

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. (Z), *Suregada multiflora* Baill. (S) and *Siphonodon celsastrineus* (S). The DZSS formula has been used in an oral dosage form for the treatment of muscle pain. However, the phytochemical profiles and the pharmacological activities of the combined DZSS formula have not been clearly investigated. **Objective:** This study aimed to investigate the phytochemical profiles and the anti-inflammatory activity of the DZSS formula. **Materials and Methods:** The ethanolic extracts of the formula (50% and 95% ethanolic extracts) 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 celsastrineus* (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.^{1,2} 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.^{1,2} 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*.³⁻⁵ *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 vicenin-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*).⁶ 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.⁷ 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)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) and (E)-3-(3',4'-dimethoxyphenyl)-4-[(E)-3',4'-dimethoxystyryl]cyclohex1-en (DMPDMS) that isolated from *Z. cassumunar* extract.^{8,9} The stem of *S. multiflora* and *S. celsastrineus* 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-

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diol and five diterpenes: *ent*-16-kaurene-3 β ,15 β -diol, abbeokutone, helioscopinolide A, helioscopinolide C and helioscopinolide I.¹⁰ Triterpene and pristimerin including a oleanane-triterpene, 3 β -acetoxy-11 α -benzoyloxy-13 β -hydroxyolean-12-one (1), was isolated from the root bark of *S. celastrineus*. Moreover, ten poly-*O*-acylated β -dihydroagarofuran sesquiterpenoids, siphonagarofurans A-J, were obtained from the fruits of *S. celastrineus*.¹¹ 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 \rightarrow 6) β -glucopyranoside from *D. scandens*, compound D or (E)-4(3',4'-dimethoxyphenyl)but-3-en-1-ol (Figure 1) and DMPBD or (E)-1-(3,4-dimethoxyphenyl) butadiene from *Z. cassumunar* and helioscopinolide A from *S. multiflora*.^{3,9,12} The 50% ethanol and water extract of *D. scandens* at dose 0.5, 1 and 2 mg/ear significantly reduced rat's ear edema and the highest dose demonstrated the best reduction of inflammation.¹³ DMPBD has been reported about the anti-inflammatory activity through the inhibition of cyclooxygenase (CO) and lipoxygenase (LO) pathway and analgesic action.⁷ The extract of *S. multiflora* possessed potent NO inhibitory effect with an IC₅₀ value of 8.6 μ g/ml. Among the isolated compounds, helioscopinolide A exhibited the highest activity against NO release with an IC₅₀ value of 9.1 μ M, followed by helioscopinolide C and suremulol D with IC₅₀ values of 24.5 and 29.3 μ M, respectively.¹² 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 *Suregada 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 I¹⁴ and identified by Assistant Professor Sombat Appamaraka,

Walairukhavej 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.*, 2012¹⁵ with some modifications. The cell suspension (1×10^5 cells/well, 100 μ L) 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 μ g/mL of lipopolysaccharide (LPS) with the test sample at various concentrations (100, 50, 10 and 1 μ g/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

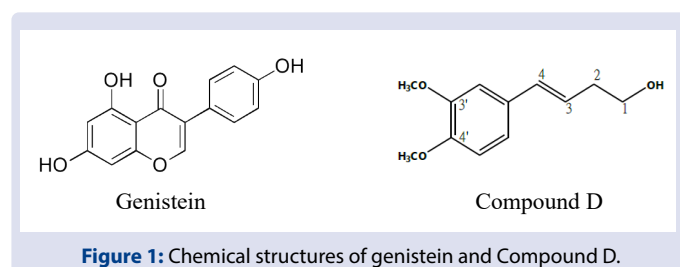
Cytotoxicity was determined by using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay¹⁵ Briefly, after 24-hour incubation with the test samples, 10 μ L of MTT solution (5 mg/mL) was added into each well. After 2-hour incubation, the medium was removed and 10 μ g/mL of isopropanol 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^{9,16}

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 μ g/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 coupled with a quaternary pump, an injector with a 20 μ L loop, an autosampler, a degasser, an automatic thermostatic column compartment, and a DAD-UV detector from Hitachi (Japan) was used. The HPLC system consisted of a column (Phenomenex C18 (250 x 4.6 mm, 5 μ m) 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: $y = 39311x + 4696.8$, $R^2 = 0.9999$.

