This was interfaced with an Ion trap mass spectrometer fitted with an ESI source and operating in zoom scan mode for the accurate determination of parent ion m/z, MS2 mode to obtain fragment ion m/z. MS operating conditions (negative ion) had been optimized with a nebulizer pressure of 15.0 psi, a capillary voltage of 3.5 kV, a dry gas flow rate of 8 L/min, and a dry temperature 350°C. Sample analysis was conducted by using a C18 reverse-phases column (0.5 μm; 4.6 × 150 mm, Rainbow, China) and detected at 310 nm. The mobile phase was water-acetonitrile (containing 0.1% formic acid, pH 3.0: 86:14, v/v) filtered through a 0.45 μm filter membrane used at a flow rate of 0.2 mL/min and room temperature (25°C).

Experiment procedures

Kinetic behavior and blood sample preparation

After overnight fast, the mice were orally administered with EAL aqueous solution at single dose of 300 mg/kg. At the time points (15, 30, 60, 120 and 240 min after intragastric administration), blood samples were collected and transferred to a heparinized Eppendorf tube and centrifuged at 4500 rpm for 10 min. Plasma (400 μL) was taken and mixed with 4 mL methanol (1:10, v/v). This mixture was oscillated for 30 s and centrifuged at 12000 rpm at 4°C for 10 min. The supernatant was collected and dried at room temperature. Meanwhile, the small intestines were taken and washed with 5 mL methanol. The eluate ion was transferred in a vial and dried at room temperature. The residue of blood samples was dissolved in 100 μL methanol, whereas that of the small intestine solution was dissolved in 500 μL methanol. 10 μL of each sample was used for HPLC analysis.

Absorption through the intestines in vivo and in vitro

In vivo

Male Wistar rats were anesthetized by injection of urethane (1 g/kg, i.p.) and kept in a plat with supine position. The small intestines were stored at 80ºC until use. All parts of the intestines were turn down from inner to outer. The bag was taken by ingaturing the both ends of the intestines.

After that, the bags from the intestines were taken into the incubation liquid containing EAL at 37°C for 30 min. The bags were taken out and washed in normal saline three times. The absorption liquid was got from the bag and stored at -80°C until to detect. Normal saline was used as a negative control.

Conversion experiment in vitro

The mice were killed by cervical dislocation, and the liver and small intestines were taken out for the microscope solution containing metabolite enzymes (Ren et al., 2009). The homogenates were centrifuged at 15 000 × g for 10 min at 4°C. 7 ml of supernatants was collected and 1.25 ml of 52 mM CaCl₂ was added. Tubes were gently shaken for 5 s and allowed to stand in an ice bath for 15 min. The solution was then centrifuged at 25 000 × g for 15 min. Pellets were finally resuspended in 0.3 ml of 0.1 M potassium phosphate buffer containing 20% glycerol. The microsome solution was aliquoted and stored at -80°C until use. The quantity of protein in microsomal solution was acquired by total protein (TP) assay kit (Zhongsheng Biotechnology Company, Beijing, China) and the concentration of protein was set at 7.5 mg/ml. From the solutions of both liver and small intestine, 100 μL samples were taken, and EAL (10 μg) was added in. After the mixture was incubated at 37°C for 30 min, 1 mL cold methanol was added to stop the reaction. The sample handling for this experiment was the same as with the plasma sample.

Caffeic acid was added to 100 μL microsome incubations of liver and small intestine. The mixture was incubated at 37°C for 30 min, and 1 mL cold methanol was added to stop the reaction. Then, the sample handling was the same as with the plasma samples.

Data analysis

All data are expressed as mean ± S.D. Data were statistically analyzed by using one-way analysis of variance (ANOVA) with F value determination. Values obtained at P<0.05 were considered statistically significant. All statistics were carried out by using the software for Office2007 (Microsoft, U.S.). The content of four compounds was determined by their standard curve and the equations. Ratio of each compound among these compounds was conducted by the equation, [content of each compound / (sum of these four compounds)] × 100%.

RESULTS

HPLC and LC/MS/MS analysis

By HPLC, four compounds of EAL, 1-O-caffeoylglycerol, caffeic acid, 1-O-p-coumaroylglycerol and p-coumaric acid, were shown in accordance with the standard compounds (Figure 1 and 2). Their retention times were 4.651 min (1-O-coumaroylglycerol), 8.227 min (1-O-p-coumaroylglycerol) and 9.335 min (p-coumaric acid). There were no interference in the detecting time. All the compounds were identified by LC/MS/MS (Figure 3). The standard curves of four compounds were conducted and the equations for quantity were as follows: 1-O-caffeoylglycerol: y (ng) = 20000x +11512 (r²=0.9976); Caffeic acid: y (ng) = 3322.19x +1614.9 (r²=0.9949); 1-O-p-coumaroylglycerol: y (ng) = 2781x + 4366.4 (r²=0.9979) and p-coumaric acid: y (ng) = 5586x+14290 (r²=0.9998).
Figure 2. Typical chromatograms for the conversion of extract of *A. comosus* leaf (EAL) in samples. Chromatograms of (A) 1-O-caffeoylglycerol (I), (B) caffeic acid (II), (C) 1-O-p-coumaroylglycerol (III), (D) p-coumaric acid (IV) and (E) four compounds of EAL.
Kinetic behavior and the compounds of EAL in mice plasma

After 15 min oral administration of EAL, \( p \)-coumaric acid was detected with higher peak concentration. Thereafter, it came down gradually near to the bottom at 240 min (Figure 4A). The concentration of four compounds of EAL in plasma at the time of 15 and 30 min was detected. The ratio of \( p \)-coumaric acid to the total compounds was highest, implying \( p \)-coumaric acid was maintained in major (Figure 4B).

Intestinal absorption of the compounds of EAL in vivo and in vitro

The absorption of four compounds in EAL was not adequate in rat small intestines. Generally, 1-O-caffeoy and 1-O-p-comar were found to be less than caffeic acid and \( p \)-coumaric acid. The content of \( p \)-coumaric acid was found to increase highly than that of three compounds (Figure 5A). The ratio of \( p \)-coumaric acid in the total content of all the four compounds became larger up to 89.36%. The ratio of caffeic acid was found to decrease to 9.61% compared to that of 35.37% of EAL. The ratio of 1-O-\( p \)-comar was 1% down from 4.89% of EAL. The ratio of 1-O-caffeoy was 0.03% down from 3.81 of EAL. Therefore, it is clear that the good absorption of \( p \)-coumaric acid was confirmed by small intestines (Figure 5B).

By the assay of different part of small and large intestines, the four compounds was found to be able to absorbed effectively through the duodenum and jejunum. The available absorption of ileum was weaker than duodenum and jejunum. Colon was poor for the absorption of four compounds (Figure 6). In duodenum,
Figure 4. Kinetic concentration of $p$-coumaric acid (A) and the ratio of four compounds (B) in blood after oral administration of extract of A. comosus leaf (EAL) in mice. 1-O-caffeoyl: 1-O-caffeoylglycerol; Caffeic: caffeic acid; 1-O-$p$-coumar: 1-O-$p$-coumaroylglycerol; $p$-Coumar: $p$-Coumaric acid. The ratio of $p$-coumaric acid either in 15 min blood sample and or in 30 min blood sample is higher than that of other three compounds ($F_{15\text{min}}$, 3,8 =431.7, $P<0.001$; $F_{30\text{min}}$, 3,8 =80.2, $P<0.001$). Each value represents the mean±S.D. from three independent mice (n=3). ** $P<0.01$.

Figure 5. The absorption of the four compounds through the small intestines after extract of A. comosus leaf (EAL) oral administration in rats. $F(3,8)=463.84$, $P<0.001$. Each value represents the mean±S.D. from three independent samples (n=3). ** $P<0.01$ v.s. the control groups.

detected in small intestine solution and plasma. We hypothesized that other processes remained during the absorption. We therefore conducted a conversion experiment of EAL in vitro. After the microsome incubation, the contents of four compounds were altered. 1-O-caffeoylglycerol and 1-O-$p$-coumaroylglycerol decreased in both liver and small intestines compared to the control of EAL (Figure 8A and C). Caffeic acid in liver incubation decreased compared to that of control samples. There was no difference of caffeic acid in the small intestines and control groups (Figure 8B). The content of $p$-coumaric acid in both liver and small intestines incubations were higher than that of control groups (Figure 8D).

Getting the four ingredients together as total content, the ratio of each compound to the total content was calculated. Figure 9 shows the ratios of four compounds of EAL, microsome in liver and small intestines. Two analogues of 1-O-caffeoylglycerol and 1-O-$p$-coumaroylglycerol showed little ratio to total content compared to EAL. Speculating, caffeic acid in liver incubation was little ratio to total content distinctly implying there must be a metabolism of caffeic acid in.

the absorbed ability of $p$-coumaric acid was stronger than in the other two parts of small intestines, in which its ratio nearly equated to that of EAL (Figure 7A). The ratios of caffeic acid to the total compounds in jejunum and ileum were higher than that of EAL (Figure 7B and C). The ratio of both 1-O-caffeoylglycerol and 1-O-$p$-coumaroylglycerol in the parts of small intestines were similar to that of control (EAL). In colon, the ratio of 1-O-$p$-coumaroylglycerol in the total compounds was decreased relatively to that of EAL (Figure 7D).

Conversion of the compounds of EAL

During the study, some of the phenolic acids could not be
Liver Figure 10. The ratio of p-coumaric acid in liver and small intestines was increased compared to EAL, suggesting there must be a biotransformation in small intestines while the ingredients were absorbed through the intestines, specially 1-O-p-coumaroylglycerol (Figure 9).

LC-MS-MS results supported our hypothesis. After incubation with the microsome incubation of both liver and small intestines, 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol were found to convert into p-coumaric acid and caffeic acid, respectively. It is easy to distinguish 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol by its MS base peak at m/z~237 and 253, supported by strong MS2 ions at m/z=119, 129 and 135, 160.9, respectively. The ion at m/z~163 is characteristic for p-coumaric acid, supported by strong MS2 ions at m/z~119,1. The ion at m/z~163 is characteristic for caffeic acid, supported by strong MS2 ions at m/z~135 (Fig.10). All these indicated that 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol were metabolised to p-coumaric acid and caffeic acid, respectively.

Metabolism of caffeic acid

By adding caffeic acid standard with the microsome incubation of liver and small intestine, the content of caffeic acid attenuated after the microsome incubation of liver comparing to normal saline incubation. There was no difference of caffeic acid between normal saline and small intestine incubation (Figure 11A).

Quasi-molecular ion [M−H]− of caffeic acid standard at retention time 15.8 min was 179 with product ion 135. One metabolite at the retention time of 14.4 min was found with Quasi-molecular ion [M-H]- 359. The MS2 of the metabolite included 135 and 179, indicating it was from caffeic acid. The product ions of it included 135 [M−H−Glucuronide−CO2]−, 179 [M−H− Glucuronide]−, prototype of CA (Figure 11B). All that suggested caffeic acid was glucuronized conjugate in liver microsome (Zhang et al., 2008).

This result suggested that caffeic acid might be transformed into a new compound with the effect of the microsome incubation of liver. The metabolism of caffeic acid occurred in the microsome incubation of liver but not small intestine, this might due to the different metabolic enzymes present in the liver and small intestine. All the transformation of the ingredients after oral administration of EAL is displayed in Figure 12.

DISCUSSION

In this work, we proved that phenolic acids in the extract of A. comosus leaf (EAL) can be absorbed in intestines...
Figure 7. Ratio to the compounds through the different sizes of the rat intestines in vitro. 1-O-caffeoy, 1-O-caffeoylglycerol; Caffeic, caffeic acid; 1-O-p-coumar, 1-O-p-coumaroylglycerol; p-Coumaric, p-coumaric acid. Each value represents the mean ± S.D. from three independent samples (n=3). * \( P<0.05 \) v.s. the control groups.

Figure 8. Concentration of four compounds in extract of A. comosus leaf (EAL) (1 mg/ml) in the incubation of normal saline and the microsomes of both liver and small intestines. 1-O-caffeoylglycerol: F(2,6)=5836.48, \( P<0.001 \). Caffeic acid: F(2,6)=439.53, \( P<0.001 \). 1-O-p-coumaroylglycerol: F(2,6)=5673.44, \( P<0.001 \). p-Coumaric acid: F(2,6)=81.72, \( P<0.001 \). Each value represents the mean ± S.D. from three independent samples (n=3). ** \( p<0.01 \) v.s. the control groups.
Figure 9. Ratio of each compound among these four compounds after incubation in vitro. Each value represents the mean ± S.D. from three independent samples (n=3). * P<0.05, ** P<0.01, v.s. the control groups.

Figure 10. Liquid chromatography–tandem mass spectrometry (LC-MS) of four compounds of EAL after incubation in the liver microsome solution.
after oral administration. There existed biological transformation when the phenolic acids were absorbed into the bloodstream by passing through the intestines. 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol could be converted into p-coumaric acid and caffeic acid. p-Coumaric acid and caffeic acid were the master and active components in blood after oral administration of EAL. Besides, caffeic acid could be converted into a

Figure 11. Contents of caffeic acid with normal saline (control) and microsome incubations of liver and small intestine (A) and liquid chromatography–tandem mass spectrometry (LC-MS) of the metabolite of caffeic acid in liver microsome (B). (A): F (2, 6) = 243.78, P<0.001. Each value represents the mean ± S.D. from three independent samples (n=3). ** P<0.01, v.s. the control groups.
metabolite in liver during this process.

The phenolic acids in A. comosus leaves are mainly p-coumaric acid, caffeic acid, 1-O-p-coumaroylglycerol, and 1-O-caffeoylglycerol. Throughout our experiments, we found that there were not other components in the plasma besides p-coumaric acid and caffeic acid after oral administration of EAL. We proved that there existed conversion of p-coumaric acid and caffeic during the absorption after oral administration of EAL. 1-O-p-coumaroylglycerol and 1-O-caffeoylglycerol could convert into p-coumaric acid and caffeic acid, respectively. Caffeic acid might be transformed into a new compound which was in agreement with previous report (Martignoni et al., 2006). These results suggested that p-coumaric acid and caffeic acid were the main functional substances of EAL. There might be a better efficacy to improve p-coumaric acid and caffeic acid ratio in medicinal research and practice in clinic.

In our study, there might be a relationship between the above conversion and the enzymes present in the liver and small intestine. According to previous literature, many phenolic acids were the substrates of cytochrome P450 (CYP) (Martignoni et al., 2006). CYP1, CYP2, CYP3, and CYP4 families play a crucial role of the metabolism functions (Uno et al., 2008). The phenolic acids in EAL might become the substrates of CYPs during absorption because of the presence of CYPs in the small intestine and liver (Martignoni et al., 2004; Nakanishi et al., 2010). We found that the 1-O-caffeoylglycerol and 1-O-p-coumaroylglycerol levels in EAL decreased, and p-coumaric acid increased in the microsome incubations of liver and small intestine compared with that of normal saline. We may therefore conclude that the phenolic components were absorbed mainly in the form of p-coumaric acid and caffeic acid.

CYP subtypes present in liver and small intestine are different (Ramiro et al., 2009). For example, CYP1A2, CYP2U1, and CYP2V1 are found only in the liver; CYP1B1, CYP2R1, and CYP2W1 are found only in the small intestine, although other CYPs have similar tissue distributions, their expressions are more or less different (Nelson et al., 2004; Choudhary et al., 2005). The modulation of cytochrome P450-linked enzyme activity by caffeic acid revealed that it was the substrate for CYP1A2 which only presented in liver (Teel et al., 1998; Huang et al., 2009). It is suggested that certain CYP enzymes of metabolizing caffeic acid are presented in the liver, not in the small intestines. We therefore might draw a word that 1-O-p-coumaroylglycerol could convert into p-coumaric acid and 1-O-caffeoylglycerol could convert into caffeic acid when it was absorbed through the small intestine.

Conclusion

In the present study, we first proved that there existed biotransformation among phenolic acids when they pass through the intestines and were absorbed into blood when the EAL was orally administrated. p-Coumaric acid and caffeic acid are the major ingredients of EAL when
absorb orally. Since A. comosus leaves are the huge products all over the world, this study will be benefit for understanding its in vivo behavior and developing of its pharmacology of anti-hyperlipidemia and anti-hyperglycemia.

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ABBREVIATIONS

EAL, Ananas comosus leaves; HPLC, high performance liquid chromatography; TP, total protein.

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Ciprofloxacin inhibits proliferation and synergistic effect against hepatocellular carcinoma cancer lines with cisplatin

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Combination therapy has generated significant interest in the clinical setting since it increases the therapeutic potential of anticancer drugs. The potential therapeutic benefits in combination of cisplatin and ciprofloxacin have not been fully explored in hepatocellular carcinoma cancer therapy. In the results to be presented here, cellular viability of Hep G2 and Bel-7402 was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and the assessment of synergism, additivity or antagonism was carried out by the median effect analysis. Mechanism of cytotoxicity of the drugs was probed by reverse transcriptase-polymerase chain reaction (RT-PCR). Cellular DNA breaks were evaluated by comet assay. In addition, the effect of the drugs on topoisomerases was also investigated. The results show that ciprofloxacin could affect viability of Hep G2 and Bel-7402 lines, its combination with cisplatin resulted in mixed interactions; synergistic and additive effect. Further investigation indicated that the synergistic effect or additive was correlated to Fas involved apoptotic path, but the effects in combination on topoisomerases were not evident. The results obtained in this study suggest that the therapeutic benefit of cisplatin anti-hepatocellular carcinoma (HCC) may be influenced by ciprofloxacin.

Key words: ciprofloxacin, cisplatin, combination, synergistic effect, hepatocellular carcinoma cell line, apoptosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the sixth most common malignancy and the third most common cause of cancer related death worldwide and accounts for as many as one million deaths annually (Varela et al., 2003). Surgical resection, local ablation therapies, and liver transplantation are regarded as potentially curative treatment modalities for HCC patients depending on the size and number of tumors. However, surgical prognosis for many patients with HCC is not favorable due to a higher likelihood of intrahepatic and remote recurrences (Yu et al., 2012). The effective treatment of this kind of disease in clinics is of higher priority.

Ciprofloxacin and its derivatives belong to the class of 4-fluoroquinolone antibiotics which are commonly used in the therapy of many bacterial infections (Koziel et al., 2010). It has been shown that the fluoroquinolones (FQs) target the bacterial enzyme DNA gyrase and also stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV, but the FQs have lesser affinity for the eukaryotic DNA gyrase homologue, topoisomerase II (topo II). Normally, at concentrations higher than normally achieved in blood, inhibition of topo II can occur resulting in the formation of stabilized cleavage complexes and ultimately the production of DNA double-strand breaks.
In addition to the antibacterial activity of ciprofloxacin, well documented evidences have shown that it has substantial antiproliferative activity against various cancer cells, such as bladder, colorectal, human prostate cancer, osteosarcoma and leukemic cell lines in vitro (Aranha et al., 2002a; Aranha et al., 2002b; Herold et al., 2002; Miclau et al., 1998; Theodore et al., 1998; Somekh et al., 1989). Aranha et al. also observed that ciprofloxacin could inhibit growth of human hepatocellular carcinoma cell line Hep G2 at higher doses, similar inhibition against Bel-7402 cell line was found in our laboratory. However, in order for ciprofloxacin to act as an antitumor agent, it has to be used at a much higher concentration than those used for the treatment of infectious diseases (Smart et al., 2008). At high concentrations (usually 200-300 μg/ml), ciprofloxacin can effectively induce apoptosis of bladder carcinoma cells and lead to cell cycle arrest at the S/G2 stage (Aranha et al., 2000). Prompted by this, it is worthwhile to try ciprofloxacin in anticancer therapy. Cisplatin is one of the first metal-based anticancer drugs discovered in the early 19th century and it is also of the most useful antineoplastic agents. Cisplatin mediates its anticancer effect by covalently binding to DNA so as to form adducts that interfere with transcription and DNA replication, and thereby triggers programmed cell death (Victoria et al., 2007). Clinical use of cisplatin determined that many patients with different types of cancer have been successfully treated, including sarcoma cancers, cancers of soft tissue, bones, muscles, and blood vessels. Such cancers received better prognosis and therefore became less life threatening these days (Rosenberg, 1980; Desoize et al., 2002). However the side-effects of cisplatin, such as nephrotoxicity, neurotoxicity and hearing loss were limited to widely use in clinics (Tsang et al., 2009; Shah et al., 2009). To reduce the side-effects of cisplatin, dose-reducing is one of options by different drug combination. Although cisplatin has been used in combined therapy, its combination with ciprofloxacin in vitro was not evaluated. In work to be presented here, we investigated whether ciprofloxacin works in synergy with cisplatin and the possible molecular mechanism as well as its potential application in clinics.

