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# *In Vitro* and *In Vivo* Activity Evaluations, LC-MS Profiling, Stability Testing, and Capsule Formulation of a Thai Folk Analgesic Herbal Formula

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**Abstract**

The Thai folk analgesic herbal formula, Ya Ka Sai Sen (YKSS), has been traditionally used among patients in rural Thailand, yet its scientific basis remains unexplored. This study evaluated the *in vitro* antioxidant, anti-inflammatory and cytotoxic properties, along with *in vivo* acute toxicity and analgesic activities of YKSS. Stability studies and capsule formulation development were also undertaken. Two batches of herbs, harvested at different times, were decocted to yield eight extracts. Total phenolic and flavonoid contents ranged from 100.50 to 179.89 mg GAE/g and 51.43 to 96.19 mg CE/g, respectively. Antioxidant assays demonstrated DPPH $IC_{50}$ values of 8.74 - 96.19 µg/mL and FRAP values of 2,148.20 - 3,032.97 µmol Fe(II)/g. However, nitric oxide scavenging was below 50%, while inhibition of nitric oxide production in cell-based assays showed $IC_{50}$ values of 820.93 - 969.13 µg/mL. Anti-lipoxygenase activity was moderate ($IC_{50}$: 15.50 - 72.75 µg/mL) and cytotoxicity against HepG2 cells varied between 1 - 10 µg/mL. LC-MS analysis identified *N*-trans-feruloyltyramine and maltose as major compounds in positive and negative ionization modes, respectively. Stability studies showed some samples degraded after one year under ambient conditions. Acute toxicity testing revealed no adverse effects at 2,000 mg/kg in rats. Analgesic evaluation via acetic acid-induced writhing tests showed significant reductions in writhing episodes, whereas no effects were noted in the hot plate test, indicating peripheral nociception inhibition. YKSS was successfully developed into a capsule formulation, demonstrating antioxidant, anti-inflammatory, and analgesic activities with a favorable safety profile. These findings support further exploration of YKSS as a potential therapeutic agent.

**Keywords:** Antioxidant, Anti-inflammation, Analgesic, Toxicity, Lipoxygenase

## Introduction

Musculoskeletal pain is a prevalent and complex condition that poses significant challenges to both patients and healthcare providers. It is experienced by individuals across all demographics, including age, gender and socioeconomic status, with approximately 47% of the global population affected at some point in their lives. Of these, an estimated 39% - 45% experience persistent symptoms requiring medical intervention. Inadequately managed musculoskeletal pain can lead to

a marked decline in quality of life and substantial socioeconomic burdens. Traditional and herbal medicines have long been explored as complementary approaches for pain management, particularly in regions with a strong history of traditional healing practices. There are several methods to treat musculoskeletal pain, incorporating pharmacological, non-pharmacological and interventional therapies within a multidisciplinary framework. These approaches are essential for optimizing patient outcomes, including pain relief, functional recovery and overall quality of life [1].

Regarding pharmacological treatment, non-steroidal analgesics and antipyretics (such as aspirin and paracetamol), non-steroidal anti-inflammatory drugs (NSAIDs) (such as ibuprofen, naproxen, meloxicam, celecoxib, parecoxib, etoricoxib, etc.), opioids and combinations with anticonvulsants, antidepressants, local anesthetics, topical agents, anxiolytics and other medications may be used [1]. However, concerns over adverse effects, including gastrointestinal complications and opioid dependence [2,3], have led to increased interest in traditional remedies with potential analgesic and anti-inflammatory properties. While pharmacological medications can be effective, traditional or folk medicines may provide a safer and more effective alternative for pain management, particularly for long-term use [4-6]. The use of herbs presents a promising and cost-effective alternative for the prevention and treatment of inflammatory conditions. This approach is supported not only by the accessibility and affordability of these natural sources but also by their inherent potential to reduce the risks associated with the adverse effects of conventional treatments [7].

Thai traditional medicines are treatment remedies native to Thailand. It is defined as “The medicinal procedures concerned with examination, diagnosis, therapy, treatment or prevention of, or promotion and rehabilitation of the health of humans or animals, obstetrics, traditional Thai massage and also includes the production of traditional Thai drugs and the invention of medicinal devices, base on knowledge or text that has been passed on from generation to generation” [8]. Thai folk medicine, a branch of traditional Thai medicine, relies on locally practiced knowledge and herbal formulations developed over centuries. This practice has been widely utilized in rural areas for treating musculoskeletal pain and inflammation. This traditional wisdom is derived from experience rather than from systematic medical theory [9].

The Ya Ka Sai Sen (YKSS) formula, developed by Mr. Arj Ramadthong, has been traditionally used as a folk medicine in Buached District, Surin Province, Thailand, for the treatment of back pain, wrist pain, abdominal tightness and as a general tonic. This herbal remedy reflects the deep-rooted wisdom of local traditional medicine and has been relied upon by communities for its claimed therapeutic benefits. This formula is composed of 18 herbal ingredients, as detailed in **Table 1**. However, the formulation is inherently flexible, allowing for adjustments based on the availability of specific herbs. For instance, as noted in **Table 1**, *Ventilago denticulata* Willd. was absent in Batch 1, while *Cladogynos orientalis* Zipp. ex Span. was absent in Batch 2. The underlying principle of this formulation relies on balancing herbs categorized by traditional knowledge as having “hot” and “cool” tastes. A small quantity of “hot-tasting” herbs is combined with a larger quantity of “cool-tasting” herbs to achieve a therapeutic balance, reflecting the wisdom of folk medicine practices. A high amount of “hot-tasting” herbs should be avoided, as it can be toxic or induce abortion in pregnant users and may cause dizziness, headache, or vomiting in non-pregnant individuals. Additionally, the quantities of individual herbs in the formula are not fixed or measured precisely by weight. Instead, they are mixed based on estimation, leading to variations in the ratio of ingredients between different batches. In this study, the authors intentionally replicated the practical variability observed in traditional use to closely reflect real-world applications of the YKSS formula. The preparation of the YKSS formula begins with thoroughly cleaning all herbs with water. The cleaned herbs are then boiled in water, ensuring that the water level completely covers the herbs. Once the mixture reaches a boil, it is removed from heat and allowed to warm or cool before use. The recommended dosage regimen varies among users. Traditionally, one teacup of decoction is consumed throughout the day as a substitute for water. The herbs are reboiled daily with added water to restore approximately the same volume as the initial preparation. The decoction can be used for four to five

consecutive days. This traditional knowledge and detailed preparation method for YKSS were kindly provided by Pra Ajan Dhanes Jattabhayo, a monk of Wat Pacha Ban Kraisorn in Buached District, Surin Province, Thailand, reflecting the cultural and medicinal heritage of the region. However, there is no scientific evidence to support the safety and efficacy of this Thai folk medicine; therefore, pre-clinical and clinical evaluations are required to validate the use of this formula [10].

This study aimed to investigate the *in vitro* biological activities of YKSS, with a particular focus on its antioxidant, anti-inflammatory and cytotoxicity properties, alongside its *in vivo* acute toxicity and analgesic effects. The primary objective of this research was to evaluate the biological activities, safety and stability of YKSS, which have not been thoroughly studied despite its widespread use in traditional medicine. To achieve these objectives, *in vitro* assays were conducted to assess the biological properties and toxicity of YKSS, while *in vivo* studies were designed to evaluate its toxicity profile and analgesic effects in animal models. These methods were chosen to ensure a comprehensive assessment of the formulation's therapeutic potential and safety, aligning with the study's aim of bridging traditional knowledge with scientific validation. Although YKSS has been traditionally used, there has been limited scientific investigation into its biological activities and safety profile. By addressing this gap, our research provides empirical evidence to support the therapeutic potential of YKSS. To the best of our knowledge, this study is the first to comprehensively investigate the biological activities, safety and formulation stability of YKSS, establishing a scientific basis for its traditional use. Additionally, the stability of the formulation was assessed and a capsule formulation was developed to enhance its usability and practicality. The findings from this study contribute valuable insights into the scientific understanding of YKSS, highlighting its potential as a safe and effective alternative for managing musculoskeletal pain and related conditions while preserving the wisdom of traditional Thai medicine.

## Materials and methods

### Materials

Acetonitrile (LC-MS grade), ferric (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), formic acid (LC-MS grade) and water (LC-MS grade) were purchased from Merck KGaA, Darmstadt, Germany. 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), acetic acid, caffeic acid phenethyl ester (CAPE), dimethyl sulfoxide, Folin-Ciocalteu reagent, gallic acid monohydrate, indomethacin, L-nitroarginine (L-NA), lipopolysaccharides (LPS), linoleic acid sodium salt, lipoxygenase from soybean, methylthiazolyldiphenyltetrazolium bromide (MTT) and nordihydroguaiaretic acid (NDGA) were purchased from Sigma-Aldrich, Inc., MA, USA. Tramadol hydrochloride was purchased from Central Polytrading Co. Ltd., Nonthaburi, Thailand. Hydrochloric acid (37%), naphthylethylenediamine dihydrochloride, orthophosphoric acid (85%), sodium acetate, sodium hydroxide, sodium nitroprusside and sulfanilamide were purchased from Carlo Erba Reagents, Cornaredo, Italy. Aluminum chloride and sodium carbonate were purchased from Ajax Finechem Pty. Ltd., New South Wales, Australia. Sodium nitrite was purchased from Loba Chemie Pvt. Ltd., Mumbai, India.