Quantitative HPLC of genistein¹⁴

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 μ g/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 $\mu\text{g}/\text{mL}$) and expressed as % inhibition against NO production. At the concentrations of 100 and 50 $\mu\text{g}/\text{mL}$, 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 $\mu\text{g}/\text{mL}$ and 40.08 ± 2.78 $\mu\text{g}/\text{mL}$, 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 ($0.07 \pm 0.00\%$) and 18.89 ± 0.24 mg/gram extract ($1.89 \pm 0.22\%$), 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.¹⁷ 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* Baill. (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.¹⁸ HPLC equipped with UV-Visible detector is the most common tool to qualitative and quantitative analysis of formula because of its precision and accuracy.¹⁹ 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 $90.42 \pm 2.46\%$ found at the concentration of 100 $\mu\text{g}/\text{mL}$. 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 $\mu\text{g}/\text{mL}$. 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/gram extract ($1.89 \pm 0.22\%$) as follow in HPLC chromatogram

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

DZSS extract	Concentrations ($\mu\text{g}/\text{mL}$)	% inhibition of NO	% cell viability
50% ethanol	100	56.64 ± 2.33^a	95.56 ± 15.78
	50	38.58 ± 6.61^b	105.37 ± 12.04
95% ethanol	100	$90.42 \pm 2.46^{a*}$	113.10 ± 11.88
	50	50.80 ± 2.38^b	121.27 ± 5.53

*a, b compared to between two concentrations of DZSS extract using t -test ($p < 0.05$)

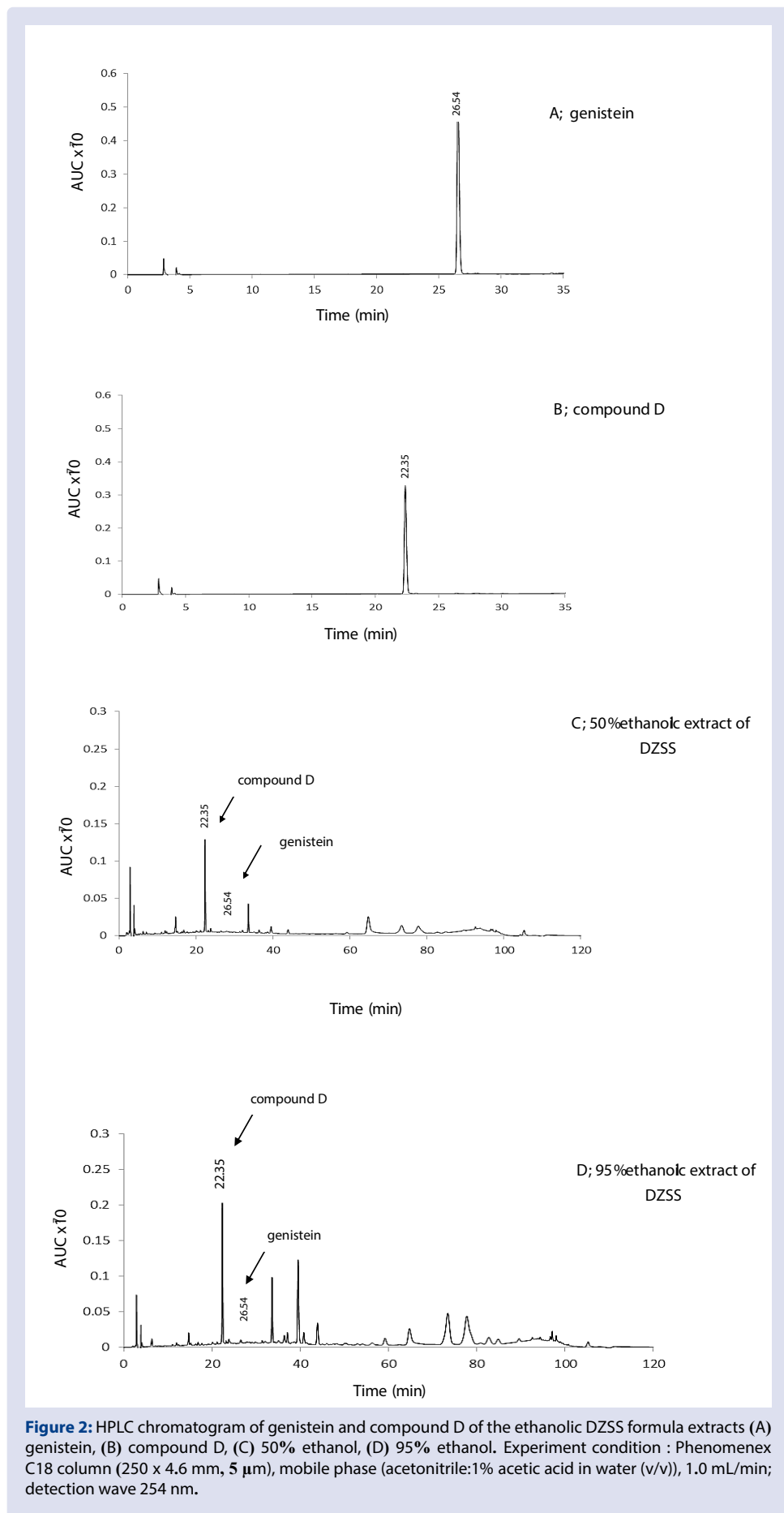
Table 2: Inhibitory effect against LPS-induced NO production of 95% ethanolic extract of DZSS and genistein (n=3).