**MATERIALS AND METHODS**

All chemical reagents used were of analytical grade. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Sigma. Ciprofloxacin hydrochloride (Sangon Biotech, Shanghai, China) and cis-dichlorodiammine platinum (II) (cisplatin) were obtained from Dezhou drug Pharmaceutical Co., Ltd.

**Cell culture and treatment**

Bel-7402 and Hep G2 human hepatoma (Y-S Biotechnology Inc., Shanghai, China) were propagated continuously in RPMI 1640 cell culture. Cells were grown at 37°C in RPMI 1640 medium supplemented with 10% freshly inactivated fetal calf serum, 100 units/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air. The cells harvested from exponential phase of growing were used for biological evaluations.

**Cytotoxicity assay**

The stock solutions used in these studies were 1 mg/ml cisplatin (in physiological saline) and 20 mM ciprofloxacin (in H₂O). Bel-7402 (or Hep G2) cells in exponential phase were washed three times with phosphate buffered saline (PBS), followed by detaching with 0.25% trypsin and centrifugation. The cells were re-suspended in RPMI-1640 and were seeded (5×10⁵/well) into 96-well plate. The cells were incubated in a humidified atmosphere of 5% CO₂ for at least 6 h after which the drugs to be evaluated were added at final concentrations 1000, 500, 250, 125, 60, 30 and 15 μM for ciprofloxacin or 40, 30, 18, 15, 12, 9, 6 and 3 μM for cisplatin and incubated respectively for 48 h at 37°C. After 48 h, 10 μl MTT solution (1 mg/ml) was added to each well, followed by further incubation of 4 h. Following incubation, the cell culture was removed by aspiration and 100 μl dimethyl sulfoxide (DMSO) was added in each well to dissolve the formazan crystals. The measurement of absorbances of the solution that was related to the number of live cells was performed on a microplate reader (MK3, Thermo Scientific) at 492 nm. Percent growth inhibition was defined as percent absorbance inhibition within appropriate absorbance in each cell line. The same assay was done in triplicate.

**Cytotoxicity studies with simultaneous drug combinations**

After an initial pre-screening assay to evaluate single drug IC₅₀, dose range was chosen to cover the concentrations below and above the IC₅₀ of each drug to explore the potential interaction between the individual drugs in the given cell lines. The exposure of ciprofloxacin and cisplatin simultaneously to the cell lines were investigated at non-equivalent drug ratios. In the experiments, concentration of ciprofloxacin was at 30, 60 and 120 μM, respectively. Meanwhile the tests at constant equipotent ratio (molar ratio of ciprofloxacin/cisplatin = 5) were conducted too. The cell viability in different combination was determined using same procedure as in individual drugs.

**Median effect plot analyses**

The combined effects of two drugs in terms of synergism, summation, or antagonism were analyzed by the median effect plot (Chou et al., 1984; Chou et al., 2010). If the observed effect of two drugs acting simultaneously is larger or smaller than that calculated from the product expression, it is assumed that synergism or antagonism, respectively, has occurred. The procedure was performed by plotting the dose-effect curves for each drug and different combinations of the drugs using the "median effect" equation 1.

\[
fa / fu = (D / Dm)^m
\]  

Where, D is the dose, Dm is the dose required for 50% effect, fa is the fraction affected by the drug(s), fu is the fraction unaffected, and m is a coefficient signifying the sigmoidicity of the dose-effect curve. The dose-effect curve was plotted using a logarithmic conversion of equation 1 and the values of m and D_m were determined. Based on the slope of the dose-effect curves, it can be determined whether the agents have exclusive effects or mutually nonexclusive effects. Combination indices (CI) was then determined using the equation 2, below:
Preparation of nuclear extracts

Crude nuclear extract was prepared according to the published procedure (Ewhm et al., 1995) with some modification. Briefly, cells were detached using trypsin followed by centrifugation and washing twice with cold nucleus buffer (NB) (150 mM sodium chloride, 1 mM potassium dihydrogenphosphate, 5 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiotreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 6.4) at 4°C. The supernatant was discarded and the cell pellet was re-suspended in 300 μl NB with an 0.3% Triton X-100 and placed on ice for 10 min, then transferred into a glass Dounce homogenizer with ten up-and-down strokes using a loose-fitting pestle. The suspension solution was centrifuged at 150 g for 10 min at 4°C, the pellet was washed with Triton X-100-free cold NB. 150 μl of cold NB solution containing 0.35 M NaCl was added to the pellet and allowed to stand 30 min at 4°C in order to extract the nuclear proteins. The supernatant was obtained by centrifugation at 10,000 g for 10 min at 4°C. The protein concentration was determined using the Bradford method.

Topoisomerase I activity assay.

To evaluate the effect of the individual or combined drug on DNA topoisomerase I (topo I) activity, the topo I assay was conducted. Briefly, nuclear extract (0.4 μg) treated by the drugs was added to the topo I reaction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM spermidine, 5% glycerol and 0.4 μg supercoiled DNA plasmid (pMD18-T) at a final volume of 20 μl and incubated at 37°C for 30 min. Following incubation, the reaction was terminated by adding 5 μl of stopping buffer (10% SDS, 0.025% bromophenol blue and 5% glycerol). The reaction products were analyzed by electrophoresis on 1% agarose gel using a TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2 mM EDTA) at 45 V for 3 h, stained by ethidium bromide (0.5 μg/ml) and photographed using a short wavelength UV lamp on Tocan 360 gel scanner (Shanghai Tiancheng Technology Inc, China).

DNA topoisomerase II assay

Relaxation activity of DNA topo II was determined by measuring the conversion of supercoiled pMD18-T plasmid DNA to its relaxed form as described in topo I activity assay except in the assay in the presence of ATP (Ting et al., 2003; Oksuzoglu et al., 2008; Emine et al., 2008).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

To explore the underlying mechanism, especially at early phase, the Hep G2 cells were treated with 1/4 or 1/2 IC50 dose and a short time period incubation. The RT-PCR was conducted to determine the changes of apoptotic genes, such as Fas, caspase-3 and caspase-8 at mRNA level. The basal gene expression of caspase and Fas were set as control in untreated cells and was measured after 12 h incubation with different concentrations of ciprofloxacin, cisplatin and their combinations. Total RNA was extracted from the cells using Trizol reagent (Sangon, Shanghai, China) according to the manufacturer’s protocol. Three micrograms of total RNA were used for reverse transcription in a total volume of 20 μl with the M-MLV reverse transcriptase (RNase H) system (LifeFeng biological technology corporation, Shanghai, China). Aliquots of 2 μl CDNA were subsequently amplified in a total volume of 25 μl using the 2xTaq PCR kit (LifeFeng biological technology corporation, Shanghai, China) following conditions recommended by the manufacturer. The sense and antisense primer (primers were synthesized by Shanghai generay bioengineering corporation company in the study, Shanghai, China) for beta actin were 5’-ACACTGTGCCCATCTCAGAGG-3’ and 5’-CGGACTGCTCATACTCTGCT-3’ (516 bp) that were used as an internal control; the sense and antisense primers for Fas were 5’-GGATCAAGGCACTTACCA-3’ and 5’-GAAGCCCTCCTCAACA-3’ (289 bp); the sense and antisense primers for caspase 3, 5’-GAAGGGAATCAATGGACTCTG-3’ and 5’-ACATCAAGCCTCAATTCACA-3’ (241 bp); the sense and antisense primers for caspase 8, 5’-AATTTGAGCCCTGCCTGGTGTCT-3’ and 5’-ATTGAGCCCTGCTGGTGTCT-3’ (227 bp) respectively. The cycling conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min, and a final extension of 72°C for 10 min. PCR products were separated on the 1.5% agarose gel viewed by DNAgreen staining. These data were acquired with Tocan 360 gel imager (version 3.2.1 software).

Comet assay

The single-cell gel electrophoresis (comet assay) was adapted from the method of Singh et al. (1988). Bel-7402 cells with (half IC50) or without treatment of the drugs were harvested by centrifugation at 1000 rpm and then embedded in 0.5% low-melting-point agarose at a final concentration of 10^6 cells/ml. 20 μl of this cellular suspension was then spread onto duplicate frosted slides that had previously been covered with 1% normal coverslip. The photography of the stained slides was taken on fluorescent microscopy.

Statistical analysis

The data were analyzed using the Sigmaplot version 10.0. Statistical comparisons among groups were performed by Student’s t-test. P values indicate level of significance compared to CI = 1.0. Degree of interaction is defined as significant at p<0.05.

RESULTS

Single-drug cytotoxicity studies

The dose-response curves of cisplatin and ciprofloxacin
Figure 1. Effectiveness of different concentrations of cisplatin and ciprofloxacin on cell growth in the Bel-7402 and Hep G2 cell lines, as described in MTT assay in methods: a, Growth inhibition of cisplatin; b, ciprofloxacin growth inhibition in the cell lines. Each value represents the mean ± SE of three independent experiments.

determined against Bel-7402 and Hep G2 cells are depicted in Figure 1. As shown in Figure 1, both drugs (ciprofloxacin and cisplatin) had significant growth inhibition in the hepatocellular carcinoma cell lines and exhibited dose-dependence compared to cells untreated by the drug. Interestingly, ciprofloxacin inhibited proliferation of Bel-7402 and Hep G2 cell lines at higher concentration (> 100 μM). The IC50 values were acquired based on the median-effect plot after 48 h of ciprofloxacin treatment. These were 187 ± 11.3 and 290 ± 18.4 μM for Bel-7402 and for Hep G2 cells, respectively. The IC50 values after 48 h treatment of cisplatin were 9.4 ± 1.4 and 18.9 ± 2.4 μM for Bel-7402 and Hep G2 cell, respectively.

**Median effect analysis of combined effect**

The different combinations of ciprofloxacin with cisplatin were applied to treat the cell lines, and the changes of combination indices (CIs) values calculated based on method of Chou and Talalay (2010) are shown in Figures 2 and 3. Figure 2 shows the plots of combination indices versus cisplatin concentration at known concentration of ciprofloxacin when the drugs were expose to Bel-7402 cell line. As shown in Figure 2A, the presence 30 μM of ciprofloxacin displays two kinds of interactions namely; synergistic (P < 0.05, four combinations) and additive effects (P < 0.05, five combinations). With increasing concentration of ciprofloxacin (Figures 2B and 2C), the antagonistic effect was displayed, one in 60 μM ciprofloxacin (P < 0.05, CI = 2.58 >1), three in 120 μM ciprofloxacin (P < 0.05, CI = 5.4, 4.1 and 1.9 >1). In the experiment of constant molar ratio of ciprofloxacin/ cisplatin (Figure 2D), the CI values indicated synergistic effects at lower concentration (P < 0.05, four combinations at CI (mean) = 0.3471, 0.3568, 0.4825 and 0.7170 <1) and additive effects (P > 0.05, three combinations at CI (mean) = 0.7950, 0.7209 and 0.8258) in the combinations (Figure 2D). For Hep G2 cells, in the presence of 30 ~120 μM ciprofloxacin, there were additive effects between the two drugs (all P > 0.05), but at constant molar ratio, the synergistic and additive (last two combinations, P > 0.05) effects can be seen in Figure 3. The results demonstrated synergistic effect could be achieved at lower molar ratio of ciprofloxacin/cisplatin for both cell lines. And the slight difference between the HCC cell lines can also be seen.

**Effect of the drugs in individual or in combination on topoisomerase catalytic activity**

The effect of ciprofloxacin, cisplatin and their combination on expression regulation of topo I and II are shown in Figure 4. As shown in Figure 4, the different DNA cleavage pattern was also included in Figure 4. Some differences were obvious, the DNA topo I activities of drug treated nucleic extracts were stronger than that of control, but no significant differences were observed between the drug treated groups. However, DNA topo II in the nucleic extract in the combination was different from among individuals and there were two more relaxed DNA.

**Evaluation of DNA fragmentation in vitro by Comet assay**

Ciprofloxacin (IC50 = 187 μM) and cisplatin (IC50 = 9.4 μM) had definite antitumor activity against Bel-7402 cell line and caused the cellular DNA break in a time and dose dependent manner. Figure 5 shows the DNA fragmentation of Bel-7402 cells after exposure to individual drug at half IC50 or in combination for 48 h. Compared to control (non-drug treatment), both cisplatin
Figure 2. Combination index plots for Bel-7402 cells exposed to cisplatin and ciprofloxacin. Combination index (CI) versus cisplatin concentration plots obtained from median-effect analysis of Chou–Talalay. The effects of ciprofloxacin concentration on CI are indicated in the figure panel is shown in the figure. CI < 1, = 1 and > 1 indicates synergistic, additive and antagonistic effect, respectively (constant ratio, last two P > 0.05; 30 μM cip: all P > 0.05; 60 μM, all P > 0.05; 120 μM, all P > 0.05). The data were derived from HepG2 cell line.

and ciprofloxacin led to the fragmentation of genetic materials of the cells at different degrees, ~30% DNA damaged for cisplatin, ~50% for ciprofloxacin and ~100% for in combination. From comet assay, it could be seen that normal comet images account for more than 90%, ~2% cells undergoing apoptosis that has a small head and a spread tail (Figure 5) (Huang et al., 2010). In the combination group, the synergistic effect between drugs was evident for highest percentage of cellular DNA breaks.

Effect of ciprofloxacin and cisplatin on caspases and Fas

The effects of the drugs investigated in individual or combined on the genes regulation are presented in Figure 6. As shown in Figure 6b, the Fas gene expression was significantly increased in the drug(s) treated group compared to control. And higher gene expression in combined than that in individual could be seen. In contrast to Fas gene expression, the response of caspase 3 and caspase 8 was irregular (Figure 6a), the changes of caspase 3 was not significant, but caspases 8 showed a down-regulation trend. The differences in apoptotic molecular response further indicated the induced cytotoxicity by the drugs was time and dose dependent.

DISCUSSION

Although the cisplatin-induced cytotoxicity and nephrotoxicity have been very well studied in both clinical and animal studies, hepatotoxicity has not been well documented. To reduce the side effects of cisplatin, a large number of studies have been focused on the ways for prevention of cisplatin side effects by supplementing with preventive agents (Liao et al., 2008). This study aimed to explore their optimal combination of ciprofloxacin used along with cisplatin to achieve higher therapy index and decrease the hepatotoxicity and nephrotoxicity of cisplatin. And their non-overlapping toxicities could also provide the basis for the investigation
Figure 3. Combination index (CI) versus cisplatin concentration plots obtained from median-effect analysis of Chou–Talalay. The effect of ciprofloxacin concentration on CI is shown in the figure. CI < 1, = 1 and > 1 indicates synergistic, additive and antagonistic effect, respectively (constant ratio, last two P > 0.05; 30 μM cip: all P > 0.05; 60 μM, all P > 0.05; 120 μM, all P > 0.05). The data were derived from HepG2 cell line.

Figure 4. The DNA relaxation assay carried out in the presence of supercoiled pMD18-T DNA and nucleic extracts from the drug treated or untreated cells. For topo I assay: Lane 1 was DNA marker; lane 6 was pMD18-T DNA alone; lanes 2-5 were control, cisplatin, ciprofloxacin and combination, respectively; for topo II assay, 1 mM ATP was added in the reaction buffer, lanes 7-10 were corresponding to the nucleic extract from control, cisplatin, ciprofloxacin and combination group. Topo I activity was getting stronger than that of control; the supercoiled, relaxed and nicked circular DNA are indicated by I, II and III.
Figure 5. The apoptotic cells were detected by comet assay method, as described in materials and methods section. After cell electrophoresis and staining with EB, Bel-7402 cells were photographed by fluorescent microscopy: a, Control; b, cisplatin; c, ciprofloxacin; d, combination.

Figure 6. A. Effects of ciprofloxacin and cisplatin and their combination on the expression of caspase-3 (a), caspase-8 (b), Fas (c) and actin(d) in Hep G2 cells after exposure to the drugs for 12 h: 1, control; 2, 5 μM cisplatin; 3, 9 μM; 4, 25 μM ciprofloxacin; 5, 45 μM ciprofloxacin; 6, 5 μM cisplatin plus 25 μM ciprofloxacin; 7, 9 μM cisplatin plus 45 μM ciprofloxacin; 8, 72 μM ciprofloxacin; 9, 5 μM cisplatin plus 72 μM ciprofloxacin. Figure 6A: RT-PCR product of the genes as indicated. B. quantification of the mRNA expression of the indicated genes. Data represents the mean ± SD of three independent experiments.

of a potential therapeutic concurrent against HCC cell lines in vitro. To probe the potential interaction of the drugs, the cytotoxicity of ciprofloxacin against HCC cell lines were evaluated, indicating that ciprofloxacin exhibits definite anti-proliferation at higher concentration, which was slightly different from that reported (Herold et al., 2002). This difference derived from two groups was due to the different incubation time: in our experiments, 48 h was chosen, but in latter 18 or 24 h exposure to the ciprofloxacin were used. This also demonstrates that the cytotoxicity of ciprofloxacin is both dose and time dependent. Meanwhile, the CIs were calculated using the median-effect analysis of Chou and Talalay (2010). Since the median-effect plots obtained for the drugs individually
were not parallel (different slopes, data not shown), exclusivity of the drug interaction effects could not be specified (Ting et al., 2003), but only the ones obtained under the second assumption were considered in our study. This has been reported as a more conservative criterion to determine the type of interaction effects since the addition of a third term in Equation 2 slightly increases the CI value (Ana et al., 2009). As shown in Figure 2, when ciprofloxacin fixed, a varied cisplatin were applied to inhibit growth of Bel-7402 cells, a mixed manner, synergistic, additive and antagonistic effect occurred. With increase of ciprofloxacin, interaction between them was gradually changed, which can be reflected by combination indices. In the presence of 30 μM ciprofloxacin, the CI value being less 1 was more, and CI increased with increase of ciprofloxacin. When a constant ratio of ciprofloxacin/cisplatin (< 5) was used, there were more CI values less one. The information indicated the interactions displayed between the drugs were a dose and ratio dependent. Similar results were obtained when the drugs combined were applied on Hep G2 cells (Figure 3). The synergistic and additive effects were predominated under the studied condition. A slight difference between the two cell lines was due to the difference in cellular sensitivity exposure to the drugs. It was clearly shown that ciprofloxacin had a definite function to influence therapeutic index of cisplatin in the current study. Dose reduction of cisplatin in clinic will be a good option when same therapeutic index was achieved by addition of less toxic supplements, such as ciprofloxacin. Although, ciprofloxacin has significant cytotoxicity at higher dose, its side-effect is much less compared to cisplatin. For HCC patients, especially liver partial resection, administration of ciprofloxacin could be beneficial because the lower dose cisplatin applied could reduce its nephrotoxicity and neurotoxicity.