### Plant samples and extraction

All herbs composed in the YKSS formula were obtained from Phra Ajarn Dhanes Jattabhayo, the monk at Wat Pacha Ban Kraisorn, Buached District, Surin Province, Thailand. The plant compositions of YKSS, including Thai common name, scientific name, family, part used and mass are shown in **Table 1**. The first and the second batches were obtained in August 2021 and March 2022, respectively. The first batch contained four samples coded as YKSS 1-1 to 1-4. The second batch contained four samples coded as YKSS 2-1 to 2-4, as shown in the diagram in **Figure 1**. They were identified by a plant taxonomist at the Department of Pharmacognosy, College of Pharmacy at Rangsit University to ensure the correct plant species. The voucher specimens were coded and deposited at the Drug and Herbal Product Research and Development Center, College of Pharmacy, Rangsit University. They were cleaned with tap water, boiled in 1.5 L of water for 15 min, filtered using cheesecloth and cooled before

being freeze-dried by a freeze dryer for 18 - 20 h. The dried extracts were kept in a desiccator until used.

**Table 1** Plant compositions of YKSS, including Thai common name, scientific name, family, part used, voucher specimen number and mass used.

| No. | Thai common name | Scientific name | Family | Part used | Voucher specimen no. | | Mass (g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Batch 1 | Batch 2 | YKSS 1-1 | YKSS 1-2 | YKSS 1-3 | YKSS 1-4 | YKSS 2-1 | YKSS 2-2 | YKSS 2-3 | YKSS 2-4 |
| 1 | Krachab Nok | *Euonymus cochinchinensis* Pierre | Celastraceae | Stem | TMRC 075 | TMRC 092 | 55.43 | 62.00 | 66.18 | 56.26 | 55.15 | 58.30 | 59.60 | 63.25 |
| 2 | Kamphang Ched Chan | *Salacia chinensis* L. | Celastraceae | Stem | TMRC 076 | TMRC 093 | 60.61 | 72.17 | 73.32 | 86.33 | 62.35 | 53.94 | 52.32 | 19.40 |
| 3 | Sae Ma Talai Rong | *Erycibe paniculata* Roxb. | Convolvulaceae | Stem | TMRC 077 | TMRC 094 | 54.73 | 42.73 | 53.95 | 49.31 | 49.26 | 60.37 | 50.69 | 40.72 |
| 4 | Phaya Fhai/Hang Hon | *Diospyros lanceifolia* Roxb. | Ebenaceae | Stem | TMRC 078 | TMRC 095 | 11.41 | 13.83 | 9.12 | 18.02 | 33.21 | 23.23 | 13.72 | 22.96 |
| 5 | Chetta Pangkee | *Cladogynos orientalis* Zipp. ex Span. | Euphorbiaceae | Root | TMRC 079 | - | 1.96 | 3.40 | 3.71 | 4.85 | - | - | - | - |
| 6 | Plao Noi | *Croton fluviatilis* Esser | Euphorbiaceae | Root | TMRC 080 | TMRC 096 | 4.89 | 11.06 | 9.39 | 4.10 | 10.76 | 9.19 | 8.23 | 10.55 |
| 7 | Thaowan Priang | *Derris scandens* (Roxb.) Benth. | Fabaceae | Stem | TMRC 081 | TMRC 097 | 11.50 | 14.18 | 9.77 | 9.39 | 15.40 | 14.10 | 14.80 | 17.05 |
| 8 | Mueay Daeng | *Gnetum macrostachym* Hook.f. | Gnetaceae | Stem | TMRC 082 | TMRC 098 | 11.24 | 4.78 | 8.67 | 6.57 | 7.44 | 6.00 | 10.73 | 10.98 |
| 9 | Mueay Khao | *Gnetum montanum* Markgr. | Gnetaceae | Stem | TMRC 083 | TMRC 099 | 6.34 | 5.11 | 5.39 | 4.99 | 12.09 | 11.68 | 17.34 | 14.48 |
| 10 | Takrai Ton | *Litsea cubeba* (Lour.) Pers. | Lauraceae | Root | TMRC 084 | TMRC 100 | 7.41 | 14.00 | 16.16 | 28.60 | 39.18 | 24.04 | 26.42 | 18.42 |
| 11 | Khamin Khruea | *Arcangelisia flava* (L.) Merr. | Menispermaceae | Stem | TMRC 085 | TMRC 101 | 17.07 | 17.22 | 19.25 | 20.72 | 40.75 | 36.46 | 34.58 | 9.41 |
| 12 | Ham | *Coscinium fenestratum* (Goetgh.) Colebr. | Menispermaceae | Stem | TMRC 086 | TMRC 102 | 3.63 | 1.83 | 1.51 | 1.70 | 3.48 | 3.37 | 5.30 | 14.87 |
| 13 | Ma Krathub Rong | *Ficus foveolata* Wall. | Moraceae | Stem | TMRC 087 | TMRC 103 | 53.81 | 67.33 | 52.75 | 43.12 | 38.10 | 40.00 | 47.41 | 11.16 |
| 14 | Kamlang Luead Ma/Pradong Luead | *Knema angustifolia* (Roxb.) Warb. | Myristicaceae | Stem | TMRC 088 | TMRC 104 | 69.42 | 47.16 | 55.77 | 72.89 | 59.83 | 64.00 | 66.45 | 39.10 |

| No. | Thai common name | Scientific name | Family | Part used | Voucher specimen no. | | Mass (g) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Batch 1 | Batch 2 | YKSS 1-1 | YKSS 1-2 | YKSS 1-3 | YKSS 1-4 | YKSS 2-1 | YKSS 2-2 | YKSS 2-3 | YKSS 2-4 |
| 15 | Rang Daeng | *Ventilago denticulata* Willd. | Rhamnaceae | Stem | - | TMRC 105 | - | - | - | - | 35.36 | 34.71 | 37.87 | 44.90 |
| 16 | Kamlang Suea Khrong | *Ziziphus attopoensis* Pierre | Rhamnaceae | Stem | TMRC 089 | TMRC 106 | 25.85 | 35.65 | 24.48 | 21.80 | 28.19 | 32.93 | 30.77 | 29.65 |
| 17 | Khruea Ngu Hao/Dee Ngu Hao | *Toddalia as iatica* (L.) Lam. | Rutaceae | Stem | TMRC 090 | TMRC 107 | 10.52 | 10.54 | 13.33 | 14.41 | 23.77 | 22.67 | 22.10 | 21.58 |
| 18 | Nom Wua/Nom Sao | *Scleropyrum pentandrum* (Dennst.) Mabb. | Santalaceae | Stem | TMRC 091 | TMRC 108 | 76.71 | 59.11 | 58.66 | 57.39 | 60.50 | 74.48 | 63.54 | 45.92 |

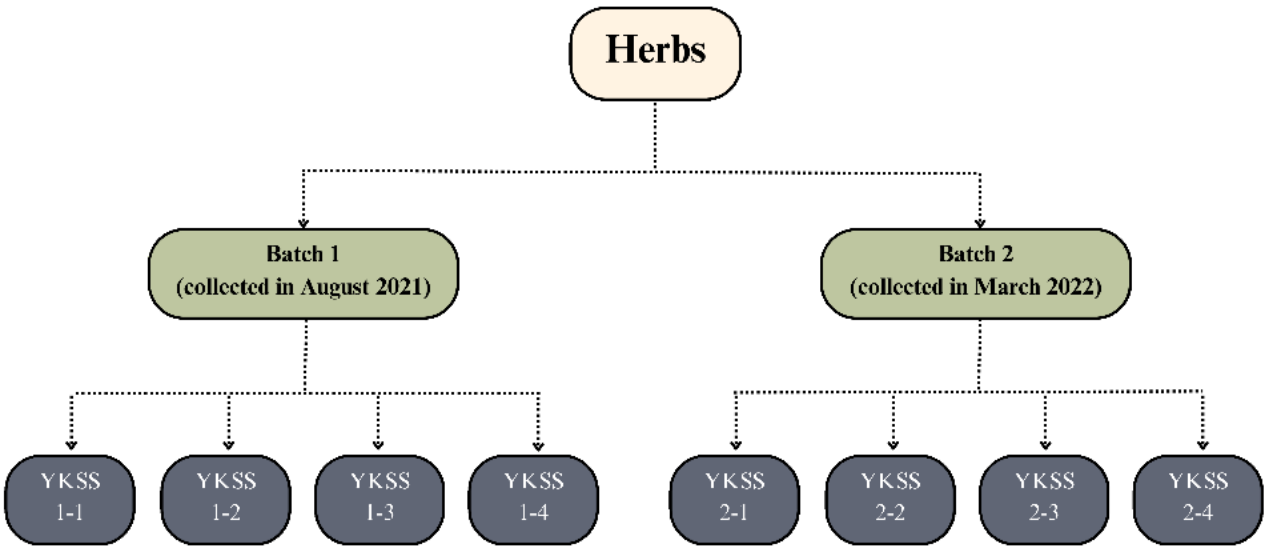


**Figure 1** Schematic representation of the YKSS samples used in this work.

**Determination of total phenolic content**

The total phenolic content (TPC) of YKSS extracts was measured using the Folin-Ciocalteu method [11]. Gallic acid standards, ranging from 6.25 to 300 µg/mL, were added in 20 µL aliquots to a 96-well plate (n = 3). Then, 100 µL of 0.2 N Folin-Ciocalteu reagent was added and the mixture was thoroughly mixed. The extracts were incubated for 6 min, followed by the addition of 80 µL of 7.5% sodium carbonate and further mixing. After an additional 1-h incubation at room temperature, the absorbance was measured at 765 nm using a microplate reader (Bio-Rad Laboratories, Inc., CA, USA). A calibration curve was constructed using gallic acid. The YKSS extracts were processed similarly, with a concentration of 0.5 mg/mL used. The TPC of the YKSS extract was then calculated using the gallic acid calibration curve and expressed as gallic acid equivalents (GAE) in mg per g of extract.

