Anti-Inflammatory Activity and Quantitative Analysis of Major Compounds of the Mixtures of *Derris scandens* (DZSS) Formula

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### ABSTRACT

**Background:** The mixtures of *Derris scandens* (DZSS) formula is a Thai traditional medicine, which consists of 4 medicinal plants, including *Derris scandens* (Roxb.) Benth. (D), *Zingiber cassumunar* Roxb. (D), *Suregada multiflora* Baill. (Z), *Siphonodon celastrineus* (S), *Siphonodon celastrineus* (S), *S. multiflora* (Benth). (Fam.Zingiberaceae) (Z), *S. multiflora* (Benth). (Fam.Zingiberaceae) (Z), *Celastraceae* (S), and *Celastraceae* (S). According to the Thai National List of Essential Herbal Medicine (2013), there are two traditional formulas of DZSS formula, DZSS1 and DZSS2, which contains a different amount of *Zingiber cassumunar* Roxb. rhizome. The ratios of 4 herbs (D:Z:S:S) in the DZSS1 and DZSS2 formulas are 1:1:1:1 and 1:2:1:1, respectively. The DZSS formula has been traditionally used for relieving muscle pain. The formula is given orally at the dosage of 900-1,500 mg three times a day immediately after a meal. This medicinal herb formula is contraindicated in pregnant women and should be carefully used in patients with peptic ulcer since *D. scandens* showed a similar mechanism of anti-inflammation as that of non-steroidal anti-inflammatory drugs (NSAIDs) with an inhibition against prostaglandin production. Gastrointestinal side effects are thus the major disadvantage of the oral DZSS formula. It is therefore interesting to develop the DZSS formula in a topical dosage form.

**Materials and Methods:** The ethanolic extracts of the formula (50% and 95% ethanolic extract) were prepared by using soxhlet extraction and which were analyzed by using HPLC. The anti-inflammatory activity of the DZSS formula was tested for its inhibitory effect against nitric oxide (NO) production in Raw 264.7 cells macrophage. The cytotoxic effect of the formula was determined by using the MTT assay. **Results:** The 95% ethanolic extract of the DZSS formula exhibited a pronounced anti-inflammatory activity with the IC₅₀ of 40.08 ± 2.78 μg/mL. The 95% ethanolic extract possessed a more potent anti-inflammatory activity than that of the 50% ethanolic extract and with no cytotoxicity. HPLC analysis indicated that the 95% ethanolic extract also had a higher yield of genistein and compound D, which are obtained the amount of 0.71 ± 0.00 and 18.89 ± 0.24 mg/g extract, respectively. **Conclusion:** Our data suggest that the 95% ethanolic extract of the DZSS formula possessed a significant anti-inflammatory activity but which is still required to investigate about biological activity *in vivo* and clinical study.

**Key words:** Anti-inflammatory activity, Genistein, Compound D, HPLC, Nitric oxide (NO), *Derris scandens*.

**INTRODUCTION**

The mixtures of *Derris scandens* (DZSS) formula is a Thai traditional medicine, which consists of *Derris scandens* (Roxb.) Benth. (Fam.Leguminosae-Papilioideae) (D), *Zingiber cassumunar* Roxb. (Fam.Zingiberaceae) (Z), *Suregada multiflora* Baill. (Fam.Euphorbiaceae) (S) and *Siphonodon celastrineus* (Fam.Celastraceae) (S). According to the Thai National List of Essential Herbal Medicine (2013), there are two traditional formulas of DZSS formula, DZSS1 and DZSS2, which contains a different amount of *Zingiber cassumunar* Roxb. rhizome. The ratios of 4 herbs (D:Z:S:S) in the DZSS1 and DZSS2 formulas are 1:1:1:1 and 1:2:1:1, respectively. The DZSS formula has been traditionally used for relieving muscle pain. The formula is given orally at the dosage of 900-1,500 mg three times a day immediately after a meal. This medicinal herb formula is contraindicated in pregnant women and should be carefully used in patients with peptic ulcer since *D. scandens* showed a similar mechanism of anti-inflammation as that of non-steroidal anti-inflammatory drugs (NSAIDs) with an inhibition against prostaglandin production. Gastrointestinal side effects are thus the major disadvantage of the oral DZSS formula. It is therefore interesting to develop the DZSS formula in a topical dosage form.