DNA topo I and II are essential nuclear enzymes that modulate DNA topology during multiple cellular processes, they play a crucial role in chromosome structure, condensation/decondensation and segregation during mitosis (Wang, 1996). Effects of cisplatin and ciprofloxacin on DNA topo I expression and their activities were also evaluated in our study. In general, tumor cells with high topo I activity are sensitive to topo I inhibitors (Andoh et al., 1987; Barry et al., 1990). Beyond our expectation, the DNA topo I activity of nucleic extract after 48 h exposure of cisplatin was stronger than that of control, showing a up-regulation of DNA topo I (Figure 4), which was not in agreement with the previously published results by Keisuke et al. (2004). Their research found the topo I activity in the given cell lines began to decrease at ~1-2 h after cisplatin exposure, but gradually recovered after 4-5 h. The up-regulation of topo I activity after cisplatin exposure may reflect the mechanism of cellular repair. However, the relationship between the effect of cisplatin on topo I activity and its sensitivity to topo I inhibitors is still unclear (Keisuke et al., 2004).

It has been demonstrated that futile attempts to repair cisplatin-induced DNA damage may result in the triggering of apoptosis (Miguel et al., 2003). And the effects of cisplatin on caspases and Fas of cancer cells have well been documented in many literature, however, the information revealed were mostly from treatment by after 48 h incubation. How the apoptotic molecules in early phase respond to the treatment of ciprofloxacin and cisplatin was paid less attention. We investigated the changes of the apoptotic molecules in the early phase after 12 h treatment of the drugs in individual or in combined model, indicating the Fas gene can be up-regulated when treatment of Hep G2 cells with the drugs in individual or in combined. It was clearly shown that synergistic and additive effects on Fas gene expression in the combined treatment, indicating the induction of apoptosis by cisplatin and ciprofloxacin were both involved in Fas-mediate signal path.

It has been observed that addition of ciprofloxacin did not result in any difference in Fas expression compared to that of control Jurkat T cells (Jun et al., 2003), but ciprofloxacin effected on Fas gene expression of malignant cells was less reported. In our study, it was clear that ciprofloxacin induced an up-regulated Fas gene expression, which might indicate the cytotoxic effects of ciprofloxacin on Hep G2 cell was involved in Fas/FasL death path instead of inhibition of topo II. Recently E reported heat shock factor 1 is a transcription factor of Fas gene (E et al., 2010), whether the up-regulated Fas gene expression by ciprofloxacin was due to recruitment of heat shock factor 1 or other transcription promoter is not clear. Cisplatin induced cell death may result from both Fas receptor dependent pathway and mitochondria-dependent pathway (Amélie et al., 2010). We presume that the two drugs exhibited a synergistic and additive effect came from their regulation action on Fas gene expression.

In conclusion, ciprofloxacin promotes cisplatin-evoked Hep G2 cancer cell apoptosis via up-regulated Fas gene expression synergistically. Different molar ratio of ciprofloxacin/cisplatin may have a different interaction pattern, suggesting that any concurrent supplementation of ciprofloxacin during the course of cisplatin-based chemotherapy could be beneficial for patients. The mechanism of ciprofloxacin induced the up-regulated Fas gene expression via recruitment of heat shot factor 1 or other transcription factor was not clear, a further study to probe the underlying path was required.

ACKNOWLEDGMENTS

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ABBREVIATIONS

HCC, Hepatocellular carcinoma; FQs, fluoroquinolones; top II, topoisomerase II; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; NB, nucleus buffer; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; CIs, combination indices.

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Full Length Research Paper

Evaluating the anti-fertility activity of *Talinum paniculatum* (Jacq.) Gaertn in female wistar rats

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*Talinum paniculatum* (Jacq.) Gaertn (*T. paniculatum*) root and leaf methanolic extracts exhibited significant estrogenic activity in female Wistar rats. Both extracts (100 and 1,000 mg.kg⁻¹ B.W) produced anti-implantation activity and early abortifacient activity in a dose dependent manner (*P*<0.001). The phytochemical screening of the plant extracts showed presence of various phytosterols: campesterol, β-sitosterol, stigmasterol, stigmastan-3-ol, stigmast-22-en-3-ol and stigmastanol. The results suggested that *T. paniculatum* root and leaf extracts possess estrogenic activity and anti-fertility effect. This may be mainly due to the presence of phytosterols.

Key words: *Talinum paniculatum* (Jacq.) Gaertn, estrogenic activity, anti-implantation activity, early abortifacient activity, anti-fertility.

INTRODUCTION

Fertility control is a critical issue for women worldwide. About 1% of pregnant women lose their lives due to the unintended pregnant or obtain an abortion in order to avoid having unwanted child (Glasier et al., 2006). Generally, the serious adverse effects such as depression, gastrointestinal disturbance, massive painful uterine contraction, systemic illness, permanent infertility or death are frequently reported in women who utilized synthetic drugs or steroid contraceptions (O’Connell et al., 2007; Sanchez-Criado et al., 1997). Although herbal contraceptives could never reach the level of classical contraceptive pills, they are commonly cheaper and may be used with minimum undesirable side effects. Hence, there is a need for suitable medicinal plants with anti-implantation and abortifacient activity that could be both safe and effective to use to control pregnancy.

*Talinum paniculatum* (Jacq.) Gaertn (*T. paniculatum*) or Som Java is recognized as having various medicinal properties (Jung et al., 2006). *T. paniculatum* is a wild deciduous perennial herb with well-developed root system. The medicinal-prepared *Talinum* spp. has long been used in folk medicine; particularly, in the treatment of type-2 diabetes, inflammatory skin problems, gastrointestinal disturbance, general weakness and reproductive disorders (Shimoda et al., 2001; Pak et al., 2005), for aphrodisiac effect and to increase vitality (Manuhara et al., 2012). The root has active constituents such as steroidal saponins and tannins. However, only tannins can be detected in the leaf (Yulia et al., 2005). Additionally, Filho et al. (2010) reported that campesterol, β-sitosterol, stigmasterol could be extracted from the leaf of *T. paniculatum*. In general, these compounds are known as phytosterols, and they possess both estrogenic and anti-estrogenic effects due to their estrogen-like structure. Despite these traditional medicinal properties, no scientific data are available regarding anti-fertility effect of the plant *T. paniculatum*. Therefore, this study was designed for evaluating the anti-fertility activity of

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Table 1. Treatment regimen for estrogenic activity evaluation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment and dosage</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. OVX</td>
<td>Vehicle control (1 mL/rat)</td>
<td>Orally</td>
</tr>
<tr>
<td>2. OVX</td>
<td>Root extract (100 mg.kg⁻¹ BW)</td>
<td>Orally</td>
</tr>
<tr>
<td>3. OVX</td>
<td>Root extract (1,000 mg.kg⁻¹ BW)</td>
<td>Orally</td>
</tr>
<tr>
<td>4. OVX</td>
<td>Leaf extract (100 mg.kg⁻¹ BW)</td>
<td>Orally</td>
</tr>
<tr>
<td>5. OVX</td>
<td>Leaf extract (1,000 mg.kg⁻¹ BW)</td>
<td>Orally</td>
</tr>
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</table>

Estrogenic activity evaluation

Female immature Wistar rats were anesthetized using thiopental sodium (25 mg.kg⁻¹ BW) intraperitoneally, and OVX was carried out via paralumbar incision just caudal to the 13th rib. After 14 days of endogenous hormonal decline (Tanee et al., 2007), OVX rats were divided into 5 groups of 5 rats in each group. First group received the vehicle (Tween 80 in sesame oil, 10% v/v) and served as control. Group 2-5 were treated with different doses of *T. paniculatum* root and leaf extracts (100 and 1,000 mg.kg⁻¹ BW, respectively). Treatment regimen for the experiment is shown in Table 1. The extracts were given for 5 consecutive days; vaginal opening and vaginal smear were observed daily between 9:00-10:00 am.

Vaginal smear was performed to examine cellular differentiation and to evaluate the presence of leukocytes, nucleated cells, or cornified cells. Vaginal smear samples were collected between 9:00-10:00 am daily by gently inserting the tip of dropper into the vagina, flushing normal saline (0.9% NaCl) in and out, and placing the fluid onto microscope slides and stained by methylene blue dripping (Urasopon et al., 2008). The appearance of cornified cells was used as an indicator of estrogenic activity (Cook et al., 1933; Parhižkar et al., 2011). The obtained vaginal cells were counted in 3 randomly chosen areas of the slide, and the percentage of cornified cells (%Co) was calculated using the following formula:

\[
\text{Percentage of cornified cell (%Co) = } \frac{\text{Cornified cells x 100}}{\text{Cornified cells + nucleated cells + leucocytes}}
\]

Fertility evaluation

The experimental protocols were designed by evaluating the anti-implantation activity and abortifacient activity as described previously (Mukhram et al., 2012). Briefly, adult female rats of proestrous stage were selected and left overnight with fertile male (1 female : 1 male). After 24 h of intercoursal period, the rats were separated and the spermatic clumps were observed. The rat that showed thick clumps of spermatozoa in vaginal smears was designated as in 1st day pregnancy.

Pregnant rats were randomly separated into 5 groups of 5 rats each. First group received the vehicle (Tween 80 in sesame oil, 10% v/v) and served as control. Group 2 through 5 were treated with different doses of *T. paniculatum* root and leaf extracts (100 and 1,000 mg.kg⁻¹ BW, respectively). Treatment regimen for the anti-fertility experiment is shown in Table 2. All groups were orally administered the vehicle and plant extracts during 1st -7th day of pregnancy. On the 8th day, the bilateral laparotomy was carried out under surgical stage of anesthesia (pentobarbital sodium 15 mg.kg⁻¹ BW) in sterile conditions. The numbers of implantation sites and corpora lutea in ovaries were observed in order to evaluate the anti-implantation activity. The lateral abdomens were sutured and rats were left in cages for recovery. The vehicle and plant extracts were further treated for 7 days (9th -14th day of pregnancy). On the 15th day, pregnant rats were sacrificed to evaluate the early abortifacient
activity.

The percentages of antiimplantation and early abortifacient activities were calculated. The summation of antiimplantation and early abortifacient activity gives percentage of anti-fertility activity of the tested materials. The calculation formulas are shown below:

\[ \text{% Anti-implantation activity} = 100 \times \left( \frac{\text{No. of implantations}}{\text{No. of corpora lutea}} \right) \]

\[ \text{% Abortifacient activity} = \left( \frac{\text{No. of resorptions}}{\text{No. of corpora lutea}} \right) \times 100 \]

\[ \text{% Anti-fertility activity} = \text{% Antiimplantation activity} + \text{% Abortifacient activity} \]

Statistical analysis

Statistical analysis of the differences between the group were analyzed by one-way analysis of variance (ANOVA) followed by the Turkey's multiple comparison tests. \( P<0.001 \) was considered as statistically significant. All data are expressed to the mean value ± SD.

RESULTS

GC/MS Analysis

The GC/MS analysis of the root extract showed the presence of 5 phytosterols which were β-sitosterol (17.37%), stigmasterol (4.23%), stigmaster-3-ol (4.10%), stigmast-22-en-3-ol (1.84%) and campesterol (1.56%), respectively. 12 known compounds were fatty acids (0.50%-11.32%) and 2 unknown compounds were detected. The leaf extract showed the presence of 4 phytosterols which were β-sitosterol (10.60%), stigmastanol (2.76%), stigmasterol (0.85%) and campesterol (0.80%). 11 known compounds; phytols (69.32%), α-tocopherol (0.99%), fatty acids (0.43-3.41%) and 2 unknown compounds were identified.

Estrogenic activity

The effects of \( T. \)paniculatum root and leaf extract on vaginal epithelial cell differentiation in OVX rats are shown in Figure 1. Oral administration of the plant extracts caused significant increase in the percentage of cornfield cells compared with control group \( (P<0.001) \). When the extracts were given, the opened vaginas were observed (data not shown).

Antiimplantation and early abortifacient activity

Dose dependent response of antiimplantation and early abortifacient activity to \( T. \)paniculatum extracts on pregnant rats is illustrated in Table 3. With an increase in the dose of both root and leaf extracts (100 and 1,000 mg.kg\(^{-1}\) BW), the percentage of anti-implantation activity was significantly increased as confirmed by decreasing the number of implantation site on 8th day of pregnancy \( (P<0.001) \). The extracts also produced a significant early abortifacient activity which is indicated from the implantation scars in the uterine horn on the 15th day of pregnancy (Figure 2).

Anti-fertility activity

Among the different dosages of the plant extracts, the significant dose dependent effect of anti-fertility activity was observed \( (P<0.001) \). The data showed that dose-related responses of anti-fertility activity produced by the leaf extracts are more effective than the root extracts. Compared with control group, the percentage of anti-fertility activity of the root extracts at the dose of 100 and 1,000 mg.kg\(^{-1}\) BW were found to be 47.11 ± 11.95 and 71.65 ± 9.98%, respectively; whereas the percentage of anti-fertility activity of the leaf extracts at the dose of 100 and 1,000 mg.kg\(^{-1}\) BW were found to be 81.21 ± 15.78 and 97.29 ± 13.95% (Table 3).

DISCUSSION

The estrogenic substances are well known for their adverse effect on the maintenance of pregnancy by affecting the equilibrium of reproductive hormones to regulate the hypothalamus-pituitary-gonadal axis (Havranex et al., 1973; Iguchi and Sato, 2000). Any disturbance in the level of these hormones may cause infertility by affecting ovulation, implantation and obstructing the uterine milieu (Hughes et al., 1991; Mcgarvey et al., 2001; Abu and Uchenda, 2011). The large consumption of estrogenic substances as well as phytoestrogens can enhance the luteolytic activity (Shibeshi et al., 2006). They also increase the sensitivity.
Table 3 Anti-fertility activity of \textit{T. paniculatum} root and leaf extracts in female Wistar rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NIS</th>
<th>NER</th>
<th>Anti-implantation Activity (%)</th>
<th>Abortifacient Activity (%)</th>
<th>Anti-fertility Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>12.20 ± 3.11</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Root extract 100 mg.kg(^{-1})BW</td>
<td>10.40 ± 1.52</td>
<td>3.60 ± 1.34*</td>
<td>18.97 ± 4.94</td>
<td>28.15 ± 9.57*</td>
<td>47.11 ± 11.95*</td>
</tr>
<tr>
<td>Root extract 1,000 mg.kg(^{-1})BW</td>
<td>8.40 ± 0.55</td>
<td>5.20 ± 1.30*</td>
<td>27.28 ± 5.17</td>
<td>44.38 ± 7.28*</td>
<td>71.65 ± 9.98*</td>
</tr>
<tr>
<td>Leaf extract 100 mg.kg(^{-1})BW</td>
<td>6.60 ± 0.55*</td>
<td>4.60 ± 1.52*</td>
<td>41.44 ± 8.47*</td>
<td>39.77 ± 9.20*</td>
<td>81.21 ± 15.78*</td>
</tr>
<tr>
<td>Leaf extract 1,000 mg.kg(^{-1})BW</td>
<td>6.00 ± 1.87*</td>
<td>5.20 ± 0.84*</td>
<td>45.42 ± 12.66*</td>
<td>51.57 ± 5.09*</td>
<td>97.29 ± 13.95*</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± SD of 5 rats in each group. NIS= number of implantation sites, NER= number of embryonic resorptions. * indicates statistically difference at \(P<0.001\) in comparison to vehicle control group.

**Figure 1.** The chronic differentiation of vaginal epithelial cells of OVX rats treated by various doses of \textit{T. paniculatum} root and leaf extracts. Data expressed as mean ± SD of 5 rats in each group. Since day 2, all dosages of the plant extracts caused significant increase in the percentage of cornfield cells compared with control group (\(P < 0.001\)). The measurement on the X axis of the graph represent day of the experiment. D0, 1 day before the experimental start; D1, first day of the experiment; D2-5 = day 2-5 of the experiment.

**Figure 2.** The 15\(^{th}\) day of pregnant uteri show embryonic resorption scars (arrow) after the oral administration of \textit{T. paniculatum} root extract (A, 1,000 mg.kg\(^{-1}\) BW) and \textit{T. paniculatum} leaf extract (B, 1,000 mg.kg\(^{-1}\) BW) for 15 consecutive days.
of the response of the uterus to prostaglandins, which leads to the failure of implantation and increase the abortion rate (Woclawek-Potocka et al., 2005).

In this study, the evaluation of the anti-fertility of T. paniculatum root and leaf extracts was conducted during the times before and after implantation process. It was found that, T. paniculatum extracts enhanced the antimplantation activity in a dose dependent manner in pregnant rats. The extracts also affected the conceptus after implantation period as illustrated by the increase in abortifacient activity. Among the tested groups, the group that was treated with leaf extract at the dose of 1,000 mg.kg

−1 BW exhibited the most potent anti-fertility activity, which is confirmed by the decreasing implantation sites and increasing abortifacient activity. In addition, the leaf extract contained high amount of phytols which could be metabolized to phytanic acid after oral ingestion by hepatic enzyme (Mize et al., 1966). This compound has been reported to obliquely activate estrogen responsive genes via the activating nuclear receptors peroxisome proliferator-activated receptors (PPARs) and heterodimerizes with retinoid X receptor (RXR) (Nuñez et al., 1997; Elmaza and Nau, 2004; Heim et al., 2002). Hence, the potent anti-fertility effects of the leaf extract may be due to the mimicking of estrogenic-like action; which synergistic occurs in response to their phytosterols and signals integrated emanating from phytol signaling pathways (Björnström and Sjöberg, 2005; Goldstein et al., 2003).

In addition, the classical effects of estrogen and phytoestrogenic compounds, such as vaginal cornification and immature opened vagina, were used to detect and confirm the property of anti-fertility substances (Ahirwar et al., 2010). The T. paniculatum root and leaf extracts can have the same effects which strongly support anti-fertility activity of the plant. In this study, the GC/MS analysis of T. paniculatum crude extracts showed the presence of non-steroidal phytoestrogens such as campesterol, β-sitosterol, stigmasterol, stigmastan-3-ol, stigmast-22-en-3-ol and stigmastanol. These phytoestrogens have been claimed to possess estrogenic activity due to their affinity to estrogen receptors leading to infertility in animals (Dane and Patil, 2012; Suryawanshi, 2011). In conclusion, the anti-fertility activity of T. paniculatum root and leaf extracts might mainly be due to the estrogenic activity of the phytosterols.

ACKNOWLEDGEMENT

This study was supported by the Office of the Higher Education Commission of Thailand.

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Antidiabetic activity of seed extracts of *Caesalpinia crista* Linn. in experimental animals

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The present study was designed to evaluate antidiabetic activity of ethanolic and aqueous seed extracts of *Caesalpinia crista* linn. in streptozotocin (STZ) induced diabetes in 2 days old pups models. The seeds were collected, authenticated and shade dried. Shade dried seeds were then grinded into coarse powder and processed for further studies. Ethanolic and aqueous extracts were prepared and the preliminary phytochemical screening was performed. Ethanolic and aqueous both extracts were evaluated for the antidiabetic activity by using the streptozotocin induced diabetes in 2 days old pups model. After 3 months of streptozotocin administration, pups become diabetic and then further protocol was proceeded. Estimation of biological parameters like serum glucose, cholesterol and triglyceride were performed and body weight, water intake and food intake were also recorded after 3 weeks of treatment. Histopathological study was performed to study the structure of islets of pancreas in different group of animals. There was a significant decrease in the biological parameters that is, serum glucose, cholesterol and triglyceride when compared with diabetic untreated group after 3 weeks treatment plant extracts. Treatment with the extracts also affected the physical parameters like decrease in body weight, increase in demand of food intake and water intake when compared with diabetic untreated group. Histopathological study shows the changes in structure of islets of pancreas in different groups of animals. Hence, it can be concluded that in the above study, both ethanolic and aqueous seed extracts of *C. crista* linn. showed antidiabetic activity, but the aqueous extract of *C. crista* linn. showed more significant effect as compared to the ethanolic extract.

Key words: Diabetes mellitus, insulin resistance, streptozotocin (STZ), *Caesalpinia crista* linn., ethanolic and aqueous extracts.