**Determination of total flavonoid content**

The determination of total flavonoid content (TFC) was modified from a previous study [12]. Twenty-five microliters of (+)-catechin hydrate, with concentrations ranging from 4 to 500 µg/mL and 100 µL of water were added to a 96-well plate (n = 3). Next, 10 µL of 5% sodium nitrite was added and the plate was incubated in the dark at room temperature for 5 min. Then, 15 µL of 10% aluminum chloride was added,

mixed and incubated in the dark at room temperature for 6 min. Afterward, 50 µL of 1 M sodium hydroxide and 50 µL of water were added and mixed. The absorbance was measured at 510 nm using the microplate reader. A calibration curve of (+)-catechin hydrate was constructed. The YKSS extract was treated similarly to the (+)-catechin hydrate group, with extracts at a concentration of 1 mg/mL. The TFC of the YKSS extract was calculated using the calibration curve of (+)-catechin hydrate and expressed as catechin equivalents (CE) in mg per g of extract.

**DPPH radical scavenging activity**

The DPPH radical scavenging assay was slightly modified from a previous study [13]. One hundred microliters of the extracts (concentrations ranging from 1 - 500 µg/mL) were added to a 96-well plate (n = 3). Then, 100 µL of 80 µM DPPH methanolic solution was added and mixed. The mixtures were kept in the dark at room temperature for 30 min before measuring the absorbance at 517 nm using the microplate reader. The DPPH radical without the extract was used as the control. The scavenging ability of the DPPH radical and half maximal inhibitory concentration ($IC_{50}$) were calculated.

**Ferric reducing antioxidant power assay**

The ferric reducing antioxidant power (FRAP) assay was adapted from a previous study [14]. Twenty microliters of YKSS extracts (at a concentration of 200 µg/mL) were added to a 96-well plate (n = 3). Next, 180 µL of freshly prepared FRAP reagent, consisting of 30 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 volume ratio, was added and mixed. The plates were incubated at 37 °C for 15 min, after which the absorbance was measured at 593 nm against a reagent blank using a microplate reader. The reducing power was determined from a calibration curve constructed with ferrous sulfate. The FRAP values were expressed as µmol Fe(II) equivalent per g of extract.

**Nitric oxide scavenging activity**

The nitric oxide (NO) scavenging assay was adapted from the method described previously [15]. Quercetin served as the positive control. YKSS extracts were prepared in water at concentrations of 500 and 1,000 µg/mL. A 50 µL portion of either quercetin or YKSS extract solution was dispensed into a 96-well plate (n = 3). Following this, 50 µL of 10 mM sodium nitroprusside was added and the mixture was incubated at room temperature for 120 min. After the incubation period, 100 µL of Griess reagent (containing 1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) was added to each well. Absorbance was measured at 546 nm using the microplate reader and the percentage of inhibition was calculated.

**Inhibition of NO production activity**

The anti-inflammatory activity was assessed using RAW264.7 murine macrophage cells stimulated with LPS to induce NO production, a key inflammatory mediator. NO suppression was evaluated following the method described previously [16]. RAW264.7 cells ($1\times10^5$ cells/well) were treated with 100 ng/mL LPS and co-incubated with various concentrations (31.25 - 2,000 µg/mL) of the test samples (n = 3) for 24 h at 37 °C in a humidified incubator with 5% $CO_2$. Positive controls included indomethacin, L-NA and CAPE. NO levels in the culture medium were quantified by reaction with Griess reagent and absorbance was measured at 570 nm using a microplate reader. The percentage inhibition of NO production was calculated and $IC_{50}$ values were determined from the inhibition curve.

**Anti-lipoxygenase activity assay**

The anti-lipoxygenase (LOX) activity assay was modified from a previous study [17]. YKSS extract was dissolved in water to achieve concentrations ranging from 0.01 - 300 µg/mL. NDGA used as a positive control, was prepared and diluted in methanol at concentrations of 0.0000001 - 0.1 mM. Subsequently, 10 µL of each sample was added to a 96-well plate (n = 3). To this, 170 µL of soybean LOX in 0.1 M phosphate buffer (pH 8.0) was added, followed by 20 µL of 2 mM linoleic acid sodium salt in methanol. The absorbance at 234 nm was measured immediately and recorded every 50 s for 5 min to monitor the production of hydroperoxylinoleic acid [18]. Enzyme activity was evaluated to ensure the mean velocity (Vmean) ranged between 80 - 120. The mean velocity was used to calculate the percentage of inhibition using Eq. (1). The blank control consisted of the reaction mixture without

the addition of the sample. Finally, $IC_{50}$ values were calculated from the inhibition curve.

$$Inhibition\ (\%) = \frac{Vmean_{control} - Vmean_{sample}}{Vmean_{control}} \times 100 \qquad (1)$$

**Cytotoxicity test**

The mitochondrial-based MTT cell viability test was used to evaluate the possible toxicity of YKSS extract *in vitro*. In a 96-well culture plate, HepG2 cells were seeded at a density of $1\times10^4$ cells per well. They were then incubated overnight at 37 °C with 5% $CO_2$. YKSS extract at different concentrations (0 - 10 mg/mL) or a positive control of 200 µM hydrogen peroxide were applied to the cells for a duration of 24 h. The cells were then incubated for three more hours after 100 µL of 0.5 mg/mL MTT solution in culture media was added to each well. A microplate reader was used to detect absorbance at 570 nm after the formazan product was dissolved in 100 µL of dimethyl sulfoxide. The percentage of cell viability in relation to the untreated control group was calculated after the experiment was carried out in triplicate [19].

**LC-MS analysis**

The LC-MS profile of YKSS 2-4 was analyzed. The sample was prepared by dissolving the YKSS 2-4 extract in water to achieve a concentration of 1 mg/mL, followed by filtration through a 0.2-µm PTFE syringe filter before analysis using an LC-MS instrument. The LC-MS analysis was conducted using an Agilent 1290 Infinity LC instrument coupled with an Agilent 6540 series QTOF-MS, equipped with an ESI source and a diode array detector (Agilent Technologies, Inc., CA, USA). Separation was performed on a Poroshell 120 EC-C18 column (4.6×150 mm, i.d., 2.7 µm) maintained at 35 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), with a flow rate of 200 µL/min. The gradient elution system was as follows: 5% B from 0 to 1 min, increased to 17% B at 10 min and held constant until 13 min, further increased to 100% B at 20 min and maintained until 25 min, then decreased to 5% B at 27 min and held constant until 33 min. The injection volume was 1.0 µL and the total analysis time was 33 min. Chemical constituents in the YKSS extract were identified using Agilent MassHunter Workstation software (qualitative analysis, version B.08.00) and the Personal Compound Database and Library (PCDL). Identified compounds were cross-referenced with the literature using databases corresponding to each plant component. Only chemical constituents with PCDL scores above 80 were selected. Chemical formulas were assigned with a mass error of less than ±5 ppm [20,21]. Mass-to-charge ratios (*m/z*) and MS analyses were performed in both positive and negative ionization modes.

**Stability test**

The YKSS extracts were stored in glass bottles protected from light and kept under ambient conditions for one year. At predetermined intervals, samples were collected to evaluate TPC and TFC in comparison to the initial time point.

**Animals**

For the acute toxicity and analgesic activity experiments, Wistar rats weighing approximately 220 ± 20 g were selected for the acute toxicity test. To evaluate analgesic effects, Swiss albino mice weighing between 20 - 25 g were used. Both rats and mice were obtained from the National Laboratory Animal Center at Salaya, Mahidol University, Nakorn Pathom, Thailand. They were acclimatized for seven days before the experiments. The animals were maintained under standard conditions, including a temperature of 23 - 25 °C, a 12-h light/dark cycle and free access to standard pelleted feed and water, under animal welfare standards. All procedures involving animals were conducted in compliance with the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) guidelines [22] to ensure rigorous experimental design, methodology and reporting. The protocol for this study was reviewed and approved by the Ethics Committee for Animal Research at Rangsit University (RSU-AEC 003-2024).

**Acute toxicity study**

The acute oral toxicity study was conducted according to the Organization for Economic Co-operation and Development (OECD) 420: Acute Oral Toxicity - Fixed Dose Procedure [23]. The animals were randomly assigned to control and experimental groups, with five rats in each group. Rats in the control group were orally administered distilled water, while rats in the test group were given the YKSS extract (Batch 2) at a

dose of 2,000 mg/kg body weight. The rats were closely monitored for any signs of death or abnormal symptoms, including changes in skin and fur, eyes and mucous membranes, behavior patterns, tremors, salivation, diarrhea, sleep and coma during the first 24 h (day 1). Observations continued once daily for 14 days. Body weight was recorded on day 1 (before YKSS extract administration), day 7 and day 14.

