Evaluation on bioactivities of total flavonoids from Lavandula angustifolia

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Abstract: Lavandula angustifolia was used to treat flus and fevers, joint swelling and pain in Uighur medicine. This study aimed to investigate antioxidant, anti-inflammatory and antalgic activities of total flavonoids from Lavandula angustifolia (LTF). Results indicated that LTF possesses the highest total flavon power. Some flavonoids separated from LTF, and their DPPH scavenging abilities as follows: rosmarinic acid (2, near to Vit C) >luteolin (3) >apigenin (4) >luteolin 7-O-β-D-glucoside (5) >apigenin 7-O-β-D-glucoside (6) >luteolin 7-O-β-D-glucuronide (7). LTF significantly decreased malondialdehyde (MDA) level in D-galactose induced aging model compared to the control group (P<0.05), as well as significantly increased plasma superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities (P<0.05). Moreover, 17.4, 34.8 and 69.6 mg/kg doses of LTF were exhibited significant analgesic and anti-inflammatory activities in a dose dependent manner (P<0.05). Cytotoxicity of LTF on Bel-7402 and Hela cell lines were showed by MTT assay also. These results verified traditional usage of this plant and suggested also that LTF is worth developing and studying further.

Keywords: Lavandula angustifolia, total flavonoids, antioxidant, anti-inflammatory, antalgic, antitumor.

INTRODUCTION

Lavender is a perennial sub-shrub, native to the Mediterranean coast at the southern foot of the Alps, is now cultivated commonly grown around the world (Ibrahim et al., 1999). Lavender is one of the most fashionable aromatic medicinal plants in the world, and its essential oil is widely applied in perfumery and cosmetics industry (Barocellia et al., 2004). The extracts from various Lavandula spp. are traditionally used to treat diseases such as epilepsy, migraine and to reduce spasms in colic pain in folk over the world (Gilani et al., 2000). At present, pharmaceutical research of Lavender mainly focused on essential oil, and its non-essential oil parts have been little bioactive studied though it was extensively studied phytochemically. In addition to volatile compounds, lavender flowers contain also other bioactive compounds such as triterpenes and phenolic substances including cinnamic acid derivatives and flavonoids (Torras-Claveria et al., 2007; Papanov et al., 1992). Polyphenols and flavonoids in general are believed to possess antioxidantive properties, and the activities of these compounds are closely related with their antioxidant properties. Flavonoids have been reported to exert multiple biological effects including anti-inflammatory, antiallergic, antiviral, and anticancer activities (Czaplinska et al., 2012) and are used for treatment of several diseases (Romagnolo et al., 2012).

MATERIALS AND METHODS

Plant materials
Lavandula angustifolia were collected from Ili region of Xinjiang, in China, in May 2011. The plant material was identified by associate researcher Jiang He, Xinjiang Institute of Material Medica. A voucher specimen was deposited at Xinjiang Institute of Material Medica.

Cells and animals
Human hepatoma carcinoma cell lines Bel 7402 and cervical carcinoma cell lines Hela were provided by Institute of Materia Medica, Chinese Academy of Medical Science.
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Sciences, and maintained with RPMI 1640 medium containing 10% fetal bovine serum, 100ng/mL penicillin and streptomycin at 37°C in a humidified atmosphere with 5% CO2. Kunming mice weighing 18±22g were obtained from the Experimental Animal Center in Xinjiang, China. They were allowed free access to food and water under a 12h/12h light/ dark cycle with the room temperature maintained at 27°C and relative humidity of 50-70%. The study protocols were approved by the Ethics Committee on Animal Experiment, Xinjiang Material Medica, China.

Preparation of LTF
Five kilograms of L. angustifolia was extracted with 70% ethanol under reflux at 80°C for 1h in three times, and the solvent was evaporated under vacuum to afford ethanol extract. Ethanol extract was separated and purified by AB-8 resin. After deionized water eluting and cleaning impurities, 50% ethanol eluent was collected to afford total flavonoids (LTF). LTF (10g) was chromatographed over ODS RP-18 with a gradient solvent system of MeOH-H2O (0:1-1:0). Fifty-one fractions were collected after combination by TLC guidance and repeated column chromatography over Sephadex LH-20 (MeOH). Finally, seven compounds were afforded: 1 (7.6mg), 2 (67.0mg), 3 (22.0mg), 4 (13.2mg), 5 (83.0mg), 6 (59.2mg) and 7 (41.0mg), respectively.