INTRODUCTION

Diabetes mellitus (DM) is a disease characterized by chronic hyperglycaemia and glucosuria produced by an absolute or relative insufficiency of insulin (Andrew et al., 2000; Swanston et al., 1990). Hyperglycaemia and glucose intolerance are common manifestations of several types of hormonal disturbances or imbalances, of which the most important is diabetes mellitus (Forster, 1987). Type 2 DM accounts for as many as 90% of DM cases and is usually characterized by the presence of both insulin resistance and relative insulin deficiency. Insulin resistance is manifested by increased lipolysis and free fatty acid production, increased hepatic glucose production, and decreased skeletal muscle uptake of glucose. Pancreatic beta cell dysfunction is progressive.
and contributes to worsening blood glucose control over time. Type 2 DM occurs when a diabetogenic lifestyle (excessive calories, inadequate exercise, and obesity) is superimposed upon a susceptible genotype. (Barbara et al., 2006). This disease is the seventh leading cause of death in the world. Weight loss which is one of the clinical features of diabetes mellitus may be due to the degeneration of the adipocytes and muscle tissues to make up for the energy lost from the body due to frequent urination and over conversion of glycogen to glucose. Weight loss is a very serious issue in the management of diabetes mellitus (Reno and Leland, 1999; Zink and Chaffin, 1998).

Though different types of oral hypoglycemic agents are available along with insulin for the treatment of diabetes, there is an increased demand by patients to use natural products with antidiabetic activity (Venkatesh et al., 2003). Since time immemorial, patients with non-insulin dependent diabetes have been treated orally by folk medicine, with a variety of plant extracts. In India, a number of plants are mentioned in ancient literature (Ayurveda) for the treatment of diabetic conditions and others are antioxidants (Atef et al., 2013), antifungal (Cui et al., 2011), anxiolytic (Jitender et al., 2013) among others.

_Caesalpinia crista_ linn. commonly known as kanchak, Latakranja is a medicinal plant belonging to the family Fabaceae, it is a widely growing plant throughout India and tropical countries of the world. Each and every part of the plant is claimed to possess some therapeutic property but seed kernel is the most widely used part all over the world in various systems of medicine. The seeds are extremely bitter, commercially available in plenty at a very low cost and are widely used for a variety of disease, especially in cases of all types of fever including malaria. In Ayurveda, the seeds, leaves and bark of all three parts are used. The herb is useful for treatment of amenorrhoea, dysmenorrhoea, diabetes and intermittent fevers. Also, used as febrifuge, anthelmintic and expectorant (Das et al., 2010; Kirtikar and Basu, 2006; Handa and Kaul, 1996). Its anthelmintic activity (Abdul et al., 2007), nootropic activity (Kshirsagar, 2011), antioxidant and reactive oxygen species scavenging activity (Mandal et al., 2011), anticancer activity (Bodakhe et al., 2011), was already reported but the anti-diabetic activity has not been established yet. In the present study, we made an attempt to establish the anti-diabetic potential of ethanolic and aqueous seed extracts of _C. crista_ linn.

**MATERIALS AND METHODS**

**Plant material**

Plant part (seed) were collected from the local market of Ropar (Punjab) and authenticated by Mr. Madan Pal, Executive Engineer, Horticulture Division No.2 (Chandigarh). The seeds were then shade dried and graded and made a coarse powder and the coarse powder were used for further studies.

**Preparation of extract**

Extraction was done according to standard procedures using analytical grade solvents. For preparation of ethanolic extract, 300 gm powdered seed was taken in a pouch of filter paper and kept inside the soxhlet thistle then extracted with petroleum ether for 48-72 h for defatting after it reextracted with ethanol (99.99%) for 48-72 h. Aqueous extract was separately prepared by maceration process. The extracts were then concentrated until dryness under reduced pressure and controlled temperature (40-50°C). Then preliminary phytochemical screening was performed (Kokate et al., 2007). Percentage yield of extracts were calculated. The percentage yield of ethanolic and aqueous extracts was found to be 8.1 and 13.3%. The LD<sub>50</sub> determination of _Caesalpinia crista_ seed extract was reported by Kshirsagar (2011) so a dose level of 100 mg/kg b.w. is selected for the treatment.

**Preliminary phytochemical screening**

The extracts were subjected to preliminary phytochemical qualitative screening to evaluate the presence of various primary or secondary metabolites following standard procedures. In previous phytochemical studies, the presence of phytoconstituents such as flavonoids, tannins, proteins, alkaloids, saponins and triterpenoids were already reported (Gill et al., 2012).

**Experimental animals**

Evaluation of antidiabetic activity was done by using streptozotocin (STZ) induced diabetes in 2 days old pups model, in which 2 days old pups of Wistar rats were used and the animals were maintained under standard laboratory conditions with access to standard diet and water ad libitum. The experiment was carried out according to the Committee for the purpose of Control and Supervision of Experiments on Animals (CPSEA) guidelines 2013. Before starting experimentation on test animals, permission of Institutional Animal Ethics Committee (IAEC) was obtained.

**Streptozotocin induced diabetes in 2 days old pups**

2 days old pups of Wistar rats weighing 7-10 gm were used to induce non-insulin dependent diabetes mellitus in this study. A freshly prepared solution of streptozotocin (90 mg/kg) in 0.1 mol/L citrate buffer, pH 4.5, was injected intraperitoneally to 2 days old pups. Vehicle control group of pups receive only saline. The animal were weaned at 30 days and after a period of 3 months they were checked for fasting glucose level to confirm the status of NIDDM. Animals showing fasting glucose level > 140 mg/dl were considered as diabetic. The pups that receive saline were considered as control animals (Gupta et al., 2011; Portha et al., 1994; Arulmozhi et al. 2004).

**Treatment protocol**

The experimental animals were divided into five groups of six animals each. Group I was served as vehicle control and was treated with normal saline, Group II served as diabetic untreated group, Group III had diabetic animals treated with the ethanolic extract of _C. crista_ linn. at a dose of 100 mg/kg, Group IV had diabetic animals treated with aqueous extract of _C. crista_ linn. at a dose of 100 mg/kg and Group V had diabetic animals treated with standard drug, glibenclamide, at a dose of 10 mg/ kg body weight. Treatment was given daily for 3 weeks and at the end of 3 weeks of treatment the animals were kept...
on 12 h fasting and blood samples were collected from tail vein and analyzed for serum glucose, cholesterol and triglyceride level. Body weight, water intake and food intake were also being recorded.

**Histopathological studies**

For histopathological studies, animals were sacrificed at the end of 3 weeks treatment, after the collection of blood sample, pancreas was removed and washed with normal saline and preserved in 10% formalin, processed and stained with hematoxylin and eosin.

**Statistical analysis**

The significance of difference among the control group and various treated groups were analyzed by means of analysis of variance (ANOVA) with least significant difference (LSD) post hoc test was used to compare the group means and P<0.05 was considered statistically significant. The experimental results are represented as mean ± SEM (standard error mean). Statistical Package for Social Sciences (SPSS), for Windows (version 15.0, Chicago, IL, USA) was used for statistical analysis.

**RESULT**

The preliminary phytochemical screening of the ethanolic and aqueous extracts of the plant *C. cristai* showed the presence of phytoconstituents such as flavonoids, tannins, proteins, alkaloids, carbohydrates reducing sugars, phytosterols, saponins and triterpenoids. Pups that received saline were considered as control animals, ethanolic, and aqueous seed extracts of *C. cristai* were used for the experiment and glibenclamide (10 mg/kg b.w. p.o.) for three weeks was used as standard drug for the group V.

Effect of ethanolic and aqueous seed extracts of *Caesalpinia cristai* on the serum level of diabetic rats is shown in Table 1. STZ at a dose of 90 mg/kg caused elevation of serum glucose (180.30± 0.689), serum cholesterol (81.60±0.240) and serum triglycerides (123.40±0.712) in diabetic untreated group when compared with vehicle control groups levels of serum glucose (82.43±0.684), cholesterol (50.83±0.763) and triglyceride (50.92±0.265). Treatment with seed extracts of *C. cristai* (ethanolic and aqueous) significantly reduced the level of these biological parameters. But the maximum effect was shown by aqueous seed extract of *C. cristal* (55.50±0.595) and triglyceride (61.18±0.385) when compared with diabetic untreated group.

Effect of ethanolic and aqueous seed extracts of *C. cristai* on body weight, water intake and food intake of diabetic rats is shown in Table 2. STZ (90 mg/kg; i.p.) treatment led to a significant reduction in body weight (154.8±0.735) and increase in water intake (54.9±0.436) and food intake (37.3 ± 0.232) of diabetic untreated group, when compared with vehicle control groups body weight (209.8±0.684), water intake (39.9±0.366) and food intake (17.8±0.366). Treatment with the ethanolic and aqueous seed extracts of *C. cristal* significantly reversed the reduced body weight and increased demand of water and food intake when compared with diabetic untreated group. But the maximum effect was shown by the aqueous seed extract of *C. cristal* which reversed the reduced body weight (185.9±0.737), and increased demand of water intake (43.0±0.578) and food intake (23.3 ± 0.320) when compared with diabetic untreated group.

Difference in the effects of induction, standard drug and test drug can be easily seen in histopathology of pancreas of experimental animals. In pancreatic sections of diabetic rats (Group-II) (Figure 1b), the islets were less intact and their shape was destroyed as compared to Group-I with infiltration of lymphocytes (Figure 1a). In standard group in which diabetic rats treated with glibenclamide, islets resemble to vehicle control rat islets having normal round and structural intactness with their nucleus (Figure 1c). In *C. cristal* aqueous extract treated rats (Figure 1d), the islets were normal round but enlarged in size and having intact nucleus which was similar to the pancreas of vehicle control groups and in *C. cristal* (Figure 1e) ethanolic extract treated rats whose islets of pancreas were normal but enlarged in size and less improved then aqueous extract group of *C. cristal*.

**DISCUSSION**

There was a significant decrease in the biological parameters that is, serum glucose, cholesterol and triglyceride when compared with diabetic untreated group after 3 weeks treatment with *C. cristal* extracts. Treatment with the extracts also affect the physical parameters like decrease body weight, increase demand of food intake and water intake when compared with diabetic untreated group. Histopathological study also showed changes in structure of islets of pancreas in different groups of animals giving strength to the results observed by the physical and biological parameters.

Diabetes mellitus, an endocrine and metabolic disorder characterized by chronic hyperglycemia produces multiple biochemical impairments and oxidative stress especially an increased susceptibility to lipid peroxidation that play role in the progression of the symptoms of diabetes (Giugliano et al., 1996). Several hypotheses have been postulated to explain the development of free radicals in diabetes which include auto oxidation of glucose, enzymatic and non-enzymatic glycation of proteins with increased formation of glucose derived advanced glycosylation end products (AGEs), enhanced glucose flux through polyol pathway (Oberlay, 1988) and reduction of antioxidant defence (Lipinski, 2001). Despite progress in the management of diabetes mellitus by synthetic drugs, most of these drugs have side effects in
Table 1. Effect of extracts of *Caesalpinia crista* linn. on serum profile of streptozotocin (STZ) induced diabetic rats (2 days old pups).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>S. glucose</th>
<th>S. cholesterol</th>
<th>S. triglycerides</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle control</td>
<td>82.4±0.684</td>
<td>50.8±0.763</td>
<td>50.9±0.265</td>
</tr>
<tr>
<td>II</td>
<td>Diabetic untreated</td>
<td>180.3±0.689*</td>
<td>81.6±0.240*</td>
<td>123.4±0.712*</td>
</tr>
<tr>
<td>III</td>
<td>Diabetic treated with CCEE 100 mg</td>
<td>119.0±0.562***</td>
<td>61.4±0.530***</td>
<td>66.0±0.774***</td>
</tr>
<tr>
<td>IV</td>
<td>Diabetic treated with CCAE 100 mg</td>
<td>111.7±0.794***</td>
<td>55.5±0.595***</td>
<td>61.1±0.385***</td>
</tr>
<tr>
<td>V</td>
<td>Diabetic treated with 10 mg/kg of glibenclamide</td>
<td>92.0±0.801***</td>
<td>50.9±0.620***</td>
<td>55.1±0.486***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=6). *, Significantly different from vehicle control (P < 0.05); ***, significantly different when compared with diabetic untreated (P < 0.05).

Table 2. Effect of extracts of *Caesalpinia crista* linn. in streptozotocin (STZ) induced diabetic rats (2 days old pups).

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Average Body weight (grams/rat)</th>
<th>Water intake (ml/rat/day)</th>
<th>Food intake (grams/rat/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Vehicle control</td>
<td>209.8±0.684</td>
<td>39.9±0.366</td>
<td>17.8±0.386</td>
</tr>
<tr>
<td>II</td>
<td>Diabetic untreated</td>
<td>154.8±0.735*</td>
<td>54.9±0.436 *</td>
<td>37.3±0.232*</td>
</tr>
<tr>
<td>III</td>
<td>Diabetic treated with CCEE 100 mg</td>
<td>179.3±0.794***</td>
<td>45.6±0.316***</td>
<td>26.3±0.263***</td>
</tr>
<tr>
<td>IV</td>
<td>Diabetic treated with CCAE 100 mg</td>
<td>185.9±0.737***</td>
<td>43.0±0.578***</td>
<td>23.3±0.320***</td>
</tr>
<tr>
<td>V</td>
<td>Diabetic rats treated with 10 mg/kg of glibenclamide</td>
<td>197.4±0.721***</td>
<td>41.3±0.650***</td>
<td>20.0±0.474***</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM (n=6); *, significantly different from vehicle control (P < 0.05); ***, significantly different from diabetic untreated (P < 0.05).

Figure 1. a, Vehicle control islets with normal round and structural intactness with their nucleus; b, diabetic untreated rat islets damaged and shrunken in size; c, effect of glibenclamide (10 mg/kg b.w. p.o.) on islets in pancreas of streptozotocin (STZ) induced diabetic rats resembling with vehicle control rat islets with normal round nucleus and structural intactness; d, effect of aqueous extracts of *Caesalpinia crista* linn. (100 mg/kg b.w. p.o.) on islets in pancreas of STZ induced diabetic rats showing round structure of islets, and intact nucleus; e, effect of ethanolic extracts of *C. crista* linn. (100 mg/kg b.w. p.o.) on islets in pancreas of STZ induced diabetic rats showing normal structure of islets but enlarged in size.

the long run. So, the search for improved and safe natural antidiabetic agents is on-going and World Health Organization has also recommended the development of herbal medicine in this concern (Schmincke, 2003).
In all diabetic patients, treatment should aim to lower blood glucose to near normal level. The present investigation fulfills this statement by producing a significant fall in blood glucose levels. Flavonoid and terpenes possess antidiabetic action. Effect of the flavonoids on pancreatic β-cells leading to their proliferation and secretion of more insulin, the mechanism by which they reduced hyperglycaemia caused by streptozotocin in diabetic rats, was already reported (Rajalakshmi et al., 2009; Patil et al., 2011).

STZ is well known for destroying insulin secreting β-cells in the islets of Langerhans (Fisher, 1985) and has been extensively used in induction of diabetes mellitus in animals (Tialve, 1983). Experimental evidence has demonstrated that some of its deleterious effects are attributable to induction of metabolic processes, which lead on to an increase in the generation of reactive oxygen species (ROS) (Chen et al., 1990). Apart from production of ROS, STZ also inhibits free radical scavenger-enzymes (Kroncke et al., 1995).

The present study was an effort to investigate the effect of seed extracts of C. crista linn. on normal and streptozotocin-induced diabetic rats. Similar effects in streptozotocin-treated diabetic animals were reported by pancreas tonic (Rao et al., 1998), ephedrine (Xiu et al., 2001), Tinospora cordifolia stem extracts (Rajalakshmi et al., 2009) and Gymnema sylvestre leaf extracts (Shamugasundaram et al., 1990). In the current studies, the damage of pancreas in streptozotocin-treated diabetic control rats and regeneration of β-cells by glibenclamide was observed. The comparable regeneration was also shown by ethanolic and petroleum ether extracts of C. crista linn. and showed significant (ps0.5) decrease in the serum glucose, cholesterol and triglyceride level and also reversed the reduced body weight and increased demand of food and water intake when compared with the diabetic untreated group. The data obtained from this study indicates that the extracts of the plant C. crista linn. are capable of exhibiting significant antihyperglycemic activities in diabetic rats. These extracts showed improvement in parameters like body weight and serum profile as well as regeneration of β-cells of pancreas and so might be valuable in diabetes treatment.

**ABBREVIATIONS**

DM, Diabetes mellitus; STZ, streptozotocin; AGEs, advanced glycosylation end products; ROS, reactive oxygen species

**REFERENCES**


**Pharmaceutical analysis of *Euphorbia cyparissias* included on Beta-cyclodextrin complexes**

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The methods of bio-control have diversified resulting in study of new fighting measures. Studies on *Euphorbia cyparissias* active compounds as well as their *in vivo* and *in vitro* activity revealed a significant pharmacologic activity, encouraging design of a complex original conditioning. Encapsulation techniques can be of considerable help in this endeavour, being a means to protect sensitive active components (such plant’s active compounds) from the environment and from other excipients used. The aim of the present study was to evaluate *E. cyparissias* extracts inclusion in β-cyclodextrine complexes and thermo gravimetric (TG-DTG) analysing of *Euphorbia’s* extracts complexes, as a GMP pharmaceutic step, in the study of thermal behaviour of a future finite conditioning destined to demodectic mange treatment, a zoonotic ectoparasitic disease. TG-DTG analysis of pure β-cyclodextrin revealed a mass loss of 11.7%, which actually represent the β-cyclodextrin decomposition. In crude *Euphorbia* extract complexes T5 / β-CD case, the mass loss was corresponding probably to the encapsulated bioactive decomplexing phase of *Euphorbia* extracts’ compounds. A similar behaviour to heating was recorded in case of crude *Euphorbia* extract T10 / β-CD complexes. In case of *Euphorbia* concentrated extracts T5 / T10 - β-CD complexes, the loss can be translated as a degradation / loss of complexes' bioactive compounds.

**Key words:** *Euphorbia cyparissias*, β-cyclodextrine, complexes, TG-DTG analysis.

**INTRODUCTION**

In medicine, biotheraphy has become a current topic. Methods linked to the parasites’ bio-control have diversified; new fighting means being studied (fungus, entomogenous nematodes, vegetal extracts, volatile oils, among others) (Kaaya et al., 2000; Samish and Rehacek, 1999; Sanis et al., 2012; Zahir and Rahuman, 2012). In this respect, plant extracts can be an important alternative control source, being a rich source of efficient bioactive compounds. Researchers try to bring new information regarding the use of spontaneous flora plants from their countries as well as other means to enrich the antparasitary arsenal (Babar et al., 2012; Borges et al., 2011; Chagas de Souza et al., 2012; Reggasa, 2000; Tona et al., 1999).

Numerous components from extracts and latex of *Euphorbiaceae* were identified, mostly diterpenes (phorbolester, ingenole, euphorbone, piceatanole, aesculetine, jolkinol, hyperoside, kaempferol, acyiphorbol, acylingenol among others) (Appendino et al., 2000; Evanics et al., 2001; Toth-Soma et al., 1993). Previous studies on the active compounds of *Euphorbia cyparissias*, revealed in the plant’s inflorescence, thirteen compounds; (sesquiterpenoids being dominant). The *in vivo* and *in vitro* experiments, following this plant’s...
extracts activity against argasides and demodectic mange, revealed also a significant ectoparasitary activity, encouraging us to design a complex original conditioning, an *Euphorbia* ointment. Encapsulation techniques can be of considerable help in this endeavour, being means to protect sensitive active components (such plant’s active compounds) from the environment and from other excipients used, this technique being used in many fields of therapy, from antibiotics, cancer to anti-parasitic domains (Thatiparti et al., 2010; Menuel et al., 2007; Becket et al., 1999).