Samples	Concentrations ($\mu\text{g}/\text{mL}$)	% inhibition of NO	IC_{50} ($\mu\text{g}/\text{mL}$)	% cell viability
95% ethanol	1	5.25 ± 2.49	40.08 ± 2.78	98.31 ± 5.64
	10	14.98 ± 3.48		100.64 ± 4.29
	50	57.21 ± 2.33		101.31 ± 8.29
	100	90.64 ± 1.33		103.47 ± 8.16
Genistein	1	8.32 ± 0.77	$23.48 \pm 2.34^*$	104.90 ± 6.83
	10	26.95 ± 2.97		105.46 ± 5.22
	25	52.05 ± 3.13		108.80 ± 8.27
	50	71.87 ± 1.51		105.64 ± 5.99

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

Table 3: The content of chemical markers in the DZSS formula extracts (n=3).

Extract	Contents			
	(mg/g extract, mean \pm SD)		(%w/w, mean \pm SD)	
	genistein	compound D	genistein	compound D
50% ethanol	0.03 ± 0.03	13.16 ± 1.32	0.003 ± 0.00	1.32 ± 0.02
95% ethanol	0.71 ± 0.00	18.89 ± 0.24	0.070 ± 0.00	1.89 ± 0.02



(Figure 2). The results also suggested that the 95% ethanol was appropriately to be able to extract the chemical markers and other chemical constituents which conformed to their anti-inflammatory activity in the DZSS formula. From the previous reports, the several phytochemicals found each ingredient of the DZSS formula possessed the anti-inflammatory activity. Genistein showed eicosanoid synthesis inhibition for cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) with IC_{50} 100 and 80 μ M, respectively. Genistein in its glycoside form, genistein 7-O- α -rhamnosyl (1--->6) β -glucopyranoside, from *D. scandens* was shown to inhibit cyclooxygenase-1 (COX-1) and 5-lipoxygenase (5-LOX) with IC_{50} 1500 and 2500 μ M, respectively.⁴ The crude drug of *D. scandens* stem was also reported that it significantly reduced muscle pain in patients with osteoarthritis and low back pain, with the similar adverse reactions to that of NSAIDs.^{3,20,21} Moreover, chloroform extract of leaf and root and aqueous extract of stem of *D. scandens* exhibited anti-inflammatory activity in the carrageenan-induced paw oedema in rat model and they inhibited eicosanoid inflammatory mediator.^{22,23} For Clinical studied found that there were no differences in efficacy and safety in the treatments of knee osteoarthritis with *D. scandens* crude for seven consecutive days and ibuprofen.²⁴ Considering the anti-inflammatory effect of *Z. Cassumunar*, the methanolic extract of its rhizome also exhibited anti-inflammatory activity in the carrageenan-induced paw oedema in rat model.^{25,26} Compound D or (E)-4-(3',4'-dimethoxyphenyl)but-3-en-1-ol and DMPBD or (E)-1-(3,4-dimethoxyphenyl) butadiene from *Z. cassumunar* was reported to have inhibitory action against NO production with the IC_{50} of 211.1 μ M and exhibited moderate COX-2 inhibitory activity with the IC_{50} of 20.68 μ M.^{27,28} Helioscopinolide A, helioscopinolide C and suremulol D, phytochemical constituents from *S. multiflora* showed the potent anti-inflammatory activity against NO production with the IC_{50} of 9.1, 24.5 and 29.3 μ M, respectively.²⁹ However, the anti-inflammatory activity of *S. celastrius* has not been documented yet.³⁰