Determination of total flavonoids
Total flavonoid content was determined using a method described by Chinese pharmacopeia (ChP., 1999). Briefly, rutin standard solution (0.2-1.2mg/ml) and distilled water was mixed to 6.0 ml in a test tube, followed by addition of 1.0ml of a 5% (w/v) sodium nitrite solution. After 6 min, 1.0ml of a 10% (w/v) aluminium nitrate solution was added and the mixture was allowed to stand for a further 6 min before 10ml of 4%NaOH was added. The mixture was made up to 25.0ml with distilled water. The absorbance was measured at 509 nm after 15 min. Total flavonoid content was calculated as rutin (mg/g) using the following equation based on the calibration curve: y = 0.5251x-0.0223, R²=0.9996, where y was the absorbance and x was the concentration.

Cytotoxicity Assays
Cytotoxicity of LTF on human hepatoma carcinoma cell lines Bel 7402 and cervical carcinoma cell lines Hela were determined using MTT assay (Mosmann T., 1983). Cells were added to 96-well culture plates at a density of 4.5×10⁵ cell mL⁻¹ and incubated at 37ºC for 24 h. Then the culture medium was removed and replaced with fresh medium supplemented with different concentrations of LTF. 20µL MTT reagent (5mg/mL) and 200 µL DMSO was added at 48 h, and plates were oscillated for 10 min in a balance oscillator after 4 h incubation at 37°C. The extent of the MTT reduction was measured by a plate reader at a wavelength of 570 nm. Inhibitory rate of cell proliferation was calculated as follows: inhibitory rate (%) = (1-Aexperimental group/A control group) × 100%.

Anti-oxidant activity
Reducing power
Reducing power of LTF was determined according to the reported method (Zubair et al., 2012). 1.0ml methanol solutions of LTF and Vit C (0.1-0.7mg, respectively) were mixed with 2.5mL phosphate buffer (0.2 M, pH 6.6) and 2.5mL 10mg/ml potassium ferricyanide [K₃Fe(CN)₆]; the mixture was incubated at 50°C for 20 min. 2.5mL 100 mg/ml trichloroacetic acid was added to the mixture, then centrifuged at 3000 rpm for 10min. The upper layer of the solution (2.5mL) was mixed with 2.5ml distilled water and 0.5ml 1.0mg/ml FeCl₃ and the absorbance were measured at 700nm. Vit C was used as positive control. Increased absorbance of the reaction mixture indicated increased reducing power. All the measurements were made in triplicate and the results averaged.

DPPH radical scavenging activity
DPPH free radical scavenging activity was determined according to the previously described procedure (Zhao et al., 2012). Different concentrations of LTF ethanol solutions (0.5ml) were mixed with 2.5ml 6.5×10⁻³ M DPPH, and absorbance was measured at 517 nm after keeping the tubes in dark for 30min. The scavenging activity was determined by comparing the absorbance with that of control containing equal volumes of DPPH solution and ethanol. The percentage inhibition of radicals was calculated using the following formula: inhibition (%)=(Acontrol−Asample)/Acontrol×100. Vit C was used as positive control.

Superoxide radical scavenging assay
Superoxide radical scavenging ability of the extract was determined with the reported methods (Xu et al., 2003). Briefly, the mixture of LTF (0.5mL) and Tris-HCl buffer (4.5mL, 50mM, pH 8.2) were incubated at 25°C for 20 min, and then 0.3mL of pyrogallol solution (80mM, 10 mM HCl) was added. The absorbance at 329nm was recorded immediately and every 30s until the reaction time reached 4min. Vit C was the reference. The percentage inhibition of radicals was obtained as followed: inhibition (%) = [1-(A1/A2)]×100, where A₀ was the absorbance of without LTF, A₁ was the absorbance of with LTF andA₂ was the absorbance of without pyrogallol.

Hydroxyl radical scavenging activity
The hydroxyl radical scavenging ability was determined according to the reported methods (Liu et al., 2009), 1.5mL 1, 10-phenanthroline (1.0mM) and 0.5mL FeSO₄ (2.5mM) were dissolved in phosphate buffer (4.0mL pH 7.4) and mixed thoroughly. A total of 1.0mL H₂O₂ (0.2%) and 2.5mL of various concentrations of LTF were dissolved in ethanol. Subsequently, the volume was adjusted to 10mL. The mixture was incubated for 1 h at 37°C in the dark; the absorbance was measured at 536 nm. The percentage inhibition of radicals was calculated using
the following equation: inhibition (%)=(As-A1)/(A0-A1)×100. As, the absorbance of the sample; A1, the absorbance of control solution containing 2.0mL 1, 10-phenanthroline, FeSO4, and 1.0mL H2O2; A0, the absorbance of blank solution containing 1, 10-phenanthroline and FeSO4.

**Ageing model**
After 1 week of acclimatisation, the mice were randomly divided into five groups for 12 mice per group and i.p. injected with 0.1ml/10g of 5% D-galactose once daily for 6 weeks. LTF treatment group mice were p.o. administered with different dosage (17.4, 34.8 and 69.6 mg/kg). The normal control group mice were i.p. injected with 0.3 ml of physiological saline and p.o. administered with 0.1mL/10g of 20% arabic gum each (Liu et al., 2003).