Previously literature reviewed on the subject of single ingredients of the DZSS formula indicated the presence of coumarins, isoflavones, flavones, isoflavone glycosides and phenyl coumarins as chemical constituents from *D. scandens*. D. scandens, has been used in Ayurvedic, Thai and Chinese herbal medicine to treat a variety of pain-related conditions, including muscle pain, joint pain, arthritis and headaches. Dalpanitin and viccinin-3, two of the flavonoids isolated from *D. scandens* gave MICs of 23 μg mL⁻¹ against S. aureus. Dalpanitin also exhibited relevant MICs on Gram-negative bacteria (94 μg mL⁻¹ against Escherichia coli and Pseudomonas aeruginosa). For the Thai traditional medicine, volatile oil from *Z. cassumunar* has been used directly apply and penetrate on the skin for remedied for muscle stress and joint pain. Four phenylbutanoids including (E)-(4(3,4′-dimethoxyphenyl)but-3-en-1-ol (compound D), (E)-(3,4′-dimethoxyphenyl)but-3-en-1-yl acetate (D-acetate), (E)-(3,4′-dimethoxyphenyl)butadiene (DMBPD) and (E)-(3,4′-dimethoxyphenyl)-4-[(E)-3′,4′-dimethoxystyrlyl]cyclohex-1-en (DMPDMS), which were isolated from *Z. cassumunar* extract. The stem of *S. multiflora* and *S. celastrineus* have been used for treatment of skin disease. The bark of *S. multiflora* found chemical constituents such as two ent-kaurene diterpenes, ent-16-kaurene-3β,15β,18-triol and ent-3-oxo-16-kaurene-15β,18-.
diol and five diterpenes: 16-kaurene-3β,15β-diol, abbeokutone, helioscopinolide A, helioscopinolide C and helioscopinolide I. Triterpene and pristimerin including α-oleanane-triterpene, 3β-acetoxy-11α-benzoyloxy-13β-hydroxyolean-12-one (1), was isolated from the root bark of S. celastrineus. Moreover, ten poly-O-acetylated β-dihydroagarofuran sesquiterpenoids, siphonagarofurans A-J, were obtained from the fruits of S. celastrineus. However, the pharmacological activity of the DZSS formula extract has not yet been reported. In the modern scientific literatures, several phytochemical constituents in the formula have been reported to possess the anti-inflammatory activity including genistein, genistein 7-O-α-rhamnosyl (1→3) β-D-glucopyranoside from Z. cassumunar, compound D or (E)-4′(3,4′-dimethoxyphenyl)but-3-en-1-ol (Figure 1) and DMPBD or (E)-1-(3,4-dihydroxyphenyl) butadiene from Z. cassumunar and helioscopinolide A from S. multiflora. The 50% ethanol and water extract of D. scandens at dose 0.5 mg and 2 mg/kg significantly reduced rat's ear edema and the highest dose demonstrated the best reduction of inflammation. DMPBD has been reported to possess the anti-inflammatory activity through the inhibition of cyclooxygenase (CO) and lipoxygenase (LO) pathway and analgesic action. The extract of S. multiflora possessed potent NO inhibitory effect with an IC50 value of 8.6 μg/ml. Among the isolated compounds, helioscopinolide A exhibited the highest activity against NO release with an IC50 value of 9.1 μM, followed by helioscopinolide C and suremulol D with IC50 values of 24.5 and 29.3 μM, respectively. Interesting, DZSS formula has been used in Thai herbal medicine to treat for muscle pain. Although the anti-inflammatory activity of each medicinal plants consisted in the DZSS formula have been reported earlier, the phytochemical profiles and the pharmacological activities of the combined DZSS formula have not been clearly investigated.

In the present study, we aimed to investigate the phytochemical profiles, the anti-inflammatory activity and the cytotoxic effect of the DZSS formula extract. The results from the study are applicable for topically products development of the DZSS formula for relieving of muscle pain with less gastrointestinal side effects.

MATERIALS AND METHODS

Chemicals

All solutions were prepared with analytical grade chemicals. Acetonitrile and methanol used were HPLC grade from Carlo Erba (Italy). The standards of Compound D were separated and purified by Assistant Professor Somsak Nualkaew, Faculty of Pharmacy, Mahasarakham University and genistein (Figure 1) was purchased from Sigma-Aldrich (USA).

Plant materials

The crude drug of four medicinal plants in the DZSS formula were prepared as follows. The dried stem of Derris scandens (D), rhizome of Zingiber cassumunar (Z) and stems of Siregaida multiflora (S) and Siphonodon celastrineus (S) were purchased from the local herb stores in December 2018. The morphological characters of each crude drug were in consistent with the description of Thai Herbal Pharmacopoeia 2019, Vol I and identified by Assistant Professor Sombat Appamaraka, Walairukhave Botanical Research Institute, Mahasarakham University. The dried samples were cleaned, sliced into small pieces (5 cm) and dried in a hot air oven at 60°C. The dried pieces were pulverized and passed through a sieve No. 60. The dried powders were used for the following experiments.