According to Thai traditional medicine, the combination of these 4 medicinal plants was used as the herbal formula in order to achieve the synergistic anti-inflammatory effect. Although, anti-inflammatory of each medicinal plant has already been studied, the anti-inflammatory activity of the DZSS formula is explored for the first time in this study. The results from the current study thus provide scientific evidence supporting the traditional use of the DSZZ formula. In this study, the DZSS formula with the D:Z:S:S content ratio of 1:2:1:1 was selected to be examined in details for the anti-inflammatory activity and the phytochemical profiles since this formula is used widely for the treatment of muscle pain by Thai traditional practitioners. From the results two chemical markers, genistein and compound D, were used to quality control of the DZSS formula. The 95% ethanolic extract of DZSS formula produced a significant anti-inflammatory action which may result from the several chemical constituents in four plants, especially we found that the high content of compound D in this formula. This result indicated that genistein in its aglycone form also had an anti-inflammatory activity similar to that of its glycoside form, genistein glycoside (genistein 7-O- α -rhamnosyl (1--->6) β -glucopyranoside). These results confirmed that DZSS formula exhibited anti-inflammatory activity with no cytotoxicity in Raw 264.7 cell lines. However, further experiments should be performed to confirm the anti-inflammatory activity especially in an *in vivo* model of inflammation.

CONCLUSION

Our data suggest that the chemical markers, genistein and compound D were obtained in the 95% ethanolic extract of the DZSS formula that which 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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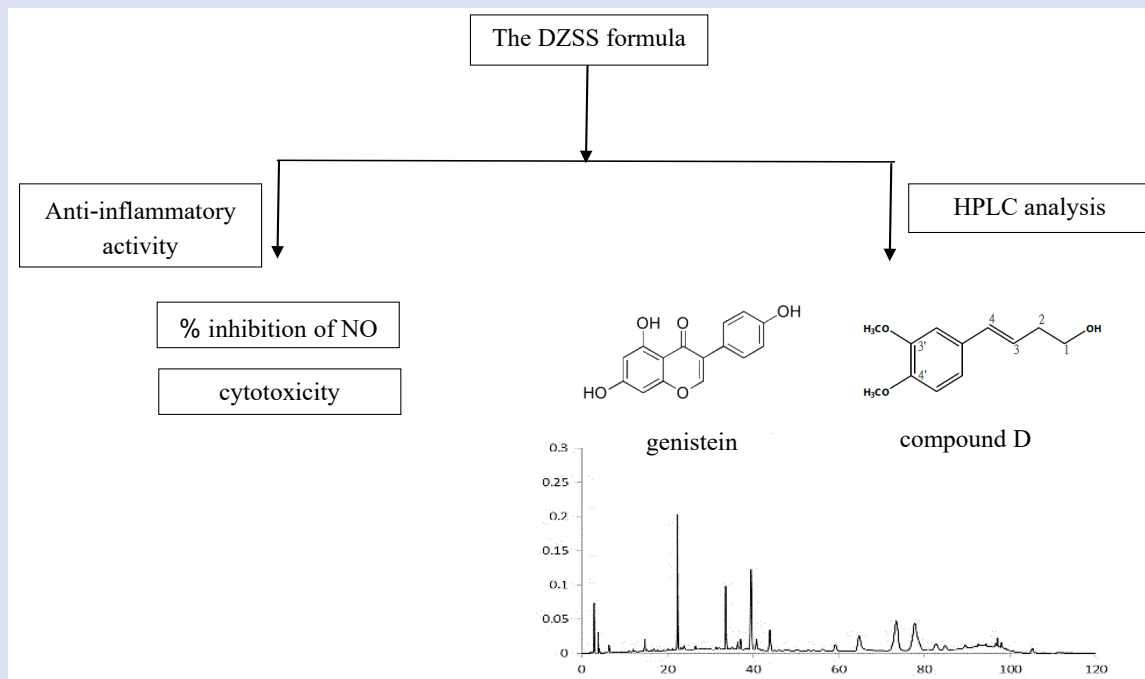
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GRAPHICAL ABSTRACT



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