**Analgesic activity study**

***Grouping and dosing***

The equivalent animal dosages of YKSS extract (Batch 2) were determined using the body surface area method [24]. The prescribed human dosage of YKSS extract is calculated for approximately one teaspoonful twice daily, with each teaspoonful weighing 1,630 mg, resulting in a total daily dose of 3,260 mg. Based on this and adjusting for body surface area, the calculated therapeutic equivalent dose for mice was approximately 670 mg/kg. However, for the study, three distinct doses were selected for the mouse experiments: a low dose of 400 mg/kg, a therapeutic equivalent dose of 800 mg/kg and a high dose of 1,200 mg/kg. Mice were divided into five groups (n=6 per group). The analgesic efficacy of YKSS extract was assessed with both single-dose administration and repeated administration over seven days. This approach aims to validate the traditional use of YKSS as a pain-relieving agent and explore its potential for long-term use.

***Acetic acid-induced writhing test***

The acetic acid-induced writhing test was conducted to evaluate visceral pain using the previously described method [25]. Each group consisted of six mice, which were orally administered YKSS at doses of 400, 800 and 1200 mg/kg and indomethacin at 10 mg/kg as a standard drug. All samples were administered to the corresponding groups by oral gavage once daily for 1 day and 7 days. On the day of the test, one h after administering the test sample, all animals received intraperitoneal injections of 0.75% acetic acid (0.1 mL/10 g body weight) to induce writhing. Each mouse was placed separately in a transparent cage and the number of writhes (contractions) was counted over 60 min following the acetic acid injection. The recorded data represented the total number of writhing episodes observed.

***Hot plate test***

The hot plate test was employed as an additional technique to evaluate analgesic activity. Using the previously mentioned methodology, it was carried out to measure latency response. Five groups of six mice each were randomly assigned. Excluded mice had a baseline latency time of more than 30 s or less than 5 s. Tramadol (100 mg/kg, the conventional medicine) and YKSS (400, 800 and 1200 mg/kg) were given orally to the mice and their reaction times were recorded at 0, 15, 30, 60 and 90 min. The temperature of the hot plate was kept at 54.0 ± 1 °C. The latency response index was defined as the number of sec that passed between the mice being placed on the hot plate and a discomfort reaction (such as leaping or licking the back of the hind paw) being observed. Complete analgesia was defined as a 45-s cutoff duration. In order to prevent paw damage, the experiment was terminated if the cutoff time was surpassed.

**Capsule preparation**

The YKSS extract was filled into hard gelatin capsule no. 0. They were evaluated for individual weight and disintegration time. In the case of individual weight, ten capsules were analyzed using an analytical balance (Model: Entris224i-1S, Sartorius AG, Göttingen, Germany). The disintegration time of six capsules was determined using a disintegration tester (Model: BJ-2, Tianjin Guoming Medicinal Equipment Co. Ltd., Tianjin, China), with water at 37 ± 0.5 °C as the disintegration medium.

**Statistical analysis**

Data were analyzed using an independent sample t-test for comparisons between two groups and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to compare differences between more than two groups using IBM SPSS Statistics v. 22 (IBM Corp., Armonk, NY, USA). A *p-value* of $< 0.05$ was considered statistically significant at a 95% confidence interval.

## Results

**Extraction yield, TPC and TFC**

The extraction yields, TPC and TFC for each batch of plants obtained from decoction are shown in **Table 2**. The extraction yields for Batches 1 (YKSS 1-1 to 1-4)

and 2 (YKSS 2-1 to 2-4) ranged from 1.26% to 2.44% and 0.88% to 1.02%, respectively. Batch 1, which contained *C. orientalis* root, yielded higher extraction rates than Batch 2, which contained *V. denticulata* stem instead of *C. orientalis* root.

The TPC of Batch 2 was significantly higher than that of Batch 1, with values ranging from 142.76 to 179.89 mg GAE/g of extract for Batch 1 and 100.50 to 156.50 mg GAE/g of extract for Batch 2. The TFC showed a broader range in Batch 1 compared to Batch 2, with values ranging from 51.43 to 96.19 mg CE/g of extract for Batch 1 and 61.90 to 76.90 mg CE/g of extract for Batch 2.

**Table 2** Extraction yield, TPC, TFC, DPPH radical scavenging, FRAP and NO scavenging activity of YKSS.

| Samples | Extraction yield (%) | TPC (mg GAE/g of extract) | TFC (mg CE/g of extract) | $IC_{50}$ (DPPH radical scavenging, µg/mL) | FRAP (µmol Fe(II) equivalent/g of extract) | NO scavenging (%) | |
|---|---|---|---|---|---|---|---|
| | | | | | | 500 µg/mL | 1,000 µg/mL |
| YKSS 1-1 | 1.64 | $100.50 \pm 0.89^{e}$ | $51.43 \pm 10.00^{c}$ | $55.31 \pm 4.69^{b}$ | $2,148.20 \pm 58.43^{c}$ | $32.62 \pm 1.35^{b,d}$ | $46.52 \pm 1.79^{b}$ |
| YKSS 1-2 | 1.62 | $101.12 \pm 3.92^{e}$ | $55.95 \pm 9.70^{b,c}$ | $68.64 \pm 5.09^{a}$ | $2,201.94 \pm 11.99^{c}$ | $33.48 \pm 2.87^{b,d}$ | $43.26 \pm 6.60^{b}$ |
| YKSS 1-3 | 1.26 | $150.25 \pm 2.15^{c,d}$ | $77.14 \pm 10.52^{a,b}$ | $41.75 \pm 1.55^{c,d}$ | $2,583.87 \pm 58.81^{b}$ | $27.85 \pm 3.65^{c,d}$ | $40.98 \pm 0.89^{b}$ |
| YKSS 1-4 | 2.44 | $156.50 \pm 1.98^{c}$ | $96.19 \pm 9.65^{a}$ | $36.52 \pm 2.24^{d}$ | $2,601.14 \pm 93.79^{b}$ | $37.14 \pm 4.18^{b}$ | $42.34 \pm 1.92^{b}$ |
| YKSS 2-1 | 0.93 | $168.09 \pm 2.15^{b}$ | $68.10 \pm 6.16^{b,c}$ | $39.19 \pm 2.29^{d}$ | $2,493.67 \pm 84.82^{b}$ | $22.51 \pm 2.87^{c}$ | $41.83 \pm 1.42^{b}$ |
| YKSS 2-2 | 0.91 | $179.89 \pm 0.18^{a}$ | $66.90 \pm 5.77^{b,c}$ | $52.75 \pm 4.25^{b,c}$ | $2,963.88 \pm 126.32^{a}$ | $34.44 \pm 1.88^{b,d}$ | $42.93 \pm 2.19^{b}$ |
| YKSS 2-3 | 0.88 | $156.71 \pm 3.49^{c}$ | $76.90 \pm 10.31^{b}$ | $61.71 \pm 7.26^{a,b}$ | $3,032.97 \pm 48.97^{a}$ | $36.47 \pm 2.18^{b}$ | $44.74 \pm 0.27^{b}$ |
| YKSS 2-4 | 1.02 | $142.76 \pm 4.42^{d}$ | $61.90 \pm 5.36^{b,c}$ | $33.77 \pm 3.06^{d}$ | $2,507.10 \pm 25.96^{b}$ | $39.21 \pm 0.61^{b}$ | $46.83 \pm 0.82^{b}$ |
| Ascorbic acid | - | - | - | $8.74 \pm 0.80^{e}$ | - | - | - |
| Quercetin | - | - | - | - | - | $70.62 \pm 1.81^{a}$ | $75.08 \pm 0.64^{a}$ |

Different letters within the same column indicate statistically significant differences ($p < 0.05$).

YKSS: Ya Ka Sai Sen, TPC: Total phenolic content, TFC: Total flavonoid content, CE; Catechin equivalent, FRAP: Ferric reducing antioxidant power, NO: Nitric oxide

**Antioxidant activities**

Antioxidant activities assessed using the DPPH radical scavenging assay, FRAP assay and NO scavenging assay are presented in **Table 2**. The DPPH radical scavenging assay, expressed as $IC_{50}$ values, ranged from 36.52 to 68.64 µg/mL for batch, which were comparable to the range of 33.77 to 61.71 µg/mL for Batch 2. The FRAP values, expressed as Fe(II) equivalents, were 2,148.20 to 2,601.14 µmol Fe(II) equivalent/g of extract for Batch 1, which were slightly lower than the range of 2,493.67 to 3,032.97 µmol Fe(II) equivalent/g of extract for Batch 2. For NO scavenging, the $IC_{50}$ values could not be determined as 50% inhibition was not achieved. Therefore, two concentrations were selected to demonstrate the percentage of NO scavenging. Batch 1 exhibited NO scavenging activity ranging from 27.85% to 37.14% at 500 µg/mL of extract, which increased to 40.98% to 46.52% at 1,000 µg/mL of extract. Similarly, Batch 2 showed NO scavenging activity of 22.51% to 39.21% at 500 µg/mL of extract, increasing to 41.83% to 46.83% at 1,000 µg/mL.

**Anti-inflammatory activity**

Anti-inflammatory activity was assessed using *in vitro* NO production inhibition and anti-LOX assays. As shown in **Figure 2**, YKSS extracts demonstrated low potency in inhibiting NO production. Batch 1 of YKSS inhibited NO production with $IC_{50}$ values ranging from 820.91 to 905.06 µg/mL, while Batch 2 exhibited $IC_{50}$ values between 883.71 and 969.17 µg/mL. Among the extracts, YKSS 1-2 was the most potent, exhibiting the lowest $IC_{50}$ value.