Preparation of the DZSS formula extracts

Four hundred grams of the dried powder of D:Z:S:S (1:2:1:1) was extracted with 50% and 95% ethanol by using soxhlet extraction for 24 hrs. The filtrate was combined and subsequently dried by using rotary evaporator.

Assay for inhibitory effect on nitric oxide (NO) production

Cell culture

The Raw 264.7 cells (ATCC® TIB-71™) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin.

Determination of NO production

Inhibitory effect on NO production in macrophage-like Raw 264.7 cells was evaluated by using the method of Sunita et al., 2012 with some modifications. The cell suspension (1 × 105 cells/well, 100 µL) were pipetted into each well of 96-well cell culture plate and incubated in the CO2 incubator for 24 hrs. After that the medium was replaced with the fresh medium containing 10 µg/mL of lipopolysaccharide (LPS) with the test sample at various concentrations (100, 50, 10 and 1 µg/mL) and incubated for further 24 hrs. For the negative control, the cells were treated with LPS and DMSO (vehicle used) instead of the test sample. NO production was indicated by the levels of nitrite accumulation in the culture supernatant, which is determined by using the Griess reagent. The absorbance of the NO products was measured at the wavelength of 520 nm by using UV-Vis microplate reader (Model 680 Reader). The experiment was carried out in triplicate.

Cell viability

Cytotoxicity was determined by using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay Briefly, after 24-hour incubation with the test samples, 10 µL of MTT solution (5 mg/mL) was added into each well. After 2-hour incubation, the medium was removed and 10 µg/mL of isopropanol was added to dissolve the formazan products in the cell. The absorbance was measured at the wavelength of 570 nm by using the UV-Vis microplate reader. The cell viability was indicated by the absorbance values obtained from the MTT assay.

Quantitative HPLC of compound D

Compound D (1.0 mg) was exactly weighed and dissolved in 5 mL methanol to give serial concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL. Three injections were performed for each dilution. The Compound D concentrations in the samples were calculated according to the regression parameters derived from the standard curves. The HPLC system consisted of a column (Phenomenex C18 (250 x 4.6 mm, 5 µm) with acetonitrile (A)/1% acetic acid in water (v/v) (B) in the gradient, flow rate 1.0 mL/min; detection at 254 nm. Compound D: y = 39311x + 4696.8, R2 = 0.9999.

Quantitative HPLC of genistein

Genistein (1.0 mg) was exactly weighed and dissolved in 5 mL methanol to give serial concentrations of 100, 50, 25, 12.5 and 6.25 µg/mL.
Three injections were performed for each dilution. The genistein concentrations in the samples were calculated according to the regression parameters derived from the standard curves. The HPLC condition was similar to that of compound D analysis, genistein: $y = 55737x + 195078, R^2 = 0.9960$.

**RESULTS**

**Determination of nitric oxide (NO)**

The anti-inflammatory activity of the DZSS formula extracts was initially screened at two different concentrations (50 and 100 µg/mL) and expressed as % inhibition against NO production. At the concentrations of 100 and 50 µg/mL, the 95% ethanolic extract of DZSS formula had a higher anti-inflammatory activity than the 50% ethanolic extract with % inhibition of 90.42 ± 2.64 and 50.80 ± 2.38, respectively ($p$-value < 0.05). (Table 1). The cell viability of macrophages exposed to both of the DZSS formula extracts were also determined. The DZSS formula extracts at every concentration tested exhibited the anti-inflammatory activity with no cytotoxicity detected (Table 1). The anti-inflammatory activity of the 95% ethanolic extract was subsequently evaluated and compared with genistein. The anti-inflammatory activity of genistein was more potent than that of the 95% ethanolic extract of DZSS formula with the IC$_{50}$ at 23.48 ± 2.34 µg/mL and 40.08 ± 2.78 µg/mL, respectively ($p$<0.05) (Table 2).

**HPLC quantitative analysis of chemical markers in the DZSS formula extracts**

Genistein and compound D were used as the chemical markers for phytochemical analysis of the DZSS formula extracts. The high yield of genistein and compound D were obtained from the 95% ethanolic extract of DZSS formula. The amount of genistein and compound D obtained were 0.71 ± 0.00 mg/gram extract (0.07 ± 0.00%) and 18.89 ± 0.24 mg/gram extract (1.89 ± 0.22%), respectively (Table 3 and Figure 2).