**Anti-inflammatory and analgesic activity**

**Acetic acid-induced writhing response in mice**
The study was carried out as described by reported methods (Muhammad et al., 2012). Fifty male mice were used and randomly divided into four groups including normal control group (5% tween-80), LTF groups (17.4, 34.8 or 69.6mg/kg body wt). Each mouse was given an injection of 0.7% acetic acid aqueous solution in a volume of 0.1ml/10g body weight into the peritoneal cavity, and then was placed in a transparent plastic box. The number of writhes was counted for 15min beginning from 5min after the acetic acid injection. Test drugs and control vehicle were administered 1h before acetic acid injection. Percentage of inhibition of writhing response was calculated.

**Xylene-induced ear edema in mice**
Anti-acute inflammatory activity was determined by xylene-induced mice ear edema (Hossein et al., 2000). Fifty mice were equally divided into four groups including normal control group (5% tween-80), LTF groups (17.4, 34.8 or 69.6mg/kg body wt). The vehicle and LTF were administered orally once per day for 3 days, respectively. One and half-hour after the last administration of LTF, inflammatory response was induced on the inner and external surface of the right ear (surface: about 1cm²) by application of 20μl xylene. 30 min later, mice were sacrificed by cervical dislocation and a section (Ø 6 mm) of ears were removed from both the treated (right) and the untreated (left) ears. Edema rate was measured as percentage of the weight difference between the two ear discs compared to the untreated (left) ears. The anti-inflammatory activity was expressed as percentage of inhibition in treated mice compared to the normal control mice.

**STATISTICAL ANALYSIS**
The data obtained were computed using SPSS 13.0 software and later analyzed using ANOVA of variance. The Duncan test with significance level of 0.05 between means was used.

**RESULTS**

**Phytochemical analysis**
Flavonoids were the major characteristic components in LTF whose content amounted to 53.01%. Seven flavonoids were isolated from LTF and elucidated as caffeic acid (1), rosmarinic acid (2), luteolin (3), apigenin (4), luteolin 7-O-β-D-glucoside (5), apigenin 7-O-β-D-glucoside (6), luteolin 7-O-β-D-glucuronicide (7) by various spectroscopic methods including NMR experiments and physicochemical characteristic as well as by comparison of the data with literature values. The structure of the compounds 1-7 from LTF are shown in fig. 1.
Reducing power
As an indicator of electron-donating activity, Fe^{3+} reduction is closely related with antioxidant properties and often used in research of antioxidant mechanism. Fig. 2 shows the dose-response curves of LTF. The reducing power of LTF increased from 0.340±0.072 at 0.1mg/ml to 1.319±0.061 at 0.7mg/ml.

DPPH radical scavenging activity
Fig.3 shows the DPPH free radical scavenging activities of LTF and its IC_{50} value was 6.48mg/ml. Furthermore, some compounds from LTF also showed stronger antioxidant activities, and their orders as follows: Vit C (IC_{50}, 1.62µg/ml) > rosmarinic acid (2; IC_{50}, 7.66µg/ml) > luteolin (3; IC_{50}, 14.62µg/ml) > apigenin (4; IC_{50}, 34.88 µg/ml) > luteolin 7-O-β-D-glucoside (5; IC_{50}, 64.04 µg/ml) > luteolin 7-O-β-D-glucuronide (7; IC_{50}, 71.4µg/ml) > apigenin 7-O-β-D-glucoside (6; IC_{50}, 103.42µg/ml).

Scavenging activities on superoxide anion and hydroxyl radicals
The scavenging effects of LTF on superoxide anion radicals and hydroxyl radicals are shown in fig. 4. LTF showed lower activity on superoxide anion and hydroxyl radicals, and its scavenging activity on two radicals were 33.53±1.08% at 0.14mg/ml and 49.15±1.54% at 0.5 mg/ml respectively.

Cytotoxicity activity
Human hepatoma carcinoma cell lines Bel 7402 and cervical carcinoma cell lines Hela were used to test effect of LTF on cell proliferation. LTF showed significant inhibitory effects on these two cell lines in a dose-dependent manner. LTF at the dose of 10mg/mL showed 80.68% and 71.64% inhibition of the growth of Bel-7402 and Hela cells, respectively (fig. 5).

Antioxidative effects for ageing mice
The results of antioxidant enzyme (SOD and GSH-Px) activities and level of MDA in serum are listed in fig. 6. After treatments, different dose group of LTF (17.4, 34.8 and 69.6mg/kg) inhibited significantly the formation of
MDA in serum and markedly raised the activities of SOD and GSH-Px in a dose-dependent manner ($P<0.05$).