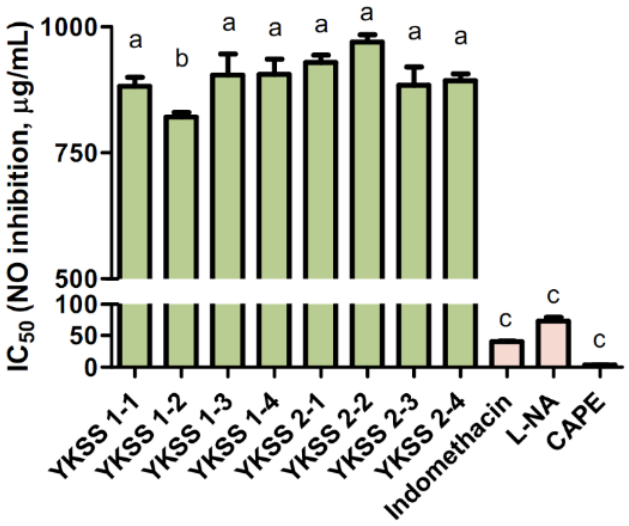


**Figure 2** Inhibition of NO production by YKSS samples, expressed as $IC_{50}$ values. Data are presented as mean ± SEM (n = 3). Comparisons made with positive controls: Indomethacin, L-NA and CAPE. Different letters signify significant differences ($p$-value < 0.05).

In the case of anti-LOX activity, YKSS extract demonstrated strong inhibition of LOX, an enzyme that regulates inflammatory responses. Batch 1 of YKSS exhibited $IC_{50}$ values ranging from 16.92 to 56.70 µg/mL, while Batch 2 showed $IC_{50}$ values between 15.50 and 72.75 µg/mL. YKSS 1-4 from Batch 1 and YKSS 2-2 from Batch 2 exhibited the lowest $IC_{50}$ values, indicating that they possess the most potent LOX inhibition activity.

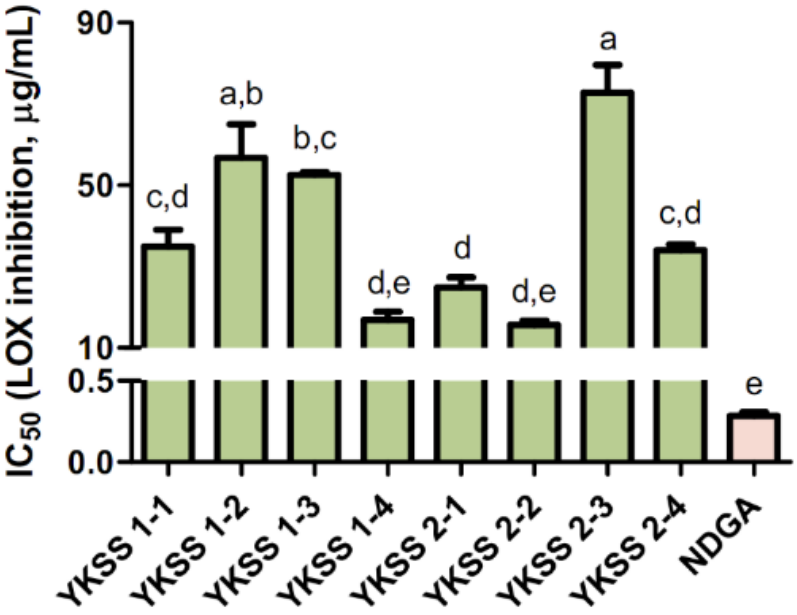


**Figure 3** Inhibition of LOX by YKSS samples, expressed as $IC_{50}$ values. Data are presented as mean ± SD (n = 3), with comparisons to the positive control, NDGA. Different letters signify significant differences ($p$-value < 0.05).

**Cytotoxicity**

Cytotoxicity was evaluated by treating HepG2 cells with the samples and measuring cell viability. Cell viability significantly decreased upon treatment with YKSS extracts, depending on the sample: YKSS 1-3 exhibited decreased viability at a concentration of 0.00001 mg/mL, YKSS 1-1 and 2-1 at 0.001 mg/mL and most other samples at 0.0001 mg/mL (**Figure 4**).

According to ISO 10993-5:2009, a sample is considered toxic if cell viability is less than 70% compared to the control group. Although YKSS extracts caused a significant reduction in cell viability, the concentrations at which toxicity (viability < 70%) was observed were relatively high: 1 mg/mL for YKSS 1-3; 5 mg/mL for YKSS 1-2, 1-4 and 2-2; and 10 mg/mL for YKSS 1-1, 2-1, 2-3 and 2-4.

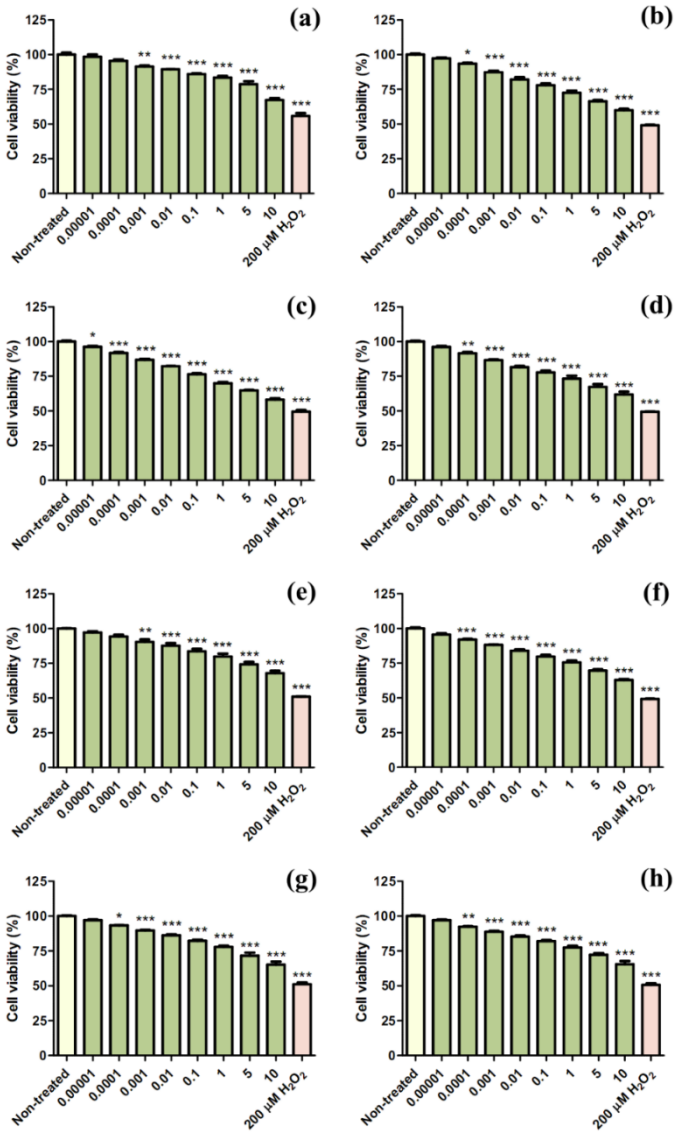


**Figure 4** The cytotoxicity of YKSS samples, represented by cell viability, at different concentrations (mg/mL) after 24 h of treatment. The data are presented as mean ± SEM (n = 3). Symbols *, ** and *** indicate significant differences compared to the non-treated group, with $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

**LC-MS profile**

Total ion chromatograms (top) and base peak chromatograms (bottom) obtained in positive and negative ionization modes are shown in **Figure 5**. The YKSS 2-4 extract composed of several classes of compounds, including alkaloids, anthraquinones, benzaldehydes, chalcones, coumarins, disaccharides, fatty acids, flavanone glycosides, flavonoids, glycosides, organic acids, phenolic acids, phenolic amides, phenolic compounds, phenylpropanoids, quinones, resveratrols, steroids, stilbenoids and terpenoids.

The YKSS 2-4 extract comprised 66 compounds previously reported in the literature: 13 compounds identified in positive ionization mode and 53 compounds identified in negative ionization mode (**Table 3**). In the positive ionization mode, *N*-trans-feruloyltyramine was the most abundant compound

(percent peak area (PA) = 88.38%), followed by derrisscandenon E (percent PA = 4.01%), isodomesticine (percent PA = 2.29%) and others. In the negative ionization mode, maltose was the most abundant compound (percent PA = 26.84%), followed by gluconic acid (percent PA = 19.26%), citric acid (percent PA = 14.32%) and others.

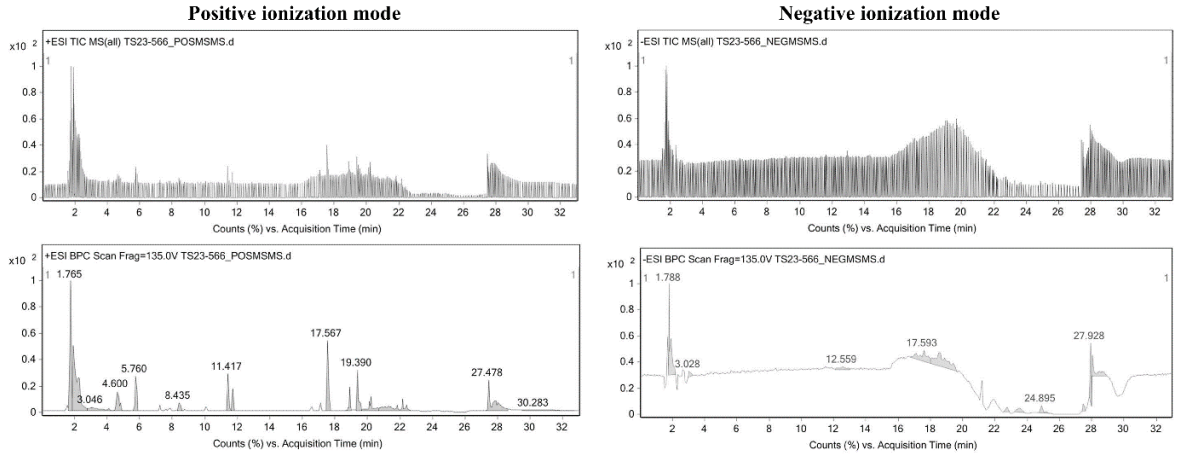


**Figure 5** Total ion chromatograms (top) and base peak chromatograms (bottom) obtained in positive and negative ionization modes.