Cyclodextrin (CD) is the general term of amyllose produced by bacillus cyclodextrin glycosyltransferase enzyme generating a series of cyclic oligosaccharides usually containing 6 to 12 D - pyran glucose units. Studied more, and of great practical significance for medicine, molecules containing 6, 7, 8-glucose units are called alpha - , beta - and gamma – cyclodextrin. Cyclodextrins are allowing the encapsulation of active substances, drugs, flavours, enzymes, among others at the lowest possible that is, molecular encapsulation. In this process, each constituent is surrounded by a cyclodextrin ring, which provides almost perfect protection against the harmful effects of the environment (Del Valle, 2004; Biwer et al., 2002). Also cyclodextrins can enhance topical drug delivery by increasing the drug availability at the barrier surface. At the surface, the active molecules partition from the cyclodextrin cavity into the lipophilic barrier, thus, drug delivery from aqueous cyclodextrin solutions is both diffusion controlled and membrane controlled. Also cyclodextrins can enhance topical drug delivery in the presence of water (Loftsson and Masson, 2001). So, cyclodextrins are suitable active substances delivery systems, because of their ability to modify the physical, chemical, and biological properties of the guest molecules through labile interactions by formation of inclusion and/or association complexes becoming an important choice for new conceived drugs including therapeutic active substances from plants (Denadai et al., 2006; Karioti et al., 2011). The aim of our study was to accomplish, as novelty, inclusion of *E. cyparissias* extracts in β-cyclodextrine (β-CD) complexes (7-membered sugar ring molecules) and the thermo gravimetric analysis (TG-DTG) of *Euphorbia*’s extracts complexes, as a compulsory GMP pharmaceutic step, in the study of thermal behaviour of a future finite conditioning components destined for treatment of demodectic mange, a zoonotic ectoparasitic disease found in dog and humans.

**MATERIALS AND METHODS**

**Obtaining *Euphorbia* / β-cyclodextrine (β-CD) extract complexes**

Quantities of β-CD presented in Table 1 (corresponding to 0.5 mmols β-CDs), were weighed and then dissolved in 4 ml distilled water at 50 ± 1°C. After dissolution on this solution, *E. cyparissias* raw or concentrated extract ethanolic solutions (4 ml) were introduced drop wise, corresponding to a 1:1 molar ratio, calculated according to the known major component of the extract (quercitine), within 30 min, under continuous stirring. Resulted solution was slowly stirred for another 15 min, then cooled for 4 h in a water bath and finally stored in refrigerator, at 4°C for 24 h, to complete the crystallization of the newly formed complexes. This suspension was subjected to filtration, being washed with 1 ml of 96% ethanol and dried in desiccators. The obtained samples were subjected then to thermo gravimetric analyze.

**Thermo gravimetric analysis (TG-DTG)**

A thermo gravimetric thermo-microbalance TG 209 F3 *Tarus* analyzer from Netzsch Instruments apparatus was used. It measures mass change as a function of temperature and has an operating temperature of between 10 and 1100°C. It is also vacuum tight enabling runs to be performed in vacuum, as well as in flowing gases, all measurements being performed in a nitrogen atmosphere to a temperature program; between 20-500°C, to a heating rate of: 10°C /min. Data acquisition was accomplished with the help of Netzsch TG 209-Acquisition program Soft/2000 and them processing with Netzsch Proteus - Thermal Analysis, program ver. 4.0/2000.

Thermo gravimetric analysis (TGA) is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture). The principle is to measure the mass change of a sample as a function of temperature or time, under a defined and controlled environment with respect to heating rate, gas atmosphere, flow rate, crucible type, among others. Common applications of TGA are materials characterization through analysis of characteristic decomposition patterns, studies of degradation mechanisms and reaction kinetics, determination of organic content in a sample, and respectively, determination of inorganic (for example, ash) content in a sample (Brown, 2001).

To establish aspects of thermal behaviour of the involved components, plants extract different concentrations and the associations and influence of rough β-cyclodextrine, different TG-
DTG tests were done after thermal analysis procedure known in pharmaceutical sciences, with the aim of mass degradation / loss of complexes bioactive compounds behaviour establishing of the conceived associations.

RESULTS

**Pure β-cyclodextrin TG-DTG analysis**

In Figures 1 and 2, pure β-cyclodextrin TG-DTG analysis is presented. TG-DTG analysis of pure β-cyclodextrin showed a mass loss of 11.68% up to 100°C, this corresponds to the release of crystalline water, and a 76.27% loss, in the 100 to 500°C temperature range, which represents the decomposition of β-cyclodextrin.

**Crude Euphorbia T5 / β-cyclodextrine extract complex TG-DTG analysis**

In the case of *Euphorbia* complex T5 / β-CD crude extract, mass loss up to 100°C was only of 8.9%, but up
to a temperature of 225°C, there was an additional mass loss of 2.8%, mass loss decomposition being approximately equal (Figures 3 and 4).

**Crude Euphorbia T10 / β-cyclodextrine complex extract TG-DTG analysis**

A similar behaviour to heating had also the crude *Euphorbia* T10/β-CD extract complex, so that up to 100°C, mass loss was 8% and between 100 and 225°C range of 2.9%, the decomposition determining a mass loss of ~ 78% (Figures 5 and 6).

**T5 and T10 Euphorbia / β-cyclodextrine concentrated complex TG-DTG analysis**

In the case of *Euphorbia* complexes T5/T10 - β-CD concentrated extracts, mass loss up to 100°C, was more than 11.91% and from 100 to 225°C range, below to 1%. This probably means the initial extracts complexes' bioactive compounds loss / degradation (Figures 7, 8 9 and 10). Differences between thermal behaviour of crude
DISCUSSION

Studies of numerous macromolecule vehicles on topical delivery and understanding the basic relationship between solvent and solute penetration is an important issue to the researchers focussed on finding more effective pharmaceutical conditionings (Karande and Mitragotri, 2004; Magnusson et al., 2001).

Cyclodextrins can effectively increase the water-soluble adverse drug solubility in water and dissolution rate. They can improve the drugs stability and bioavailability of intestinal granules of volatile oil, reduce irritation and toxicity of drugs and drugs releasing and improving formulations (Cross et al., 2001; Inamori et al., 1994). In the recent years many researchers have reported on the topic of active substances' delivery potential of cyclodextrins and their possible applications in medicine. For example Liu et al. (2012) explored the formation of inclusion complex between puerarin and glucosyl-β-
cycloexdetrin (G-β-CD) to improve the aqueous solubility of puerarin. Results showed clearly that the process led to the formation of a supramolecular complex in which the guest molecule, puerarin, was entrapped inside the cavity of the host, G-β-CD enhancing its therapeutic potential.

Sudha and Enoch (2011) studied interaction of curculigosides, phyto-constituents of plant Curculigo orchioides, and their β-cycloexdetrin complexes with bovine serum albumin. As results, curculigoside–cycloexdetrin complexes were found to bind more weakly to the bovine serum albumin molecule than their free forms. Petrović et al. (2010) studied the inclusion complexes between the Cinnamomum verum essential oil and β-cycloexdetrin, prepared by co-precipitation method, in order to determine the effect of the ratio on the inclusion efficiency for encapsulating oil volatiles. Results revealed that the chromatographic profile of the

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**Figure 7.** The *Euphorbia* T5 / β-CD concentrated complex TG-DTG analysis.

**Figure 8.** The *Euphorbia* T5 / β-CD concentrated complex (low-temperature domain) TG-DTG analysis.
surface adsorbed oil was different. The thirteen major compounds, found in the commercial *C. verum* essential oil, were present in all of the extracts, but in the different proportions between the total and surface oil extracts indicating the best therapeutic choice to choose. Yadav et al. (2009) prepared and evaluated the anti-inflammatory activity of cyclodextrin complex of curcumin for the treatment of inflammatory bowel disease (IBD) in a colitis-induced rat model. Results revealed that curcumin β-CD complexes proved to be potent angioinhibitory agents. It was concluded that the degree of colitis caused by administration of dextran sulphate solution (DSS) was significantly attenuated by CD of curcumin, recommending this nontoxic natural dietary complex as useful means in the therapeutic strategy for IBD patients. Finally, Panichpakdee and Supaphol (2011) studied the
Figure 11. Pure β-CD thermograms overlap, pure (green) and Euphorbia T5 / β-CD (red) and T10 / β-CD (blue) crude extract complexes.

Figure 12. Pure β-CD thermograms overlap (green) and Euphorbia T5/β-CD (red) and T10/β-CD (blue) (low-temperature domain) crude extract complexes.

Figure 13. Pure β-CD thermograms overlap (green) and Euphorbia T5/β-CD (red) and T10/β-CD (blue) concentrated complexes.
inclusion complex between an active substance from the medicinal plant *Centella asiatica* L. asiaticoside and 2-hydroxypropyl-cyclodextrin, respectively, demonstrating the practical and therapeutic advantages of the analyzed complexes. The thermo gravimetric analysis (TG-DTG) of *Euphorbia* extracts / β-cyclodextrine complexes was important to establish that the actives’ compounds are complexed on cyclodextrin nuclei and different aspects of its behaviour. This has added further to the utility of *in vitro* studies on the lines of modern pharmacophytotherapy.

**Conclusions**

TG analysis of pure β-cyclodextrine showed a mass loss of 11.7%, which actually represent the β-cyclodextrin decomposition. In crude *Euphorbia* extract complexes T5 / β-CD case, the mass loss was corresponding probably to the encapsulated bioactive decomplexing phase of *Euphorbia* extracts’ compounds. A similar behaviour to heating was recorded in case of crude *Euphorbia* extract T10 / β-CD complexes, demonstrating a normal decomposition and loss of water of crystallization and
also expected normal physical transitions: initial vaporization, evaporation, sublimation, desorption and finally drying. In case of Euphorbia concentrated extracts T5 / T10 - β-CD complexes, the loss can be translated as a degradation / loss of complexes' bioactive compounds weight being an expected and true mass change.

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ABBREVIATIONS

CD, Cyclodextrin; TGA, thermo gravimetric analysis; IBD, inflammatory bowel disease; DSS, dextran sulfate solution.

REFERENCES


Effect of cholic acid on colonic motility in mice

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The role of bile acids on the gastrointestinal motility are contradictory, especially the role of mast cells mediators in this effect. Thus, cholic acid (CA) was examined for its in vitro action on the motility of the mice colon using different doses of CA (0.3, 30, 50, 100, 200, 300 and 500 µM). The contractile activity of the colon segment was recorded as changes in intraluminal pressure under isovolumetric conditions. The mean amplitude of the peristaltic motor complexes and the frequency (interval) of phasic contractions were determined. In other experiments, to study the CA mode of action, tissues were preincubated with 5-HT3 antagonist (Gransetrone hydrochloride), 5-HT4 antagonist (GR113808), H1 antagonist (Pyrilamine maleate salt) and protease activated receptor (PAR1) antagonist (BMS-200261) prior to challenge with CA (300 µM). CA inhibitory effect on contractile activity might be via its antagonistic action on the 5-HT3, 5-HT4, H1 receptors and PAR1 with variable levels. In conclusion, CA perfusion, at certain concentration levels, induced significant physiological changes in colon motility that might propose its antagonistic action on the receptors of the mast cells neuromediators.

Key words: Cholic acid, colonic motility, receptor antagonists, peristaltic motor complexes.

INTRODUCTION

Bile acids (BAs) control intestinal motility and secretion, in addition to their role in the digestion and absorption of dietary fat. The primary bile acid, cholic acid (CA) is synthesized from cholesterol in hepatocytes, secreted into canaliculi, and stored in the gallbladder. After secretion into the intestine, CA is actively absorbed in the ileum. The small amounts of CA that normally reach the colon is deconjugated and dehydroxylated by bacteria to form the secondary BAs which are passively absorbed (Alemi et al., 2013).

Although BAs have marked effects on intestinal motility and secretion by poorly understood mechanisms. Diminished secretion of bile during cholestatic disease causes constipation, whereas excess delivery of bile due to impaired intestinal BA absorption causes diarrhea (Bunnett et al., 2012).

Irritable bowel syndrome (IBS) is an extremely common disorder that affects up to 20% of the general population around the world (Astegiano et al., 2008). It is responsible for almost half of the referrals to gastroenterologists. Surprisingly, the cause of IBS is poorly understood and several pathophysiological mechanisms have been implicated. Bile acid malabsorption (BAM) is generally not regarded as a cause of IBS. However, recent improvements in the techniques employed for assessing BAM have demonstrated that it may promote and contribute to a low-grade mucosal inflammation in IBS (Camilleri et al., 2008) and diarrhea in patients with inflammatory bowel disease symptoms (IBD) (Surawicz, 2010). Although the cell types that mediate these actions of BAs have not been identified, studies using neurotoxins and neurotransmitter antagonists suggest that BAs control motility and secretion through effects on the enteric nervous system (Alemi et al., 2013).

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Mast cell is a resident cell of several types of tissues and contains many granules rich in neuromediators such as histamine, serotonin and proteases. High circulating levels of BAs result in mast cell activation to release its neuromediators (Boyer et al., 2006) which are implicated in alterations in gastrointestinal motility during acute intestinal infection with pathogenic bacteria (Barbara et al., 2006; Wang et al., 2007). Serotonin is produced to stimulate enteric smooth muscle contraction via 5-HT3 and 5-HT4 receptors (Kim, 2009), while histamine activates enteric neurons and regulate intestinal motility (Umoren et al., 2013) via interaction with H1 receptors (Liu et al., 2003). In other conditions mast cells are associated with impaired motility, often due to deleterious effects of mast cell proteases on the interstitial cells of cajal (Wang et al., 2007).

The H4 receptor is a histamine receptor belonging to the family of rhodopsin like G-protein-coupled receptors. This receptor, which is activated by the biogenic amine histamine, is expressed throughout the body, to be specific, in smooth muscles, on vascular endothelial cells, in the heart, and in the central nervous system. Antihistamines, which act on this receptor, are used as anti-allergic drugs (Shimamura et al., 2011). Pyrilamine, also known as Mepyramine, is a first generation antihistamine, targeting the H4 receptor (Parsons and Ganellin, 2006). It also has anticholinergic properties.

Serotonin or 5-hydroxytryptamine (5-HT) is found widely distributed throughout the gut and the central nervous system. In the gut, 5-HT is found mostly in mucosal enterochromaffin cells which are sensory transducers that release 5-HT to activate intrinsic (via 5-HT1P and 5-HT4 receptors) and extrinsic (via 5-HT3 receptors) primary afferent nerves.

Serotonin 5-HT4 receptors are in the alimentary tract (smooth muscle), urinary bladder, heart and adrenal gland as well as the central nervous system (Hegde and Eglen, 1996). The 5-HT4 receptor appears to mediate both inhibition and activation of smooth muscle involving myogenic as well as neural actions (Wouters et al., 2007). In the alimentary tract, stimulation of 5-HT4 receptors has a pronounced effect on smooth muscle tone, mucosal electrolyte secretion, and the peristaltic reflex (Hegde and Eglen, 1996). The 5-HT4 receptors antagonist (GR113808) is a drug which acts as a potenti and selective 5-HT4 serotonin receptor antagonist (Kaumann, 1993). It is used in researching the roles of 5-HT4 receptors in various processes (Mikami et al., 2008), and has been used to test some of the proposed therapeutic effects of selective 5-HT4 agonists.

Protease-activated receptors (PAR) are a subfamily of related G protein-coupled receptors that are activated by cleavage of part of their extracellular domain. They are highly expressed in platelets, but also on endothelial cells, myocytes and neurons. There are four known protease-activated receptors or PARs, numbered from one to four (PAR 1-4). These receptors are members of the seven transmembrane G-protein-coupled receptor super family and are expressed throughout the body.

Reports on the motor action of BAs on the gastrointestinal tract have been conflicting. The aim of this study is to examine the effect of CA as the primary BAs on colonic motility in mice and to characterize the role of mast cell mediators in this effect in controlling intestinal motility in mice.

MATERIALS AND METHODS

Experimental animals

Swiss male mice (27 to 33 g body weight) have been used in this study. Animals were caged (5/cage) ad libitum under standard conditions (light/dark cycle) in the animal house of the laboratory during the experiments. Mice were kept in the animal house for 2 weeks prior to the trial to customize the diet and the environmental conditions and their weight were monitored during this period by the investigators. Body weight of animals from all groups was monitored and measured during the whole period of the experiment. All experiments were approved by the Ethics Committee of King Fahad Medical Research Center, King Abdulaziz University.

Chemicals and drugs

CA, mast cell stabilizers, cholinergic and adrenergic blockers, mast cells mediator antibodies and antagonists were purchased from Sigma-Aldrich Corporation (St. Louis, MO USA).

The following drugs were used: CA, 5-HT3 antagonist (Granisetron hydrochloride), 5-HT4 antagonist (GR113808), H1 antagonist (Pyrilamine maleate salt) and PAR1 receptor antagonist (BMS-200261).

All drugs were dissolved in distilled water, except CA which was dissolved in dimethylsulphoxide (DMSO, 0.1%). Drugs were stored at -20°C. Freshly diluted aliquots were maintained on ice during the course of the experiments and added to the bath in microlitre volumes.

Tissue preparation for motility recordings

Randomly selected mice were killed by cervical dislocation. A midline laparotomy was performed and a segment of colon was rapidly excised and placed in gassed (95% O2 and 5% CO2) Krebs bicarbonate buffer solution (composition in mM: NaCl 117, KCl 4.7, NaHCO3 25, CaCl2 2.5, MgCl2 1.2, NaH2PO4,2H2O 1.2 and D-glucose 11). Muscles tissue segments were cleared of any mesenteric connective tissue and the lumen flushed with Krebs solution. Tissues were prepared according to the method described by Abdu et al. (2002). Colon segments approximately 5 cm in length were prepared from each animal (four in total) and were mounted horizontally in separate 20 ml perfusion chambers. Tissues were maintained at 37°C, and perfused with Krebs solution at a rate of 5 ml/min and were allowed to equilibrate for at least 30 min before recording. The oral and aboral ends of each segment were secured to two metal catheters fixed at either end of the chamber and adjusted to maintain the segments at their resting length. For each segment, the oral end was connected to a perfusion pump for infusion of Krebs solution at a rate of 0.16 ml/min, and the aboral end was attached to a pressure transducer (NL108T2) to record contractile activity as changes in intraluminal pressure under isovolumetric conditions. The experimental setup was standardized by routinely infusing Krebs solution into the closed segment to an initial intraluminal pressure of 4 to 5 cmH2O. Regular aborally propagating waves of contraction (peristaltic
pressure waves) were developed under these conditions and could be maintained for several hours.

The output from the pressure transducers (motor complex of colon) of control and CA treated colon segments were monitored and analyzed by using a NeuroLog™ System (NL900D, Digitimer Ltd., Hertfordshire, UK) and data-acquisition system (Power1401, Cambridge Electronic Design Ltd., Cambridge, UK) which connected to a computer running Spike 2 version 4 software (Cambridge Electronic Design Ltd., Cambridge, UK), that displayed the two channel pressure recordings online and also stored the data for subsequent offline analysis to compare changes of amplitude and intervals of smooth muscle motility in different treatments.

Colon motility

Isolated colon segments were distended to 4 to 5 cm/H2O to evoke peristaltic motor complexes (PMCs). Only preparations with regular maintained peristaltic motor complexes (PMCs) were used for subsequent experiments. PMCs were quantified in terms of their peak amplitude above baseline and were expressed as cm/H2O, while duration and interval between them were expressed in seconds (s). The baseline and treated mean values of the amplitude of the PMCs and the frequency (interval) of phasic contractions were determined 15 min before and after challenge.

CA or the appropriate vehicle was added to the chambers 15 min after stopping perfusion. The recording continued for a further 15 min before washing out the drug and reinstituting perfusion. Dose response curve was done for the primary BA (CA), for the following doses (0.3, 30, 50, 100, 200, 300 and 500 µM, n = 28, 4 mice for each dose).