**Fig. 6**: Antioxidant effect of LFT on MDA, SOD and GSH-Px in liver in D-galactose induced aging mice. Values are the mean ± S.D. **$P<0.01$, compared with normal group; $^*P<0.05$, **$P<0.01$, compared with model group.

**Anti-inflammatory and analgesic activities**

The oedema inhibitory rates of LTF were 13.68%, 18.09% and 21.65% at doses of 17.4, 34.8 and 69.6 mg/kg, respectively. Peripheral analgesic activity was assessed by acetic acid-induced writhing test, which showed significant ($P<0.01$ and $P<0.05$) suppression of writhes (table 1). The oral administration of LTF induced a dose dependent analgesic activity and the values ± SD for the extract are shown in table 1. Injection of acetic acid into the control mice resulted in 70.2±16.2 writhes. Pretreatment with methanol extract of LTF at doses of 17.4, 34.8 and 69.6 mg/kg reduced the number of writhes to 23.8±15.7 (40.99% inhibition), 22.8±13.1 (43.27% inhibition) and 14.3±12.3 (64.39% inhibition) respectively.

**DISCUSSION**

In this study, total flavonoids from *L. angustifolia* (LTF) were enriched and purified by AB-8 macro porous resins, and the content of flavonoids in LTF is 53.01%. Furthermore, we evaluated on bioactivities of LTF by determination of radicals scavenging, xylene induced ear edema and acetic acid-induced writhing test as well as MTT assay.

Reduction of DPPH radical was determined by the decrease of its absorbance at 517nm induced by antioxidants. DPPH reacts with antioxidants and gets converted into 1, 1-diphenyl-2-picrylhydrazine because of rapid hydrogen accepting ability and hence a quick decrease in absorbance is showed (Kai et al., 2007). The degree of decrease in absorbance indicates the scavenging potential of the anti-oxidant extract (Philip et al., 2004). LTF and its compounds (rosmarinic acid, luteolin, apigenin, luteolin 7-O-β-D-glucoside, apigenin 7-O-β-D-glucoside and luteolin 7-O-β-D-glucuronide) all show better DPPH radicals scavenging activities, of which IC$_{50}$ value of LTF was 6.48mg/ml, and IC$_{50}$ value of rosmarinic acid was 7.66µg/ml. However, LTF exhibited weak scavenging effect on superoxide anion and hydroxyl radicals compared to reference. The antioxidant system in the body comprises several important enzymes such as SOD and GSH-Px (Kasapoglu et al., 2001; Warner et al., 1996). Subacute mice ageing were induced by injection of D-galactose (Ke et al., 2009), and the biological redox substance in mice can be disturbed by long-term injection of D-galactose, such as decreasing of SOD and GSH-Px activities. LTF can inhibit significantly the formation of MDA in serum and markedly raise the activities of SOD and GSH-Px in a dose-dependent manner.

One of central feature of inflammation is the activation of macrophage cells that synthesize and release large amounts of reactive oxygen species (ROS) causing cell and tissue injury (Ansari et al., 1996; Uma et al., 2008). ROS, such as superoxide, hydrogen peroxide and hydroxyl radical, are important mediators that provoke or sustain inflammatory processes. Therefore the usage of antioxidants and radical scavengers can attenuate inflammation, and the importance of antioxidant enzymes is generally emphasized in the prevention of oxidative stresses (Vendemiale et al., 1999). Anti-inflammatory effect of LTF was evaluated by xylene induced ear edema. This model can reflect the oedematization during the early stages of acute inflammation, which was probably related with the release and inhibition of the inflammation factors (Okoli et al., 2004). The oral administration of LTF suppressed significantly ear oedema in mice.

**CONCLUSION**

In summary, our results suggest that LTF shows antioxidant, anti-tumor, anti-inflammatory and analgesic activities. The results may provide preliminary scientific evidence to support the folk medicinal utilization of *L. angustifolia* and pharmacodynamics in-depth shall be further studied.
**Table 1:** Anti-inflammatory and analgesic activity of LTF in the different tests

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>Xylene-induced ear edema</th>
<th>Acetic acid-induced writhing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Auricular swelling (mg)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Model</td>
<td>-</td>
<td>70.2±16.2</td>
<td>13.68</td>
</tr>
<tr>
<td>LTF</td>
<td>17.4</td>
<td>60.6±24.7</td>
<td>13.68</td>
</tr>
<tr>
<td></td>
<td>34.8</td>
<td>57.5±25.3</td>
<td>18.09</td>
</tr>
<tr>
<td></td>
<td>69.6</td>
<td>55.0±11.2*</td>
<td>21.65</td>
</tr>
</tbody>
</table>

*P<0.05, compared with model group.

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**REFERENCES**


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