**Table 3** LC-MS profiles of YKSS 2-4 in positive and negative ionization modes.

| No. | RT (min) | Compound name | Chemical formula | $ESI^+/ESI^-$ | Observed mass (*m/z*) | Reference mass (*m/z*) | Mass error (ppm) | Class of compound | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.712 | Gluconic acid | $C_6H_{12}O_7$ | $(M-H)^-$ | 196.0583 | 195.0510 | −0.26 | Organic acids | [26] |
| 2 | 1.749 | Maltose | $C_{12}H_{22}O_{11}$ | $(M+CHO_2)^-$ | 342.1162 | 387.1143 | −0.02 | Disaccharides | [27] |
| 3 | 2.364 | Citric acid | $C_6H_8O_7$ | $(M-H)^-$ | 192.0271 | 191.0198 | 0.43 | Organic acids | [26] |
| 4 | 3.111 | Scleropentasides C | $C_{16}H_{22}O_{11}$ | $(M-H)^-$ | 390.1149 | 389.1082 | −3.30 | Glycosides | [28] |
| 5 | 4.543 | (+)-*N*-(methoxylcarbonyl)-*N*-norglaucine | $C_{12}H_{23}NO_4$ | $(M+Na)^+$ | 245.1617 | 268.1510 | −4.18 | Alkaloids | [29] |
| 6 | 5.381 | Vanillic acid | $C_8H_8O_4$ | $(M-H)^-$ | 168.0423 | 167.0349 | 0.25 | Phenolic acids | [30] |
| 7 | 5.587 | 4-Hydroxy-3-methoxybenzyl 4-O-β-D-xylopyranosyl-(1→6)-b-D-glucopyranoside | $C_{19}H_{28}O_{12}$ | $(M+CHO_2)^-$ | 448.1577 | 493.1560 | −0.92 | Glycosides | [31] |
| 8 | 5.760 | Parvifolol B | $C_{28}H_{22}O_7$ | $(M+H)^+$ | 470.1354 | 471.1427 | −2.51 | Flavonoids | [32] |
| 9 | 5.792 | 2,5-dimethoxy-*p*-benzoquinone | $C_8H_8O_4$ | $(M-H)^-$ | 168.0417 | 167.0347 | −3.43 | Quinones | [33] |
| 10 | 5.792 | Pimpinellin | $C_{13}H_{10}O_5$ | $(M+CH_3CO_2)^-$ | 246.0529 | 305.0665 | 0.23 | Coumarins | [34] |
| 11 | 5.853 | Scleropentasides B | $C_{17}H_{24}O_{11}$ | $(M+CH_3CO_2)^-$ | 404.1313 | 463.1451 | −1.42 | Glycosides | [28] |
| 12 | 6.320 | Coniferin | $C_{16}H_{22}O_8$ | $(M+CHO_2)^-$ | 342.1308 | 387.1280 | −1.95 | Glycosides | [35] |
| 13 | 6.320 | Potalioside B | $C_{20}H_{30}O_{13}$ | $(M+CHO_2)^-$ | 478.1679 | 523.1661 | −1.58 | Glycosides | [28] |

| No. | RT (min) | Compound name | Chemical formula | $ESI^+/ESI^-$ | Observed mass (*m/z*) | Reference mass (*m/z*) | Mass error (ppm) | Class of compound | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 14 | 6.320 | 2,6-Dimethoxy-*p*-hydroquinone 1-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl unit | $C_{19}H_{28}O_{13}$ | $(M+CH_3CO_2)^-$ | 464.1522 | 523.1661 | −1.72 | Glycosides | [31] |
| 15 | 6.396 | Syringic acid β-D-glucopyranoside | $C_{15}H_{20}O_{10}$ | $(M-H)^-$ | 360.1053 | 359.0979 | −1.07 | Glycosides | [36] |
| 16 | 8.342 | Isopimpinellin | $C_{13}H_{10}O_5$ | $(M+CH_3CO_2)^-$ | 246.0525 | 305.0662 | −1.23 | Coumarins | [34] |
| 17 | 9.222 | (-)-Epicatechin | $C_{15}H_{14}O_6$ | $(M-H)^-$ | 290.0788 | 289.0712 | −0.91 | Flavonoids | [37] |
| 18 | 9.222 | Norbraylin | $C_{14}H_{12}O_4$ | $(M+CHO_2)^-$ | 244.0733 | 289.0712 | −0.97 | Coumarins | [38] |
| 19 | 9.560 | 3,4,5-Trimethoxyphenyl-β-D-glucopyranoside | $C_{15}H_{22}O_9$ | $(M+CHO_2)^-$ | 346.1256 | 391.1236 | −2.25 | Glycosides | [39] |
| 20 | 9.777 | Gnetuhainin J | $C_{29}H_{24}O_8$ | $(M+H)^+$ | 500.1465 | 501.1531 | −1.24 | Stilbenoids | [40] |
| 21 | 9.961 | Foliachinenoside $A_1$ | $C_{26}H_{32}O_{14}$ | $(M+CH_3CO_2)^-$ | 568.1779 | 627.1916 | −2.30 | Glycosides | [41] |
| 22 | 10.049 | Toddaculin | $C_{16}H_{18}O_4$ | $(M+Na)^+$ | 274.1197 | 297.1091 | −3.00 | Coumarins | [42] |
| 23 | 10.167 | Foliachinenoside H | $C_{16}H_{28}O_{10}$ | $(M+CHO_2)^-$ | 380.1674 | 425.1661 | −2.18 | Glycosides | [43] |
| 24 | 10.167 | Phthalic acid | $C_8H_6O_4$ | $(M-H)^-$ | 166.0260 | 165.0188 | −3.52 | Phenolic acids | [44] |
| 25 | 10.282 | Vanillin | $C_8H_8O_3$ | $(M+CH_3CO_2)^-$ | 152.0473 | 211.0612 | −0.53 | Benzaldehydes | [45] |
| 26 | 10.358 | Noroxyhydrastinine | $C_{10}H_9NO_3$ | $(M+CHO_2)^-$ | 191.0581 | 236.0559 | −1.00 | Alkaloids | [46] |
| 27 | 10.905 | Coumurrayin | $C_{16}H_{18}O_4$ | $(M+Na)^+$ | 274.1205 | 297.1100 | −0.13 | Coumarins | [47] |
| 28 | 11.158 | *p*-Hydroxybenzaldehyde | $C_7H_6O_2$ | $(M-H)^-$ | 122.0367 | 121.0294 | −0.81 | Benzaldehydes | [45] |
| 29 | 11.349 | (+)-Abscisyl-β-D-glucopyranoside | $C_{15}H_{14}O_6$ | $(M-H)^-$ | 290.0784 | 289.0711 | −2.06 | Glycosides | [48] |
| 30 | 11.349 | Gnetol | $C_{14}H_{12}O_4$ | $(M+CHO_2)^-$ | 244.0729 | 289.0711 | −2.50 | Stilbenoids | [49] |
| 31 | 11.502 | Reticuline | $C_{19}H_{23}NO_4$ | $(M+CH_3CO_2)^-$ | 329.1620 | 388.1758 | −2.24 | Alkaloids | [50] |
| 32 | 11.762 | Isocorydine | $C_{20}H_{23}NO_4$ | $(M+CHO_2)^-$ | 341.1621 | 386.1606 | −1.77 | Alkaloids | [51] |
| 33 | 11.762 | (+)-Isoboldine | $C_{19}H_{21}NO_4$ | $(M+CH_3CO_2)^-$ | 327.1466 | 386.1606 | −1.52 | Alkaloids | [52] |
| 34 | 11.812 | 2′-hydroxy-2,4-dimethoxy-4′-*O*-[(E)-3,7-dimethyl-2,6-octadieny] chalcone | $C_{27}H_{32}O_5$ | $(M+H)^+$ | 436.224 | 437.2313 | −2.29 | Chalcones | [53] |
| 35 | 11.948 | Foliasalacioside J | $C_{19}H_{34}O_8$ | $(M+CHO_2)^-$ | 390.2252 | 435.2231 | −0.30 | Glycosides | [43] |
| 36 | 12.273 | Xanthoplanine | $C_{21}H_{26}NO_4$ | $(M+Na)^+$ | 356.1844 | 379.1746 | −4.88 | Alkaloids | [54] |
| 37 | 12.747 | Foveospirolide | $C_{15}H_{18}O_8$ | $(M+CHO_2)^-$ | 326.0995 | 371.0979 | –1.92 | Phenolic compounds | [55] |
| 38 | 13.264 | Isorhapontigenin-3-O-β-D-glucopyranoside | $C_{21}H_{24}O_9$ | $(M+CHO_2)^-$ | 420.1417 | 465.1399 | –0.71 | Glycosides | [56] |
| 39 | 14.769 | *Cis*-syringin | $C_{17}H_{24}O_9$ | $(M+CHO_2)^-$ | 372.1419 | 417.1399 | –0.40 | Glycosides | [43] |