Colon was pre-incubated with the following antagonists, 5-HT3 antagonist (Granisetron hydrochloride, 10 µM), 5-HT4 antagonist (GR113808, 10 µM), H1 antagonist (Pyrilamine maleate salt, 10 µM) and PAR1 receptor antagonist (BMS-202621, 20 µM) for 5 min after stopping perfusion and prior to challenge with CA (300 µM, n = 16, 4 mice for each antagonist). The recording continued for a further 15 min before washing out the drug.

Statistical analysis of data

All values are reported as mean (standard error of mean (SEM)). Data were statistically compared using "student's" t test paired, unpaired or Wilcoxon rank-sum test as appropriate. Data were also analyzed using one-way analysis of variance (ANOVA) with Dunnett's test for a multiple comparison. Probability of P < 0.05 was considered significant.

RESULTS

Dose response curve of CA

Luminal distension of isolated segments of mice colon evoked a regular pattern of contractile activity. The activity consisted of periodic increases in intraluminal pressure separated by relative intervals.

The dose response effect of CA on the intervals and amplitudes of the colon motility was done using different doses (0.3, 30, 50, 100, 200, 300 and 500 µM, n = 28, 4 mice for each dose).

By using 50, 100, 200, 300 and 500 µM of CA, there was a significant increase in the PMC intervals compared to the control values (P < 0.05, Figures 1 and 3), while after administration of the 200, 300 and 500 µM, CA significantly decreased the PMC amplitudes (P < 0.05, Figures 1 and 4).

Effect of CA in the presence of pharmacological antagonists on PMC intervals

Treatment of colon segments with 300 µM of CA induced significant increase in the PMC intervals (101.6 ± 10 s) compared to the control values (90.8 ± 5 s, P < 0.05) (Figure 5). The same results were obtained after incubation of the colon with 10 µM of 5-HT3 antagonist (100.6 ± 19 s) (Figure 5A) and 10 µM of H1 antagonist (106.42 ± 23 s) (Figure 5C). Addition of CA (300 µM) following pre-incubation of colon segment with both blockers (5-HT3 and H1) augmented the interval values of PMCs significantly (122 ± 13 and 109.7 ± 20 s), respectively, compared to the control levels (90.8 ± 5 s, P < 0.05). It is important to mention that the elongation of the PMC intervals caused by the combination of (CA + 5-HT3) antagonist (122 ± 13 s) was significant compared to the values of CA alone (101.6 ± 10 s, P < 0.05) or 5-HT3 antagonist alone (100.6 ± 19 s, P < 0.05). Also, there was significant increase in the intervals of the PMCs of the colon treated with (CA + H1) compared to H1 alone (109.7 ± 20 s versus 106.42 ± 23 s, P < 0.05).

In contrast, 5-HT4 antagonist (10 µM, Figure 5B) and PAR1 receptor antagonist (20 µM, Figure 5D) significantly reduced the PMC intervals (70.6 ± 13 and 54.6 ± 3 s), respectively, compared to both control (90.8 ± 5 s, P < 0.05) and CA values (101.6 ± 10 s, P < 0.05). Colon tissues treated with the CA following treatment with both competitors (5-HT4, PAR1) showed the same noteworthy inhibitory effect on the PMC intervals (60.1 ± 11 and 55.7 ± 7 s), respectively, compared to the control (P < 0.05).

Effect of CA in the presence of pharmacological antagonists on PMC amplitudes

The response of the colon concerning the amplitudes of PMC to CA and the pharmacological antagonists is illustrated in Figures 4A, B, C, D and 2. A significant inhibitory response to the PMC amplitudes were recorded following incubation of different segments of mice colon with CA 300 µM (70.5 ± 6 versus 56.2 ± 16 cm/H2O).

5-HT3 antagonist 10 µM inhibited the amplitude to 53.3 ± 8 cm/H2O compared to control (70.5 ± 6 cm/H2O, P < 0.05). This inhibition was increased by the combination of CA + 5-HT3 receptor antagonist to 38.9 ± 15 cm/H2O, P < 0.05 (Figure 6A).

The addition of 5-HT4 antagonist (10 µM) alone or with CA into the colon segments (Figure 6B) showed a decrease in motility amplitudes (54.9 ± 5 and 56.9 ± 2 cm/H2O, respectively) when compared with the control (70.5 ± 6 cm/H2O, P < 0.05).
Figure 1. Dose response curve. Representative recording traces showing the effect of different doses (0.3, 30, 50, 100, 200, 300 and 500 µM) of CA on the intervals and amplitudes of the PMC of mice colon.

Figure 2. Effect of CA in the presence of pharmacological antagonists on colon motility. Representative recording traces showing the effect of intraluminal pressure on colon motility of mice. In vitro tissue preparations were preincubated with (5-HT3 antagonist, 10 µM, A), (5-HT4 antagonist, 10 µM, B), (H1 antagonist, 10 µM, C) and (PAR1 receptor antagonist, 20 µM, D) prior to challenge with CA (300 µM). The same inhibitory effect were noticed after applying 10 µM of H1 antagonist (38.3 ± 7 cm/H2O), and 20 µM PAR1 receptor antagonist (43.8 ± 10 cm/H2O) and after the combination of each blocker with CA (25.55 ± 7 cm/H2O, Figure 6C) and (30.24 ± 3 cm/H2O, Figure 6D), respectively compare to control (70.5 ± 6 cm/H2O, P <
Figure 3. The effect of CA on PMC intervals. Effect of different doses (0.3, 30, 50, 100, 200, 300 and 500 \( \mu \text{M} \)) of CA on the PMC intervals. Values represent mean ± SE of 4 colon segments from different animals. Significantly different compared to the control group (\( P < 0.05 \)).

Figure 4. The effect of CA on PMC amplitudes. Effect of different doses (0.3, 30, 50, 100, 200, 300 and 500 \( \mu \text{M} \)) of CA on the PMC amplitudes. Values represent mean ± SE of 4 colon segments from different animals. Significantly different compared to the control group (\( P < 0.05 \)).

Discussion

BAs affect intestinal motility, although whether their actions are stimulatory or inhibitory is controversial. Early \textit{in vivo} studies showed that BAs either stimulate motility or have no effect (Feldman and Gibaldi, 1968; Kirwan et al., 1975 and Falconer et al., 1980). \textit{In vitro} researches demonstrated that BAs inhibit contractions of rabbit and guinea pig intestine (Armstrong et al., 1993; Romero et al., 1993; Poole et al., 2010).

This study showed that CA significantly inhibited the
Figure 5. Effect of CA in the presence of pharmacological antagonists on the intervals of the PMC of mice colon. Tissue preparations were preincubated with (5-HT3 antagonist, 10 μM, A), (5-HT4 antagonist, 10 μM, B), (H1 antagonist, 10 μM, C) and (PAR1 receptor antagonist, 20 μM, D) prior to challenge with CA (300 μM). Values represents mean ± SE of 4 colon segments from different animals. *Significantly different compared to the control group, (P < 0.05). †Significantly different compared to the CA data, (P < 0.05). ‡Significantly different compared to the antagonist data, (P < 0.05).

PMC amplitudes and intervals in a dose dependent manner compared to the control. The effect of CA on these results was in agreement with the previous studies since the infusion of BAs into the intestine delays transit, supporting an inhibitory role of CA (Van Ooteghem et al., 2002). Although in this study, the mechanisms by which
CAs inhibit intestinal function have not been investigated; recent researches that refer to recent discovery of receptors that mediate their effects confirmed our results (Lefebvre et al., 2009). However, the activation of GpBAR1 by BAs inhibits large intestinal motor function, which could be due to the effects of GpBAR1 activation which mediated through activation of cholinergic and nitrergic interneurons (Keely, 2010). In these experiments, CA inhibited PMC amplitude after addition of 5-HT3 antagonist, 5-HT4 antagonist and H1 anta-gonist. These results were in agreement with previous studies which demonstrated that BAs are able to activate mast cells (Gelbmann et al., 1995; Fihn et al., 2003) and act via 5-HT3 receptor. Therefore, the inhibition of CA in the

Figure 6. Effect of CA in the presence of pharmacological antagonists on the Amplitudes of the PMC of mice colon. Tissue preparations were preincubated with (5-HT3 antagonist, 10 \( \mu \)M, A), (5-HT4 antagonist, 10 \( \mu \)M, B), (H1 antagonist, 10 \( \mu \)M, C) and (PAR1 receptor antagonist, 20 \( \mu \)M, D) prior to challenge with CA (300 \( \mu \)M). Values represents mean \( \pm \) SE of 4 colon segments from different animals. *Significantly different compared to the control group, (\( P < 0.05 \)). †Significantly different compared to the CA data, (\( P < 0.05 \)).
presence of histamine antagonist on PMCs suggested that
mast cells are playing an important role in mediating
hyperexcitability and enteric neuron depolarization via
their release of histamine from mast cell degranulation
(Sand et al., 2009).
In these experiments, the protease inhibitors, PAR1
receptor antagonist also inhibited the PMCs. These data
supported by Poole et al. (2010) who identified a novel
mechanism for the well-known effects of BAs on intestinal
motility. He found that BAs inhibit motility by a
mechanism that is consistent with activation of GpBAR1
on inhibitory motor neurons that release nitric oxide (NO).
However, in this study, the effect of PAR1 was
controversial since CA increases motility in the presence
of PAR1 antagonist. The justification for that is the mast
cells are regulated by the release of NO (Sand et al.,
2009). Therefore, increase motility in this study could be
due to the lack of releases of NO during the application
of CA, therefore, these results suggest new mechanism
concerning the possibility of antagonistic action of CA on
the receptors of the mast cells neuromediators and CA
may also act on the mucosa to release factors that
control motility.
The increase motility in this study supported also by
Kirwan et al. (1975), who found that the abnormally high
quantities of primary BAs in the colon cause diarrhea not
only by inhibiting the absorption of water and electrolytes,
but also by eliciting colonic motor activity.
This study showed that CA in the presence of PAR1
inhibited the amplitudes and is in agreement with Poole
et al. (2010). BAs induced inhibitory effects on contractile
activity were unaffected by atropine which indicates that
muscarinic receptors are not involved in these responses.
The same authors reported that a BA that is a potent
GpBAR1 agonist inhibited motility of intestinal segments
by a neurogenic and nitricergic mechanism.
In conclusion, this study has described that in vitro CA
perfusion, at certain concentration levels, induced
significant inhibitory changes in colon motility. The clear
inhibitory effect of CA on PMC amplitudes in the
presence of 5-HT3 and 5-HT4 and PAR1 antagonists
suggests that CA may act through the release of mast
cell neuromediators. This may propose its antagonistic
action on the receptors in addition to the possible direct
effect of the CA on the colon musculature.

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Full Length Research Paper

Potential antioxidant and anti-inflammatory effects of *Hyphaena thebaica* in experimentally induced inflammatory bowel disease

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Ulcerative colitis and Crohn's disease are chronic recurrent inflammatory bowel disease (IBD) of unknown origin. Oxidative stress is believed to be a key factor in the pathogenesis of the mucosal damage in IBD. The aqueous extract of *Hyphaena thebaica* fruits showed an antioxidant activity; this is due to the substantial amount of their water-soluble phenolic contents. This study was undertaken to study the effect of *H. thebaica* in experimentally induced IBD. Sprague Dawley rats were pretreated orally for 5 days with distilled water in control, *H. thebaica* 500 mg/kg in acetic acid + *H. thebaica* group and 5-aminosalislyc acid (5-ASA) 100 mg/kg in acetic acid + 5ASA groups. Colitis was induced by transrectal administration of 4% acetic acid on 5th day. All the animals were sacrificed with ether overdose 48 h after colitis induction, and 10 cm colon segment was resected from distal end. Colon was weighed (for disease activity index) and scored macroscopically and microscopically after histological staining. Biochemical assessments included myeloperoxidase (MPO) and tissue catalase (CAT), glutathione (GSH) and superoxide dismutase (SOD) measurements. *H. thebaica* showed significant prevention of increase in colon weight and disease activity index along with decrease in macroscopic and microscopic lesion score as compared to control group. Significant improvement was observed in the levels of MPO, CAT and SOD, except GSH. However, the effect of *H. thebaica* was significantly less than 5-ASA. *H. thebaica* at 500 mg/kg showed significant amelioration of experimentally induced IBD, which may be attributed to its antioxidant and anti-inflammatory properties.

Key words: Colitis, antioxidant, *Hyphaena thebaica*, myeloperoxidase, catalase, superoxide.

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are two major categories of inflammatory bowel diseases (IBDs). Although the etiology and pathophysiology of IBD still remain unclear, immune dysfunction, reactive oxygen species (ROS), inflammatory mediators and cytokines play important roles in its development and recurrence (Sellin and Pasricha, 2006). It is supposed that homeostasis is disrupted in IBD patients because of over-expression of inflammatory cytokines (TNF-α, IL-1, IL-6) and/or lower expression of regulatory or anti-inflammatory cytokines (IL-2, IL-4, IL-10, TGF-β) (Lih-Brody et al., 1996).

Development of ulcerative colitis is characterized by increased mucosal infiltration by neutrophils and monocytes which, having become activated, release proinflammatory cytokines-tumor necrosis factor α (TNF-
α), interferon-γ (IFN-γ), interleukin-1β (IL-1β) and free oxygen radicals, induce increased expression of iNOS COX-2, nuclear transcription factor NF-κB, protein p66, and enhancement of the lipoperoxidation processes. These changes cause destructive affections of the mucous membrane of the large intestine, for example impairment of mucous barrier, swelling, ulcers, erosions, and hemorrhages (Hosoi et al., 2001; Martin et al., 2005). The destructive changes the intestinal mucosa is subjected to due to ulcerative colitis are associated with increased numbers of active forms of oxygen, enhanced synthesis of NO, expression of iNOS by epithelial cells, macrophages, and neutrophil infiltrato into the damaged mucous membrane. Concomitantly, the activity of myeloperoxidase, the marker of inflammation was enhanced (Tanaka et al., 2002).

Common drugs that are administrated for the management of IBD include sulfasalazine, 5-ASA derivatives and glucocorticoids. Immunosuppressants, antibiotics and monoclonal antibodies (Infliximab) are also occasionally used for intractable disease conditions (Mcquaid, 2007). These therapeutic agents have side effects and they could not appropriately cure IBD patients (Cross et al., 2008). In many studies, it has been reported that antioxidants show beneficial effects in experimental colitis (Nosal’ova et al., 2000).

Medicinal plants and plant products are the oldest and tried health care products. Their importance is growing not only in developing countries but in many developed countries. *Hyphaene thebaica* (L.) mart is one of the plants used in ethnomedicine and belongs to the family palmae and subfamily Borassoideae. It grows commonly in both Sahel and Sahara regions of Africa (Vonmaydell, 1986). Locally, various extracts and decoction of *H. thebaica* are used in the treatment of bilharzia, haematuria, hypertension and as a haematinic agent (Adaya et al., 1977; Vonmaydell, 1986; Kamis et al., 2003).

Research on the fruit pulp of *H. thebaica* showed that it contains nutritional trace minerals, proteins and fatty acids, in particular the nutritionally essential linoleic acid (Kamis et al., 2003). The identification of compounds, by thin-layer chromatography, showed that the fruit contains significant amounts of saponins, coumarins, hydroxycynamates, essential oils and flavonoids. The fruit also lowers blood pressure in animal models (Sharaf et al., 1972). The aqueous extract of doum fruits showed an antioxidant activity; this is due to the substantial amount of their water-soluble phenolic contents (Hsu et al., 2006). Acetic acid induced colitis model is similar to human ulcerative colitis in terms of histological features, and has been used extensively in many experimental studies of IBD (Nosal’ova et al., 2000; La et al., 2003). The effect of various herbal drugs (but not *H. thebaica*) on experimental models of IBD has been reported earlier with the antioxidant potential as the main mechanism of action against IBD (Ko et al., 2005; Zeytunlu et al., 2004), as the plant *H. thebaica* is thought to possess antiinflammatory and antioxidant properties (Mohamed et al., 2010). This study was undertaken to study the effect of *H. thebaica* in experimentally induced IBD and to find its probable mechanism of action including its antioxidant potential.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals were purchased from Sigma chemical company (St Louis, MO, USA).

**Plant**

The root of *H. thebaica* (L.) mart was obtained from the plant department of faculty of agriculture, Mansoura university.

**Preparation of suspension**

The root of the plant was treated according to the method of Joslyn (1970). It was dried in an oven for about six hours at 60°C followed by sun drying for days. The dried root was ground into fine powder using mortar and pestle. The powder was sieved through a 0.25 mm sieve. Aqueous suspension was constituted by dissolving 5 g of the powdered root in 100 ml of distilled water and stored at low temperature. The suspension was shaken vigorously to obtain a homogenous mixture before administration.

**Animals and treatments**

Adult Sprague Dawley rats of both sexes (n = 24) weighing 250 to 300 g were housed individually in a light- and temperature-controlled room on a 12 to 12 h light–dark cycle, in which the temperature (22 ± 2°C) and relative humidity (65 to 70%) were kept constant. The animals were fed a standard pellet lab chow, and food was withdrawn overnight before induction of colitis, but access to water was allowed *ad libitum*. The experiments performed were approved by the Institutional Animal Care and Use Subcommittee of our university. The animals were divided into four groups with six animals in each group as follows:

1. Group A (normal control) – received distilled water 10 mL/kg/day, p.o.
2. Group B (acetic acid) – received distilled water 10 mL/kg/day, p.o.
3. Group C (acetic acid + H.thebaica) – received aqueous suspension of pulp extract of *H. thebaica* 500 mg/kg/day, p.o. (Modu et al., 2000, 2001)
4. Group D (acetic acid + 5ASA) – received 5-ASA 100 mg/kg/day p.o.

The animals were pretreated with the respective drugs (volume of drugs was kept constant at 10 ml/kg) for 5 days, along with the normal diet. On the 5th day, animals were kept fasting for 12 h (overnight) and IBD was induced next morning in Groups B, C and D by administration of 1 ml of 4% acetic acid solution transrectally (TR). Group A (normal control) animals received 0.9% normal saline (TR) instead.
Induction of colitis

For induction of IBD, an 8 mm soft pediatric catheter was advanced 6 cm from the anus under low-dose ether anesthesia. Rats were in Trendelenburg position during this process and 1 ml of 4% acetic acid or 0.9% normal saline solution was slowly administered TR. The rats were maintained in head-down position for 30 s to prevent a leakage, and the rest of the solution was aspirated. After this process, 2 ml of phosphate buffer solution with pH 7 was administered (TR) (Zeytunlu et al., 2004).

Histological analyses

All the animals were sacrificed after 48 h of IBD induction, by ether overdose. Abdomen was opened and colons were exposed. Distal 8 cm of colon was excised and opened by a longitudinal incision. After washing the mucosa with saline solution, mucosal injury was assessed macroscopically using the scale of Morris et al. (1989) as follows: no damage (0); localized hyperemia but no ulceration (1); linear ulcer without significant inflammation (2); linear ulcer with significant inflammation at one site (3); two or more sites of ulceration and inflammation (4) and two or more sites of ulceration and inflammation or one major site of inflammation and ulcer extending > 1 cm along the length of colon (5). Disease activity index (DAI) was measured as the ratio of colon weight to body weight, which was used as a parameter to assess the degree of tissue edema and reflects the severity of colonic inflammation (Ko et al., 2005). Moreover, a 6 to 8 mm sample block of the inflamed colonic tissue with full thickness was excised from a region of grossly visible damage for histological analysis. Formalin fixed tissue samples were embedded in paraffin and stained with Hand E stain. Colonic tissues were scored for histological damage using the criteria of Wallace and Keenan (1999): 0 = intact tissue with no apparent damage; 1 = damage limited to surface epithelium; 2 = focal ulceration limited to mucosa; 3 = focal, transmural inflammation and ulceration; 4 = extensive transmural ulceration and inflammation bordered by normal mucosa; 5 = extensive transmural ulceration and inflammation involving the entire section.