**DISCUSSION**

Thai medicinal plants are used for the treatment of a wide variety of diseases according to the theory of TTM which includes the symptoms and causes of diseases as well as the taste of medicinal plants. The single plants as well as their combination were used for the treatment of different diseases by traditional physicians. The mixtures of *Derris scandens* (DZSS) formula is a Thai traditional medicine, which consists of four medicinal plants, including *Derris scandens* (Roxb.) Benth. (D), *Zingiber cassumunar* Roxb. (Z), *Suregada multiflora* Bk. (S) and *Siphonodon celastrineus* (S). However, the phytochemical profiles and the anti-inflammatory activity of the DZSS formula have not been clearly studied. The anti-inflammatory activity of the DZSS formula extract was investigated in accordance with its ethnobotanical use for the treatment of muscle aches and pain. The inhibitory effect against nitric oxide (NO) production in macrophages were used to investigate the anti-inflammatory activity since NO plays an important role in mediating many aspects of inflammatory responses. HPLC equipped with UV-Visible detector is the most common tool to qualitative and quantitative analysis of formula because of its precision and accuracy. In this study, the 95% ethanolic extract of DZSS formula exhibited a higher anti-inflammatory action than its 50% ethanolic counterpart with the % maximal inhibition of 90.42 ± 2.46% found at the concentration of 100 µg/mL. Additionally, the 95% ethanolic extract possessed no cytotoxicity at every concentration tested. The 95% ethanolic extract of DZSS formula had anti-inflammatory activity with IC$_{50}$ of 40.08 ± 2.78 µg/mL. The anti-inflammatory activity of the formula is evidently linked with the contents of the phytochemicals, genistein and compound D, found in the DZSS formula extracts. Although, the DZSS formula is a lack of quality control and the absence of pharmacological activity. The results clearly showed that the 95% ethanolic extract of DZSS formula exhibited anti-inflammatory activity in vitro. The chemical markers of the 95% ethanolic extract of DZSS formula were determined, which found that the high content of compound D were obtain 18.89 ± 0.24 mg/g extract (1.89 ± 0.22%) as follow in HPLC chromatogram.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentrations (µg/mL)</th>
<th>% inhibition of NO</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>% cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% ethanol</td>
<td>1</td>
<td>5.25 ± 2.49</td>
<td></td>
<td>98.31 ± 5.64</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>14.98 ± 3.48</td>
<td></td>
<td>100.64 ± 4.29</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>57.21 ± 2.33</td>
<td>40.08 ± 2.78</td>
<td>101.31 ± 8.29</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90.64 ± 1.33</td>
<td></td>
<td>103.47 ± 8.16</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8.32 ± 0.77</td>
<td></td>
<td>104.90 ± 6.83</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>26.95 ± 2.97</td>
<td></td>
<td>105.46 ± 5.22</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>52.05 ± 3.13</td>
<td>23.48 ± 2.34*</td>
<td>108.80 ± 8.27</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>71.87 ± 1.51</td>
<td></td>
<td>105.64 ± 5.99</td>
</tr>
</tbody>
</table>

*p<0.05 significant as compared between two groups using t-test.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Contents (mg/g extract, mean ± SD)</th>
<th>Contents (%w/w, mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>genistein</td>
<td>0.03 ± 0.03</td>
<td>0.003 ± 0.00</td>
</tr>
<tr>
<td>compound D</td>
<td>13.16 ± 1.32</td>
<td>1.32 ± 0.02</td>
</tr>
<tr>
<td>genistein</td>
<td>0.71 ± 0.00</td>
<td>0.070 ± 0.00</td>
</tr>
<tr>
<td>compound D</td>
<td>18.89 ± 0.24</td>
<td>1.89 ± 0.02</td>
</tr>
</tbody>
</table>

Table 1: Screenings of the anti-inflammatory activity and cell viability of the ethanolic extracts of DZSS formula (n=3).

Table 2: Inhibitory effect against LPS-induced NO production of 95% ethanolic extract of DZSS and genistein (n=3).

Table 3: The content of chemical markers in the DZSS formula extracts (n=3).
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Figure 2: HPLC chromatogram of genistein and compound D of the ethanolic DZSS formula extracts (A) genistein, (B) compound D, (C) 50% ethanol, (D) 95% ethanol. Experiment condition: Phenomenex C18 column (250 x 4.6 mm, 5 µm), mobile phase (acetonitrile:1% acetic acid in water (v/v)), 1.0 mL/min; detection wave 254 nm.
Our data suggest that the chemical markers, genistein and compound D were obtained in the 95% ethanolic extract of the DZSS formula that possessed a significant anti-inflammatory activity. The present study may support the traditional use of DZSS formula for treatment of muscle aches and pain. However, further in vivo and clinical experiments are still required.

CONCLUSION

Our data suggest that the chemical markers, genistein and compound D were obtained in the 95% ethanolic extract of the DZSS formula that possessed a significant anti-inflammatory activity. The present study may support the traditional use of DZSS formula for treatment of muscle aches and pain. However, further in vivo and clinical experiments are still required.

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GRAPHICAL ABSTRACT

The DZSS formula

Anti-inflammatory activity

% inhibition of NO
cytotoxicity

HPLC analysis

genistein

compound D

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