| No. | RT (min) | Compound name | Chemical formula | $ESI^+/ESI^-$ | Observed mass (*m/z*) | Reference mass (*m/z*) | Mass error (ppm) | Class of compound | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 40 | 15.183 | Sorbic acid | $C_6H_8O_2$ | $(M+CH_3CO_2)^-$ | 112.0522 | 171.0661 | –2.01 | Fatty acids | [57] |
| 41 | 15.612 | Fibaruretin B | $C_{20}H_{24}O_7$ | $(M+Na)^+$ | 376.1508 | 399.1400 | –3.81 | Terpenoids | [58] |
| 42 | 16.090 | 20-hydroxyecdysone | $C_{27}H_{44}O_7$ | $(M+CHO_2)^-$ | 480.3080 | 525.3063 | –1.37 | Steroids | [59] |
| 43 | 16.291 | Gnetifolin E | $C_{21}H_{24}O_9$ | $(M+CHO_2)^-$ | 420.1416 | 465.1398 | –1.07 | Resveratrols | [60] |
| 44 | 16.608 | Foliasalacioside H | $C_{24}H_{44}O_{11}$ | $(M+CH_3CO_2)^-$ | 508.2878 | 567.3023 | –1.18 | Glycosides | [43] |
| 45 | 16.760 | Hesperidin | $C_{28}H_{34}O_{15}$ | $(M-H)^-$ | 610.1891 | 609.1819 | –1.15 | Flavanone glycosides | [38] |
| 46 | 16.815 | Foliachinenoside $B_1$ | $C_{21}H_{30}O_{11}$ | $(M+CH_3CO_2)^-$ | 458.1778 | 517.1916 | –2.27 | Glycosides | [41] |
| 47 | 17.388 | Parvifolol B | $C_{28}H_{22}O_7$ | $(M-H)^-$ | 470.1359 | 469.1285 | –1.43 | Flavonoids | [32] |
| 48 | 17.459 | 5,7-Dimethoxy-8-(3′-methylbuta-1,3′-dienyl)coumarin | $C_{16}H_{16}O_4$ | $(M+CHO_2)^-$ | 272.1050 | 317.1033 | 0.49 | Coumarins | [47] |
| 49 | 17.471 | Reticuline | $C_{19}H_{23}NO_4$ | $(M+Na)^+$ | 329.1620 | 352.1513 | –2.27 | Alkaloids | [50] |
| 50 | 17.529 | *N*-trans-feruloyltyramine | $C_{18}H_{19}NO_4$ | $(M-H)^-$ | 313.1306 | 312.1236 | –2.74 | Phenolic amides | [61] |
| 51 | 17.567 | *N*-trans-feruloyltyramine | $C_{18}H_{19}NO_4$ | $(M+Na)^+$ | 313.1310 | 336.1202 | –1.41 | Phenolic amides | [61] |
| 52 | 17.593 | Pinosylvin | $C_{14}H_{12}O_2$ | $(M+CHO_2)^-$ | 212.0837 | 257.0818 | –0.12 | Stilbenoids | [62] |
| 53 | 17.593 | Isorhapontigenin | $C_{15}H_{14}O_4$ | $(M-H)^-$ | 258.0892 | 257.0820 | –0.13 | Stilbenoids | [56] |
| 54 | 17.825 | Isodomesticine | $C_{19}H_{19}NO_4$ | $(M+Na)^+$ | 325.1310 | 348.1199 | –1.32 | Alkaloids | [29] |
| 55 | 18.100 | Parvifolol C | $C_{28}H_{22}O_8$ | $(M+H)^+$ | 486.1314 | 487.1384 | –0.20 | Flavonoids | [32] |
| 56 | 18.127 | Bergapten | $C_{12}H_8O_4$ | $(M+CH_3CO_2)^-$ | 216.0412 | 275.0550 | –4.67 | Coumarins | [63] |
| 57 | 18.340 | Citronellol | $C_{10}H_{20}O$ | $(M+CHO_2)^-$ | 156.1513 | 201.1495 | –0.59 | Terpenoids | [64] |
| 58 | 18.493 | 3,4,5-Trimethoxycinnamyl alcohol | $C_{12}H_{16}O_4$ | $(M-H)^-$ | 224.1044 | 223.0975 | –1.94 | Phenylpropanoids | [65] |
| 59 | 19.009 | Foveoeudesmenone | $C_{15}H_{24}O_2$ | $(M+CH_3CO_2)^-$ | 236.1768 | 295.1907 | –3.46 | Terpenoids | [55] |
| 60 | 19.229 | Toddanin | $C_{15}H_{16}O_5$ | $(M-H)^-$ | 276.0984 | 275.0916 | –4.95 | Coumarins | [47] |
| 61 | 19.592 | Menthol | $C_{10}H_{20}O$ | $(M+CHO_2)^-$ | 156.1509 | 201.1492 | –3.53 | Terpenoids | [66] |
| 62 | 19.924 | 17-Octadecen-9-ynoic acids | $C_{18}H_{30}O_2$ | $(M+CH_3CO_2)^-$ | 278.2244 | 337.2389 | –0.68 | Fatty acids | [67] |
| 63 | 19.998 | Citral | $C_{10}H_{16}O$ | $(M+CH_3CO_2)^-$ | 152.1197 | 211.1335 | –3.06 | Terpenoids | [68] |
| 64 | 20.123 | Derriscandenon E | $C_{25}H_{24}O_6$ | $(M+NH_4)^+$ | 420.1583 | 438.1922 | 2.49 | Flavonoids | [69] |
| 65 | 20.306 | 8-Hydroxy-r-guaiene | $C_{15}H_{24}O$ | $(M+CH_3CO_2)^-$ | 220.1818 | 279.1957 | –4.04 | Terpenoids | [70] |
| 66 | 20.505 | Islandicin | $C_{15}H_{10}O_5$ | $(M-H)^-$ | 270.0521 | 269.0449 | –2.73 | Anthraquinones | [71] |

RT; retention time, ESI; electrospray ionization, *m/z*; mass-to-charge ratio, Ref.; references

**Stability data**

The stability of YKSS extracts was evaluated by storing them under ambient conditions for one year. Stability was evaluated by comparing the TPC and TFC values at the initial time point and after one year of storage. **Figure 6** shows that both TPC and TFC

decreased after one year of storage. Specifically, TPC significantly decreased in 5 out of 8 samples, while TFC significantly decreased in 6 out of 8 samples. However, the stability of their biological and pharmacological activities was not determined in this study.

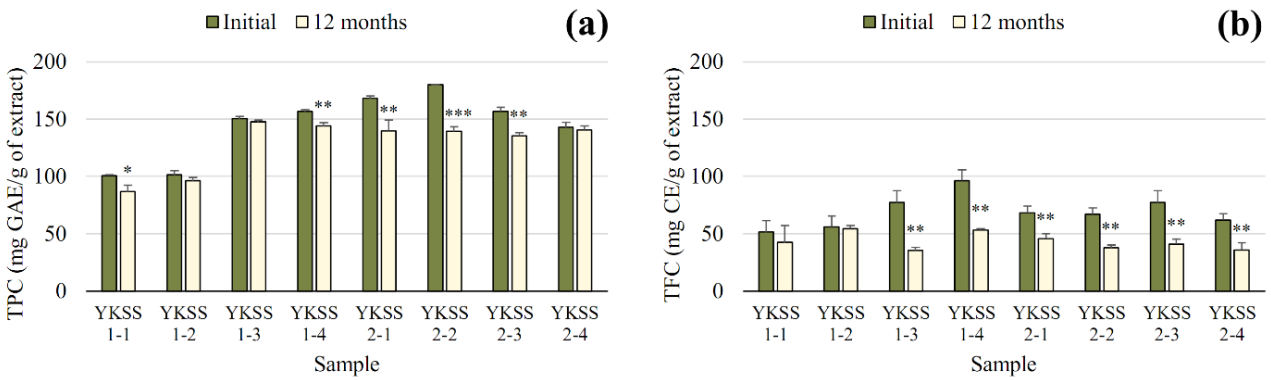


**Figure 6** Stability of TPC and TFC in YKSS samples stored under ambient conditions for 12 months.

**Acute toxicity**

After administering distilled water to the control group and 2,000 mg/kg body weight of YKSS extract to the test group, no deaths, abnormal signs, symptoms, or unusual behaviors were observed in either group during the first 24 h or throughout the 14-day observation period. Furthermore, no anomalies were detected in the rats following treatment with distilled water or YKSS extract. Additionally, there were also no significant differences in weight changes between the test and control groups.

**Analgesic activity**

The pain-relieving effectiveness of YKSS extract on acetic acid-induced abdominal constrictions and writhing is shown in **Figure 7**. The results demonstrated that YKSS at doses of 800 and 1,200 mg/kg significantly reduced the number of writhes compared to the control group, both after a single dose and following multiple doses (a seven-day repeated regimen). The standard drug, indomethacin (10 mg/kg), exhibited a pronounced analgesic effect by markedly reducing the writhing count. While YKSS extract showed an analgesic effect, its potency was lower than that of indomethacin. Notably, the analgesic effect of YKSS extract was greater with repeated dosing over seven days compared to a single dose (**Table 4**). These findings suggest that a multiple-dose regimen of YKSS is more effective than a single dose for pain relief.