Biochemical assessments

After scoring, the colonic tissue samples were homogenized with 10 volumes of ice-cold 0.25 M sucrose and centrifuged at 14,000 rpm to measure the biochemical parameters in the resulting supernatant.

Measurement of myeloperoxidase (MPO) activity

To measure MPO activity, colonic samples were minced on ice and homogenized in 10 ml of ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB). The homogenates were then sonicated and centrifuged for 20 min at 12,000 g. MPO activity was measured spectrophotometrically as follows: 0.1 ml of supernatant was combined with 2.9 ml of 50 mM phosphate buffer in 0.0005% H2O2. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the change in absorbance per minute at room temperature, in the final reaction.

Assessment of antioxidant status in colonic tissue

Glutathione (GSH) activity

Glutathione (GSH) level was determined according to method of Beutler (1975). The reaction mixture contained supernatant, phosphate buffer and 5,5'-dithio-bis 2-nitrobenzoic acid (DTNB) in a final volume of 10 ml. A blank was also prepared. The absorbance was immediately read spectrophotometrically at 412 nm before and after addition of DTNB. The values were determined from the standard curve.

Superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) was measured according to the method of Fridovich (1983). Assay medium consisted of 0.01 M phosphate buffer, 3-cyclohexilamino-1-propanesulfonic acid (CAPS), saturated NaOH with pH 10.2, solution of substrate (0.05 mM xanthine, 0.025 mM P-iodonitrotetrazolium violet) and 80 μl xanthine oxidase. Absorbance was read spectrophotometrically at 505 nm. SOD was expressed as U/mg of proteins.

Catalase activity

Catalase was measured by the method of Beers and Sizer (1952). Phosphate buffer (2.5 ml, pH 7.8) was added to supernatant and incubated at 25°C for 30 min. After transferring into the cuvette, the absorbance was measured at 240 nm spectrophotometrically. Hydrogen peroxide (650 μl) was added and change in absorbance was measured for 3 min. Values were expressed as μmol/min/mg of protein.

Statistical analyses

Statistical analyses were done using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. P < 0.05 was considered as significant.

RESULTS

Acetic acid administration to the acetic acid group caused significant macroscopic ulcerations and inflammations (P < 0.05) in rat colon along with significant mucosal injury (Figure 1A and B) microscopically (P < 0.05), when compared to the normal control group (Figure 1 and Table 1) (P < 0.05). Also, there was significant derangement of biochemical parameters including tissue levels of MPO, GSH, SOD and catalase (P < 0.05), showing oxidative stress due to colon damage and colonic inflammation (Figure 3A and B). Aqueous extract of H. thebaica showed significant activity against experimentally induced IBD when compared to the acetic acid group (P < 0.05), with near normalization of colon architecture both macroscopically as well as microscopically (Table 1 and Figure 1C). Tissue oxidative stress was reduced with significant improvement in tissue levels of SOD and CAT (P < 0.05), showing its antioxidant potential, although there was no significant
Figure 1. Group A (normal control): Normal mucosal architecture. Group B (experimental control): Extensive necrosis with transmural infiltration.

Figure 2. (a) Group C (H. thebaica) Infiltration up to submucosa, architecture maintained. (b) Group D (5-ASA): Near normalization of architecture with mucosal infiltration only.

difference in GSH levels when the two groups were compared ($P > 0.05$). Also, significant improvement in the levels of MPO was observed ($P < 0.05$) (Table 1 and Figures 2A, B, 3A and B).

As for the standard drug 5-ASA, its activity against IBD was significantly better than extract of *H. thebaica* with regard to all the parameters ($P < 0.05$).

**DISCUSSION**

The results of this study have shown that aqueous extract of *H. thebaica* has got a good potential to suppress experimental colitis in rats, as indicated by macroscopic, microscopic and biochemical evaluations. Acetic acid induced colitis model is similar to human ulcerative colitis in terms of histological features. It affects the distal colon portion and induces non-transmural inflammation, massive necrosis of mucosal and submucosal layers, mucosal edema, neutrophil infiltration of the mucosa and submucosal ulceration. The protonated form of the acid liberates protons within the intracellular space and causes a massive intracellular acidification resulting in massive epithelial damage. The inflammatory response initiated by acetic acid includes activation of cyclooxygenase and lipoxygenase pathways (Sharon and Stenson, 1985; MacPherson and Pfeiffer, 1976).

Phytochemical studies revealed the presence of flavonoids, coumarins and saponins in *H. thebaica*. Various antioxidant flavonoids and coumarin derivatives have been reported as protective products to prevent and treat intestinal inflammatory processes induced by different chemical inducers of experimental colitis (Galvez et al., 2001; Distasi et al., 2004; Luchini et al., 2008; Witaicenis et al., 2010). Several studies have shown that different saponins from *Panax ginseng* and...
Figure 3. (a) Effect of *H. thebaica* on tissue CAT (umol/min/mg protein) and GSH (nmol/mg protein) on acetic acid induced colitis in rats. Mean ± SE. (6 numbers each). (b) Effect of *H. thebaica* on tissue SOD (U/mg protein) and MPO (U/g protein) on acetic acid induced colitis in rats. Mean ± SE (6 numbers each).

Table 1. Effect of *H. Thebaica* on macroscopic score, disease activity index and microscopic score (Mean ± SE).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Microscopic score</th>
<th>Disease activity index (DAI)</th>
<th>Macroscopic score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>0±0</td>
<td>0.67±0.042</td>
<td>0.33±0.21</td>
</tr>
<tr>
<td>Experimental</td>
<td>4.7±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14±0.045&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.67±0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>H. thebaica</em></td>
<td>3.0±0.36&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.9±0.003&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>2.0±0.36&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5-ASA</td>
<td>1.5±0.22&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>0.76±0.033&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.0±0.26&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P&lt;0.05</em></td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Value expressed as mean ± SE (number 6). *p: in comparison to normal control*; *p: in comparison to experimental control.*; *p: in comparison to standard group.*

*Codonopsis lanceolata* were active compounds in experimental colitis (Joh et al., 2011; Joh and Kim, 2011). Hence, it is plausible that the presence of these classes of natural compounds in the *H. thebaica* contribute to the
observed intestinal anti-inflammatory activity.

The flavonoids found in the extract of *H. thebaica* possess anti-proliferative activity that causes a decrease in the weight and volume of contents of granuloma in inflammation (Koganov et al., 1999). Therefore, this might be its probable mechanism of anti-inflammatory action. MPO, an enzyme found predominantly in the azurophilic granules of neutrophils, is a biochemical marker of neutrophil infiltration, and measurements of its activity have been widely used to detect intestinal inflammatory processes (Yamada et al., 1992; Villegas et al., 2003). Reduction of MPO activity can be interpreted as a manifestation of the anti-inflammatory property of a given compound (Veljaca et al., 1995). The intestinal anti-inflammatory activity of the *H. thebaica* was also related to an inhibitory effect on MPO activity.

Oxidative stress is believed to play a key role in the pathogenesis of IBD-related intestinal damage (Grisham and Granger, 1988). Intestinal mucosal damage in the IBD, including Crohn’s disease and ulcerative colitis, is related to both increased free radical production and a low concentration of endogenous antioxidant defense (Kotroubakis et al., 2004). In the present study, the aqueous extract of *H. thebaica* showed an antioxidant activity; this is due to the substantial amount of their water-soluble phenolic contents (Hsu et al., 2006). Furthermore, flavonoids are a large group of naturally-occurring plant phenolic compounds that inhibit lipid oxidation by scavenging radicals or by other mechanisms such as singlet oxygen quenching, metal chelation, and lipoxygenase inhibition (Yanishlieva-Maslarova, 2001; Steenkamp et al., 2013).

As proved by the above study and also as described in literature (Mohamed et al., 2010), the extract of *H. thebaica* possesses significant antioxidant property, proving its role in the management of experimentally induced IBD. Hence, it can be concluded from this study that extract of *H. thebaica* has potent activity against experimentally induced IBD, due to its anti-inflammatory and antioxidant properties. Further investigations for its clinical utility are warranted.

REFERENCES


The efficacy of premedication with ibuprofen, gelofen and acetaminophen in the depth of anesthesia in mandibular molars with irreversible pulpitis

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Pain control is one of the major aspects in dental practice. Dental pain can usually be controlled using different techniques such as local anesthesia and medications. Acetaminophen is a pain reliever and fever reducer. However, it shows no effect on inflammation. Therefore, non-steroidal anti-inflammatory drugs are currently used to control inflammation and pain. This study sought to compare the efficacy of premedication with ibuprofen, gelofen and acetaminophen in the depth of anesthesia in mandibular molars with irreversible pulpitis. In this double-blind randomized controlled trial, 60 patients with at least one mandibular molar with symptoms of irreversible pulpitis requiring root canal therapy were recruited. Another tooth in the same quadrant was selected as the control. Patients were randomly allocated to one of the following groups: ibuprofen 400 mg, gelophen 400 mg, acetaminophen 325 mg, and placebo 500 mg. The medications were taken 30 min prior to local anesthesia and the cold test and electric pulp test (EPT) were repeated for the test teeth and the control teeth after 10 min with the development symptoms of anesthesia. Access preparation was then initiated and the patients were asked to quantify the level of pain during exposure of the dentin and pulp using a visual analogue scale (VAS). Data was analyzed using analysis of variance (ANOVA), and repeated measure ANOVA, P < 0.05 were deemed significant. There were significant differences between the mean baseline VAS score, and the mean VAS score recorded at the time of dentin and pulp exposure however revealed a significant difference in the VAS score only at the time of dentin exposure among the study groups (P < 0.005). No significant difference was observed in the mean VAS score at the time of pulp exposure among groups (P = 0.076). The EPT was significantly higher after the test compared with the baseline (P = 0.421). Premedication with ibuprofen and gelofen have significant effect in the depth of anesthesia in mandibular molars with irreversible pulpitis, and significantly decreased VAS but placebo and acetaminophen are functionally alike and had no significant effect.

Key words: Irreversible pulpitis, pain, root canal therapy.

INTRODUCTION

Pain is one of the most unpleasant feelings that a human being may experience throughout life. Pain control, especially in the early phases of endodontic treatment, is an important aspect of a successful dental procedure. Optimum pain management results in building up trust and facilitates the entire procedure (Walton et al., 2008).
Moreover, pain and anxiety is associated with increased stress, which may compromise the patient’s health leading to problems such as syncope (Eli, 2003; Maggirias and Locker, 2002). Sudden, severe pain may induce substantial changes in the cardiovascular, respiratory, endocrine and neurogenic systems, resulting in severe medical conditions (Kaviani et al., 2011).

In endodontic therapy, inferior alveolar nerve block (IANB) is the standard method of local anesthesia in the mandibular teeth. Nevertheless, clinical studies have demonstrated a notable range of failure (7 to 77%) for this technique. Moreover, the success rate of anesthesia in teeth with irreversible pulpitis is less than normal pulp (Aggarwal, et al., 2009; Tortamano et al., 2009). According to Hargreaves and Keiser (2002), some of the factors which may account for this failure include: anatomic factors, acute anaphylaxis, the effect of inflammation on the pH of the tissue, the effect of inflammation in blood circulation, the effect of inflammation on central hypersensitivity and its effect on the nociceptors.

Activation of nociceptors in the presence of inflammation is one of the strongest theories explaining the reduced efficacy of anesthesia. Inflammatory mediators reduce the stimulation threshold in nociceptor neurons to a level at which the slightest stimulators induce a severe neurogenic response (Goodis et al., 2006). This inflammatory process occurs as a result of the production of prostaglandins (PGs) as the end point product of the metabolism of arachidonic acid through the cyclooxygenase pathway (COX). PGs then result in increased sensation of pain by increasing the sensitivity of the nerve endings to bradykinin and histamine (Dray, 1995).

Researchers have studied different ways to achieve better anesthesia in teeth with pulps. Premedication with analgesic agents has been proposed as one of the alternatives with controversial results. Modarresi et al. (2006) and Parirokh et al. (2010) have reported a considerable improvement in the level of anesthesia when patients receive analgesics prior to IANB for the treatment of teeth with irreversible pulpitis. On the other hand, Ianiro et al. (2007) failed to show any significant difference between the case and control groups, although their observations were suggestive of a tendency toward better clinical results in the medication group.

Several other researchers including Aggarwal et al. (2009), Oleson et al. (2010) and Simpson et al. (2011) reported an insignificant improvement in the success of IANB accompanied by premedication with analgesics in treating irreversible pulpitis. Numerous analgesics are used for pain control in endodontics. Non-narcotic analgesics including acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and gelofen are among these drugs. We aimed to compare the efficacy of premedication with three different analgesics (Ibuprofen 400 mg, gelofen 400 mg, and acetaminophen 325 mg) in the anesthetic depth of IANB in teeth with irreversible pulpitis.

**MATERIALS AND METHODS**

This was a double-blind randomized controlled clinical trial. The study population consisted of 60 patients presenting to the Endodontics Department in Babol School of Dentistry, Babol, North Iran. The patients were to have at least one mandibular tooth with symptoms of irreversible pulpitis (spontaneous or nocturnal pain). Patients with history of systemic diseases, consumption of any type of analgesic agent at least 12 h prior to the study, any contraindication towards the use of analgesic agents, hypersensitivity to lidocaine 2% with 1:80000 epinephrine or any of the infiltration techniques, advanced periodontal diseases and periapical radiolucency associated with the study teeth and teeth with extensive restorations or previous endodontic treatments, were excluded from the study. Patients provided informed consent followed by a complete medical and dental history and clinical examination.

The pulp vitality tests including electric pulp test (EPT) and cold tests were then performed for each study tooth and a control tooth from the same quadrant. For the EPT, tooth paste was placed between the tip of the pulp tester (COXO Medical Instrument Co. LTD) and the tooth. The tip of the pulp tester was placed on an area of sound enamel at the incisal third of the tooth where there was no contact with the gingiva, restoration or any cracks in the enamel. As soon as the patient reacted to the stimulus (feeling of heat or tingling), the test was over. The EPT and cold test results were recorded for each study tooth and the respective control tooth. The patients were asked to determine their level of pain before and after taking the medications based on a visual analogue scale (VAS): 0 cm = no pain, 0 to 3: mild pain (patient can feel the pain, however does not become uncomfortable), 3 to 6: moderate pain (the pain is irritating but tolerable), 6 to 10: severe pain (the pain is intolerable) (Ianiro et al., 2007), > 10: very severe pain.

The medications included acetaminophen 325 mg (Kharazmi, Iran), ibuprofen 400 mg (Roozdaru, Iran), gelofen 400 mg (Zakaria, Iran), and placebo (capsule of 500 mg glucose). All medications were prepared identically with similar sizes and shapes in the Department of Pharmacology, Babol University of Medical Sciences and were presented to the patients as blue gelatinous capsules. The capsules were placed in identical envelopes and coded, yielding a total of 60 envelopes (four groups of 15 each).

The patients were randomly divided into four groups of 15 and were asked to take one capsule of the same drug group. The patients and the operator were blind to this procedure. After 30 min, one cartridge (1.8 mm) of 2% lidocaine, with 1:80000 epinephrine (Pharmaceutical Mfg.Co. Daroupaksh, Iran), was injected using the IANB technique. Patients who failed to meet the clinical signs of anesthesia after 10 min, that is tingling in the lower lip and the tip of the tongue, or those who reacted to the stimulus made by an explorer between the lateral incisor and the canine, were excluded. With the advent of signs of anesthesia, EPT and cold test recordings were repeated for the test and study teeth. Access preparation was then initiated and the quality and quantity of pain during dentin and pulp exposure were recorded using the VAS.

Data were submitted to the statistical package for social sciences (SPSS) software version 18, and analysis was performed using the following tests: Analysis of variance (ANOVA) and repeated measure ANOVA. P < 0.05 was considered as significant.

**RESULTS**

This double blind clinical study included 60 patients (30 men and 30 women), with ages ranging between 14 to 55 years (25.69 ± 9.50 years). Table 1 summarizes patients’ age and sex as well as their pain level prior to treatment. Statistical tests failed to show any significant difference in
Table 1. Comparison between the groups in terms of age, sex and pain at baseline.

<table>
<thead>
<tr>
<th>Group</th>
<th>Placebo</th>
<th>Acetaminophen</th>
<th>Ibuprofen</th>
<th>Gelofen</th>
<th>Total</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD)</td>
<td>22.80±8.53</td>
<td>24.53±7.44</td>
<td>26.47±10.58</td>
<td>28.80±10.91</td>
<td>25.65±9.50</td>
<td>0.353</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>7</td>
<td>6</td>
<td>10</td>
<td>7</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>8</td>
<td>9</td>
<td>5</td>
<td>8</td>
<td>30</td>
</tr>
<tr>
<td>Pain at baseline (mean±SD)</td>
<td>7.52±2.01</td>
<td>8.18±1.89</td>
<td>6.16±2.40</td>
<td>6.52±1.46</td>
<td>7.09±2.08</td>
<td>0.027*</td>
</tr>
</tbody>
</table>

SD = Standard deviation, *Significant.

**Figure 1.** Comparison between baseline VAS scores and VAS scores at the time of dentin and pulp exposure.

any of these factors between the groups. ANOVA revealed a significant difference in the mean VAS scores at baseline and during access preparation (dentin and pulp exposure) between the groups. However, accordingly, this marked difference was only seen at the time of dentin exposure (P = 0.005) and not pulp exposure (P = 0.076).

In the acetaminophen group, the mean VAS score showed a reduction rate of 70.4 and 54.52% at the time of dentin and pulp exposure, respectively compared with the baseline. In the ibuprofen group, the reduction rate at the time of dentin and pulp exposure was 100 and 82.14%, respectively; and in the gelofen group, this reduction rate was 100% at the time of dentin exposure and 70.39% at the time of pulp exposure. The reduction rate in the placebo group at the time of dentin exposure was 92.95% and at the time of pulp exposure was 55.85% compared to the baseline (Figure 1).

The success rate of anesthesia was defined by complete elimination of pain or slight pain during the endodontic treatment. The success rate among the placebo, acetaminophen, ibuprofen and gelofen groups was 20, 13.33, 66.66, and 46.66%, respectively. Table 2 presents the success and failure rates of IANB at the time of dentin and pulp exposure among the study groups. The mean EPT values increased in all groups after taking the test medications and the injection, however, according to the ANOVA and repeated measure ANOVA, this increase was not deemed significant (P = 0.421) (Figure 2).

**DISCUSSION**

The present study investigated the effect of premedication with 325 mg dose of acetaminophen, 400 mg dose of gelofen and 400 mg dose of ibuprofen in the depth of anesthesia in mandibular molars with irreversible pulpitis. EPT and cold test were used to evaluate the sensitivity of
Table 2. The success and failure rates of IANB at the time of dentin and pulp exposure among the study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Success (%)</th>
<th>Failure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dentin exposure</td>
<td>Pulp exposure</td>
</tr>
<tr>
<td>Placebo</td>
<td>3 (20)</td>
<td>11 (73.33)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>2 (13.33)</td>
<td>8 (53.33)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>10 (66.66)</td>
<td>5 (33.33)</td>
</tr>
<tr>
<td>Gelofen</td>
<td>7 (46.66)</td>
<td>8 (53.33)</td>
</tr>
</tbody>
</table>

Figure 2. Mean EPT values in all groups after taking the test medications and the infiltration.

Belonging to the category of fast-acting analgesics with minimal adverse effects, acetaminophen, gelofen and ibuprofen were chosen to be evaluated in the present study. Researchers have studied the analgesic effects of a wide range of dosages for different medications. Bjornsson et al. (2003a,b) revealed that compared to 500 mg naproxen, premedication with 1000 mg acetaminophen shows a significant reduction in pain up to 1 h post third molar surgical extraction. They further demonstrated that premedication with 600 mg ibuprofen and 1000 mg acetaminophen have similar effects in pain reduction after third molar surgical extraction. Mehlisch (2002) stated that in cases of mild to moderate pain, acetaminophen remains the most appropriate medication of choice. Seymour et al., (1996) studied the efficacy of three doses of ibuprofen (200, 400 and 600 mg) in reducing post surgical pain. Their findings suggested that the 600 mg dose renders more successful pain reduction.

Based on the doses used in the literature and the doses available in the pharmaceutical market in Iran, we provided each patient with one capsule of 325 mg acetaminophen, 400 mg ibuprofen and 400 mg gelofen. Lidocaine 2% with 1:80000 epinephrine is one of the most common local anesthetic agents used in dentistry as well as many other studies (Modaresi et al., 2006; Ianiro et al., 2007; Mikesell et al., 2005; Claffey et al., 2004). Therefore, one cartridge (1.8 ml) of this agent was used. Mikesell et al. (2005) and Claffey et al. (2004) showed no significant difference in the success of IANB using lidocaine and articaine.

In another study by Ianiro et al. (2007), there was no
significant difference in the success of IANB between the group taking 1000 mg acetaminophen and their counterparts taking 1000 mg acetaminophen + 600 mg ibuprofen. Olesen et al. (2010) and Aggarwal et al. (2009) failed to show a significant difference in the success rate of IANB when the anesthesia was accompanied by premedication with two different doses of ibuprofen (600 and 800 mg). Likewise, Simpson et al. (2011) concluded that premedication with 800 mg ibuprofen +1000 mg acetaminophen has no significant effect in the success of IANB in patients with symptomatic irreversible pulpitis. Olesen et al. (2010) further stated that while Ibuprofen can inhibit the production of new prostaglandins, early production of other inflammatory mediators and the effects of pre-activated nociceptors are likely responsible for the high failure rate of anesthesia even after taking the analgesic agent.

On the other hand, Parirokh et al. (2010) and colleagues compared the efficacy of premedication with 600 mg ibuprofen, 75 mg indomethacin and placebo in 150 patients with irreversible pulpitis. Their findings showed that premedication with the test drugs significantly increased the success of IANB compared to the placebo group (P < 0.01). According to Parirokh et al. (2010), the severity of pulpal inflammation at the time of treatment may also account for the differences in the results. In their study, only patients with asymptomatic irreversible pulpitis (patients with delayed response to cold test and absence of spontaneous pain) were included in the study. In the present study, however, the study population consisted of patients with spontaneous pain who were referred to the endodontics clinic.

The mean EPT readings at baseline were 32.87 and 40.46 in the case and control teeth, respectively. These readings increased to 53.22 and 57.37 in teeth with irreversible pulpitis and healthy teeth. These changes may explain why inflammation affects the depth of anesthesia. After taking the placebo agent and the IANB, the mean EPT readings in the control teeth and inflamed teeth was 62.07 and 51.47, respectively. This increase was solely attributed to the anesthetic agent, and the readings in the inflamed teeth marked the failure of local anesthetic agent in inducing a deep anesthesia in the presence of inflammation.

The increase in EPT readings in all groups after taking the premedication is indicative of the effect of premedication in increasing the depth of anesthesia. This increase was observed in all groups including the placebo group, with no significant difference. Modarresi et al. (2006) evaluated the effectiveness of premedication with 400 mg ibuprofen in comparison with acetaminophen codeine (600 mg acetaminophen + 40 mg codeine) 1 h prior to local anesthesia in treating irreversible pulpitis using EPT. They revealed that both medications increased the depth of anesthesia, with no significant difference.

Our findings resembled those of Modarresi et al. (2006). Although the difference between the groups was deemed insignificant, our observations were suggestive of a greater reduction in pain in the ibuprofen and gelofen group. Seven patients in the acetaminophen group, four in the ibuprofen group, seven in the gelofen group, and nine patients in the placebo group received intrapulpal infiltration because of the pain during pulp exposure.

Conclusion

Premedication with ibuprofen 400 mg and gelofen 400 mg have significant effect in the depth of anesthesia in mandibular molars with irreversible pulpitis, and significantly decreased VAS, but placebo and acetaminophen 325 are functionally alike and had no significantly effect.

ABBREVIATIONS

ANOVA, Analysis of variance; EPT, electric pulp test; IANB, inferior alveolar nerve block; PGs, prostaglandins; COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; VAS, visual analogue scale.

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Effects of forsythoside on lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages

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Forsythoside (FS) is an active component of Forsythia suspense and exhibit a certain functions, such as, anti-inflammation, anti-bacterial, anti-virus, among others. However, immunomodulation effects of FS at cell level have been unclear. This work is to investigate the effects of FS on RAW264.7 cells stimulated with lipopolysaccharide (LPS) by detecting the release of cytokines and chemokines, phagocytosis and surface molecule expression. The results showed that the absorbance value of cells in FS groups at medium and high concentration was significantly lower compared with LPS group in terms of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. There was a marked increase in the release of tumor necrosis factor (TNF)-α and decrease NO production in the experimental groups. FS and LPS-treated RAW264.7 cells strongly exhibited phagocytosis capacity to CFSE-labeled chicken red blood cells, and the number of positive cells expressing MHC-II molecules showed a significant decrease in FS together with LPS groups by flow cytometry analysis. The experiment demonstrated that FS has no significant cytotoxicity at the low concentration, markedly promoted the release of TNF-α and down-expressions of MHC-II surface molecules, and enhanced the phagocytosis capacity of macrophages, whereas inhibited NO production, which suggested that FS has cell immune regulation effect.

Key words: Forsythoside (FS), function, RAW264.7 cells, lipopolysaccharide (LPS).

INTRODUCTION

Forsythia suspense has been known as “Lianqiao” (Chinese) and this genus is widely distributed in Asia countries, such as China, Korea and Japan, and its extracts are used as a Chinese traditional medicine to treat inflammation, pyrexia, ulcer, gonorrhea and erysipelas (State Pharmacopeia Commission of P. R. China, 2005). A number of chemical constituents from this plant with diverse structures, including phenylethanoid glycosides, forsythoside, lignans and flavonoids have been reported, and the other reported that three new caffeoyl phenylethanoid glycosides and six known compounds were isolated (Tokar et al., 2004; Fu et al., 2009). At present many researchers are of the opinion that Forsythoside is the active component of pharmacological effects of forsythia suspense and exhibits anti-inflammatory, anti-bacterial, and anti-tumor effects, however, its immunomodulatory role and the mechanism at cellular level is not clear.

Macrophages are important immune cells which are the first line of host defense against bacterial infection and cancer growth, and play essential roles in the initiation, maintenance and resolution of inflammation (Yan et al., 2011). When stimulated by lipopolysaccharide (LPS), a bacterial endotoxin, macrophages produce a number of inflammation-related enzymes, cytokines and chemo-kines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, prostaglandin E2 and nitric oxide (NO), cyclooxygenase-2 and inducible nitric oxide synthase for...
the primary protection of the host (Hu et al., 2008; Tao et al., 2009). These cytokines and chemokines are essential for the inflammatory response to pathogenic germs or toxicants, as well as the elimination of tumor cells (Liew, 2003). However, overproduction of these inflammatory mediators is often associated with numerous diseases, such as rheumatoid arthritis and atherosclerosis (Isomaki, 1997; Libby, 2000). Thus, inhibition of the overproduction of inflammation-related mediators may play a beneficial role in inflammatory diseases. RAW264.7 cells derived from pristane-elicited murine peritoneal macrophages transformed with Abelson leukemia virus and the physiological studies of macrophages have been notably advanced by the availability of cell lines (Raschke, 1978). RAW264.7 cells have been particularly valuable because of their ease of culture, rapid growth rate, and the phenotypic resemblance to primary macrophages (Rouzer et al., 2005).

In the present study, we aimed to investigate the mechanisms underlying the immunoregulatory effects of FS on LPS-stimulated RAW264.7 cells by evaluating the level of NO and TNF-α, the phagocytosis and surface molecule expression.

MATERIALS AND METHODS

Reagents

Forsythoside (FS) was purchased from China institute of Veterinary Drug Control (Beijing, China). RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone Laboratories, Inc. (Logan, Utah, USA). Thiazoyl blue tetrazolium bromide (MTT) and LPS (purified lyophilized powder) from Escherichia coli O111:B4 were purchased from Sigma-Aldrich (St. Louis, USA). Rat monoclonal [NIMR-4] to MHC Class II (FITC) and TNF-α mouse ELISA Kit were purchased from Abcam (Cambridge, UK). CFSE was obtained from eBioscience (San Diego, C.A.).

Macrophage culture

RAW264.7 cells were cultured in 75-cm² plastic flasks and DMEM medium containing heat-inactivated 10% FBS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) at 5% CO₂ and a 37°C humidified atmosphere. Adherent cells were detached by scraping and then cultured in either 96-well plates (2×10⁵ cells/well) or six-well plates (2×10⁶ cells/well) for experiments at 85% confluence.

Assessment of cell viability

RAW264.7 cells were seeded into 96-well plates at a density of 1×10⁵ cells/well and incubated in the presence of LPS (1 μg/ml final concentration) and different concentrations of FS. After incubation for 24 h, 100 μl of MTT (0.5 mg/ml final concentration) was added and incubation was continued for another 4 h. Mitochondrial succinate dehydrogenase in live cells converted MTT into visible formazan crystal during incubation. The formazan crystals were then solubilized in dimethylsulphoxide and the absorbance was measured at 570 nm by using an enzyme linked immune sorbent assay (ELISA) microplate reader. Relative cell viability was calculated and compared with the absorbance of the untreated control group.

Assay of cellular NO production

RAW264.7 cells were cultured in 96-well plates using DMEM medium containing an l μg/ml final concentration of LPS for 24 h and then incubated for another 24 h by adding different concentrations of FS in the media. After incubation, the supernatant medium was collected and 100 μl of cellular supernatant media containing NO₂ (stable oxidation product of NO) was mixed with the same volume of Griess reagent for the incubation of 15 min. The absorbance of the mixture at 550 nm was measured with an ELISA microplate reader. The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in DMEM medium, and the concentrations of nitrite in the supernatant media of treated-cell groups were calculated.

Detection of tumor necrosis factor (TNF)-α release

The production of TNF-α in RAW264.7 cells was detected using ELISA kits following the manufacturer's instructions. RAW264.7 cells were stimulated by a 1 μg/ml final concentration of LPS for 24 h incubation and then treated with different concentrations of FS for another 24 h. After incubation, the supernatant medium was collected and used for the detection of TNF-α. 50 μl of TNF-α standards (prepared for calibration), or the same volume of each group medium, was added to the wells of TNF-α antibody-coated 96-well plates in triplicate. Biotinylated antibody reagent (50 μl) was added and incubated for 3 h at room temperature. The reaction mixture was aspirated and washed using PBS. Streptavidin-horseradish peroxidase conjugate (100 μl) was added and incubated for 30 min at room temperature. After a thorough washing, 100 μl of TMB substrate solution was added and incubated for 30 min at room temperature, and the reaction was stopped by the addition of 50 μl of stop solution and the absorbance was determined at 450 nm using the microplate reader. A TNF-α standard curve was used to quantify the amount of TNF-α released by RAW264.7 cells.

Analysis of phagocytic activity

A single-cell suspension of chicken red blood cells (cRBCs) was obtained freshly and washed three times with PBS. 1×10⁵ cells/ml cRBCs in PBS were labeled with 5.0 μM CFSE for 15 min at 37°C. These cells were then washed thoroughly with in-complete medium and re-suspended in the complete medium. RAW264.7 cells were adjusted to 2×10⁵ cells/well and cultured overnight in 24-well culture plate. Then 100 μl of 2×10⁵/ml CFSE-labeled cRBCs were added in the 24-well culture plate containing RAW264.7 cells and incubated for 4 h at 37°C. Non-phagocytic cells were removed by rinsing with PBS, and phagocytic cells were collected by repeated pipetting, and then flow cytometric analysis was performed on a FACSCalibur.

Evaluation of MHC-II molecule expression

RAW264.7 cells were stimulated by a 1 μg/ml final concentration of LPS for 24 h incubation and then treated with different concentrations of FS for another 24 h. Cells were collected and 5×10⁵ harvested macrophages were washed once with FACS buffer. For one-color staining, cells were stained with fluorescein isothiocyanate (FITC)-labeled mouse MHC-II antibody. Nonspecific FcR binding was blocked by anti-mouse FcR mAb 2.4G2. At least ten thousand cells were assayed using a FACSCalibur flow cytometry, and data
RESULTS

Cell viability of RAW264.7 cells treated with FS

Cell viability was detected by MTT assay and the results are shown in Figure 1. Compared with LPS group, there was significant decrease in cell absorbance value in FS+LPS groups at the 80 and 160 μg/ml of FS, respectively, however there was not marked effect on cell viability in LPS+40 μg/ml FS. The results indicated FS was helpful to cells survival and relieve effects of LPS on RAW264.7 cells at the low concentration.

NO production and TNF-α release from RAW264.7 cells

There was a significant enhancement of the nitrite concentration in RAW264.7 cells stimulated with LPS in conditioned medium compared with that in LPS non-stimulated cells (Figure 2), which indicated that a stable NO$$^2$$ product was released into the culture medium and there was an increased NO production. RAW264.7 cells treated with FS showed a reduction in the production of NO following those stimulated with LPS at the low and medium concentration of FS; however, the level of NO
production was a little more at high dose of FS. The results suggested that FS had a degree inhibitory effect on NO production from the macrophages. The amount of TNF-α released from LPS-treated RAW264.7 cells together with FS was assessed using anti-TNF-α coated ELISA plates to determine the effects of FS. There was a significant increase in TNF-α release of FS and LPS-stimulated cells compared with that of LPS non-treated RAW264.7 cells (Figure 3), which showed FS had the potential to promote the release of TNF-α from LPS-induced RAW264.7 cells.

**Phagocytic ability and MHC-II surface molecule expression in RAW264.7 cells**

To determine the expression level of MHC-II surface molecule in cells treated with FS and LPS, the positive cells were detected using FCM (Figure 4). There was a marked decrease in positive cells treated with LPS together with FS at different concentration compared with the LPS-stimulated cell group, which showed down-expression of MHC-II surface molecules. The results indicated that FS had the potential to inhibit the expression of MHC-II surface molecules. Phagocytic capacity is a very important function for macrophages. In order to analyze phagocytic activity of macrophages treated with FS, RAW264.7 cells stimulated with LPS were cultured in the presence of different concentration of FS. cRBCs were collected and labeled with CFSE. Treated RAW264.7 cells were co-cultured for 2-4 h with CFSE-labeled cRBCs, and phagocytosis was evaluated in term of the uptake of macrophages to CFSE-labeled cRBC using FCM. There was a significant increase in

![Figure 3](image3.png)  
*Figure 3. Effects of FS on TNF-α secretion from RAW264.7 cells. a vs 0 (p<0.01), b vs LPS (p<0.01), c vs LPS (p<0.05), d vs 40 (p<0.01), e vs 80 (p<0.01), f vs 160 (p<0.01).*

![Figure 4](image4.png)  
*Figure 4. Cell surface expression of MHC-II molecules in RAW264.7 cells treated with FS. a vs 0 (p<0.05), b vs LPS (p<0.05), c vs LPS (p<0.01), d vs 40 (p<0.01).*
phagocytes of macrophages cultured in the media containing FS at comparative high concentration, which showed FS improved the phagocytic capacity of macrophages (Figure 5).

DISCUSSION

Macrophages play a crucial role in the inflammatory and immune responses and are major sources of inflammatory mediators, including prostaglandins (PGs), NO and TNF-α. RAW264.7 mouse macrophage cell line has long been used as an in vitro model to study inflammatory molecules synthetic response to a wide variety of stimuli (Shin, 2008; Jeong, 2009). It is known that macrophages produce multiple inflammatory molecules such as NO, TNF-α, IL-1, IL-12, interferon-γ and chemokines in response to the stimulation of some lectins (Kesherwani et al., 2007), such as phytohemagglutinin to stimulate RAW264.7 cells could induce the production of NO (Daekyung et al., 2011). Nitric oxide is a multifunctional biomolecule involved in a variety of physiological and pathological processes, such as vascular regulation, host immune defense, neurotransmission and other systems (Paige, 2007). Vascular dysfunctions are associated with the impaired production of NO, whereas bacterial septic shock, certain inflammatory and autoimmune are associated with NO overproduction (Moncada et al., 1992). NO plays a beneficial role in anti-tumor and anti-virus replication, and in anti-inflammatory processes and the suppression of NO overproduction has become a new therapeutic strategy for the treatment of inflammatory-related diseases (Pacher, 2007). In our study, we found that the exposure of RAW264.7 cells to LPS caused a significant increase in NO production, and FS reduced to a degree NO production in LPS-stimulated RAW264.7 cells, which suggested that FS had the potential of anti-inflammatory action. This observation is consistent with the findings previously reported by other researchers (Hu et al., 2008; Lin et al., 2008).

Cytokines play important roles in the regulation of inflammation. TNF-α, IL-1β and IL-6 are multifunctional pro-inflammatory cytokines and exhibit various pro-inflammatory effects in certain inflammatory diseases, such as rheumatoid arthritis and atherosclerosis (Dayer, 2004). It has been reported that TNF-α elicits downstream pro-inflammatory events, such as the release of IL-6, certain inflammatory cytokines (Straub et al., 2000). TNF-α has long been considered as a key molecule for the induction of apoptosis and the development of the humoral immune response. In this experiment, RAW264.7 cells treated with LPS and FS showed a significant increase in TNF-α release from RAW264.7 cells, which indicated that FS is capable to promote LPS-induced production of TNF-α.

Major histocompatibility (MHC) is a large cluster of genes, which encode antigen presenting molecules and
therefore play a key role in molecular self/non-self discrimination and in the activation of adaptive immune response (Janeway et al., 2005). Its identification is considered as a major breakthrough in the identification as a key component of the adaptive immune system to respond to pathogen infection (Christophe et al., 2011). MHC-II molecules are cell surface glycoproteins that bind self and antigenic peptides and present them to T cells to initiate an immune response (Germain et al., 1993). One of the most important functions for macrophages is to present peptides derived from exogenous antigens to surrounding T cells through their cell-surface interactions and the expression of co-stimulatory molecules in antigen presenting cells (APCs), which is crucial in determining the nature and extent of the immune responses (Ma et al., 2008). Phagocytosis of macrophages is a part of innate immunity for protection against foreign pathogens, microorganism or dead cells (Gordon et al., 2005). Previous reports showed that alternatively activated macrophages have an enhanced phagocytosis ability and a lower antigen presenting ability (Bouhlel et al., 2007). In our experiment, we found that positive cells expressing MHC-II molecules in RAW264.7 cells treated with FS and LPS was significantly lower, and cells stimulated with FS and FS showed significantly higher phagocytosis capacity against RBCs compared with the control, which might be related to the activation stages of macrophages (Liu et al., 2007). These results were consistent with the concept in alternative activated macrophages the antigen presenting ability is reduced (Weigert et al., 2007).

In conclusion, the observed immunoregulatory effects of FS may be mediated partly by the promotion of TNF-α secretion and phagocytotic ability, the inhibition of NO production, down-regulation of MHC-II expression, which suggest that FS, an essential active constituent of forsythia suspens, may be a potential chemical agent for the disease treatment.

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ABBREVIATIONS

LPS, lipopolysaccharide; FS, forsythoside; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; ELISA, enzyme linked immuno sorbent assay; TNF, tumor necrosis factor; RBCs, chicken red blood cells; FITC, fluorescein isothiocyanate; PI, propidium iodide; PGs, prostaglandins; MHC, major histocompatibility; APCs, antigen presenting cells.

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