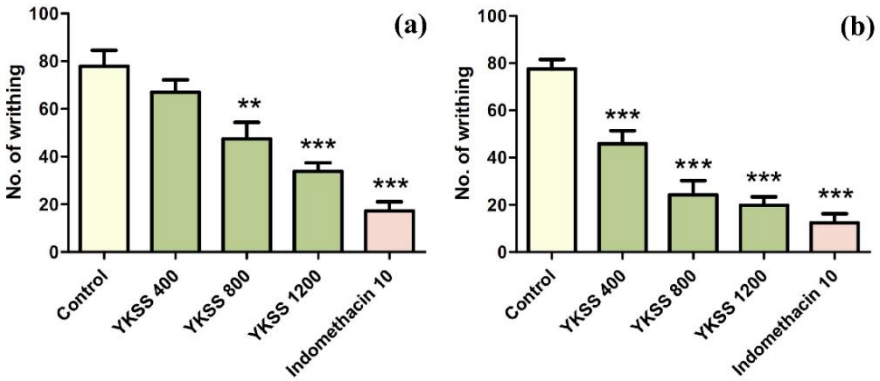


**Figure 7** Effect of YKSS on acetic acid-induced writhing in mice (n = 6 per group) after (a) a single dose and (b) seven days of repeated doses. Data are presented as the number of writhing responses recorded over 60 min post-acetic acid injection. Symbols *, ** and *** indicate significant differences compared to the control group, with $p$-value < 0.05, $p$-value < 0.01 and $p$-value < 0.001, respectively.

**Table 4** Comparison of analgesic effects between single and repeated doses of YKSS extract in the acetic acid-induced writhing test

| Treatment* | Percent pain inhibition (%) | |
|---|---|---|
| | Single dose | Repeated dose |
| YKSS 400 | 13.88 | 38.14 |
| YKSS 800 | 39.16 | 67.50 |
| YKSS 1200 | 56.52 | 74.27 |
| Indomethacin 10 | 77.94 | 83.33 |

*Indomethacin 10 refers to indomethacin at a dosage of 10 mg/kg body weight. YKSS 400, 800 and 1200 represent YKSS extract at dosages of 400, 800 and 1200 mg/kg body weight, respectively.

In the hot plate test, YKSS extract administered at doses of 400, 800 and 1200 mg/kg did not show a significant increase in latency time compared to the control group under both single-dose and seven-day repeated-dose conditions, as shown in **Figure 8**. Conversely, the standard drug, tramadol at 100 mg/kg, demonstrated a pronounced and significant increase in latency time, confirming its potent analgesic effect. This finding indicates that, while YKSS extract may have analgesic properties, it has not been shown to be as effective in thermal pain models as tramadol.

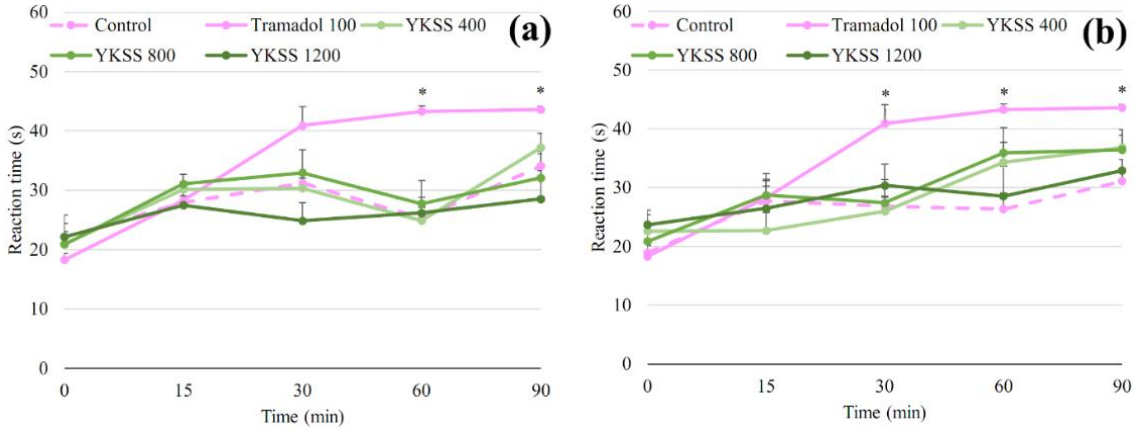


**Figure 8** The effect of YKSS on the pain threshold in the hot plate test following (a) a single dose and (b) a repeated dose (n = 6/group). Data are presented as the mean and SEM of reaction time (s) measured at 0, 15, 30, 60 and 90-min post-treatment. Symbols *, ** and *** indicate significant differences compared to the control group, with $p$-value < 0.05, $p$-value < 0.01 and $p$-value < 0.001, respectively.

**Capsule formulation**

The YKSS extracts were combined and filled into capsules, with each capsule containing approximately 360 mg of the extract. No capsule exhibited a weight variation exceeding ± 5% of the average weight. The formulation demonstrated rapid disintegration, taking approximately less than 4 min (**Table 5**). These results suggest that YKSS can be effectively prepared in capsule form, allowing for easy production and convenient administration for patients.

**Table 5** Physical properties of YKSS capsule.

| Topic | Individual weight (mg, n=20) | Disintegration time (min, n=6) |
|---|---|---|
| Mean ± SD | 361.85 ± 9.03 | 3.89 ± 0.66 |

**Discussion**

YKSS is a Thai folk herbal formula used in certain rural areas of Thailand. In practice, the plants are prepared by cutting or chopping the raw materials into small pieces, with the size depending on the original dimensions of the plant material—a larger plant results in larger pieces compared to smaller plants. Consequently, the mass ratios of the plant components in the formula are not consistent. Additionally, the composition of the formula can vary, as substitutions may be made when certain plants are unavailable. Moreover, the administration is flexible. Traditionally, one teacup of the decoction is consumed throughout the day as a substitute for water. Alternatively, some patients prefer a different regimen, consuming the decoction two or three times a day, depending on their lifestyle. For instance, farmers who are not working on a given day may consume it three or more times, whereas those engaged in farm work might limit their intake to twice daily, typically at breakfast and dinner. This formula has been shared through word of mouth, as it has not been recorded in the scientific or literary domain. This presents a significant challenge for scientific investigation of the formula. Despite these limitations, the authors have attempted to evaluate its activities by adhering to Thai folk knowledge and replicating the preparation method of this herbal formula.

Variations in the composition and mass ratios of herbal raw materials, as shown in **Table 1**, could influence the extraction yield, chemical constituents, as well as efficacy of the formulation. This is evident in the differing extraction yields of YKSS Batch 1, which contains *C. orientalis* root, compared to YKSS Batch 2, which includes *V. denticulata* stem. The higher extraction yield of Batch 1 can be attributed to differences in formula composition and the nature of the plant raw materials. Batch 2 includes a larger, harder *V. denticulata* stem, resulting in lower extractive yields. In contrast, the *C. orientalis* root, despite its smaller size and lower ratio in the formulation, is more readily extracted, leading to a higher yield. Explaining the nature of herbal formulas comprising numerous plant components is challenging due to the difficulty in proving interactions among the individual components. However, previous studies have demonstrated that plant components can interact with each other, influencing the quantity of chemical constituents extracted from Thai herbal formulations. This phenomenon has been observed in formulas such as Triphala [72], Trisamo [73], Chatuphalathika [74] and Trikatuk [75] formulas. Moreover, the synergistic effect of plant compositions enhancing activity beyond that of individual components has also been demonstrated in previous studies [76,77]. The synergistic activity of the herbal formula may arise through multiple mechanisms, including multi-target effects, enhanced oral bioavailability, reversal of drug resistance, mitigation of adverse effects and amplification of the pharmacological potency of compounds within the herbal extracts [78].

Consequently, variations in extraction yield, TPC and TFC across YKSS samples may influence their antioxidant activity. This study evaluated antioxidant activity using three assays: DPPH radical scavenging, FRAP and NO scavenging. For the DPPH radical scavenging assay, the $IC_{50}$ values of all YKSS samples ranged between 50 and 100 µg/mL, indicating strong antioxidant activity [79,80]. Consistent with the FRAP assay, the YKSS extracts demonstrated high FRAP activity, signifying the presence of potent electron-donating antioxidants capable of reducing ferric ions ($Fe^{3+}$) to ferrous ions ($Fe^{2+}$), which is indicative of strong antioxidant activity [81]. Regarding NO scavenging activity, the YKSS extract exhibited low activity, as even at high concentrations, it failed to achieve an $IC_{50}$ value.

Almost all of the plants, including *S. chinensis* [82,83], *E. paniculata* [84,85], *D. lanceifolia* [86], *C. orientalis* [87], *D.* scandens [88,89], *G. macrostachym* [85,90,91], *G. montanum* [92], *L. cubeba* [26,93-102], *A. flava* [103-105], *C. fenestratum* [46,106-108], *F. foveolata* [109,110], *K. angustifolia* [85,111,112], *V. denticulata* [85,113] and *T. asiatica* [114-117], exhibited antioxidant activity. Furthermore, several specific compounds based on LC-MS profiles, have been reported to exhibit antioxidant activity, including *N*-trans-feruloyltyramine [118-120], citric acid [121-123], vanillic acid [124-126], isopimpinellin [127], (-)-epicatechin [128], vanillin [129-131], *p*-hydroxybenzaldehyde [132-134], gnetol [135], *cis*-syringin [136], 20-hydroxyecdysone [137-139], hesperidin [140-142], pinosylvin [143], isorhapontigenin [144-146], bergapten [147,148],

citronellol [149], menthol [150] and citral [151]. It can be described that certain individual and combined plant compositions, along with antioxidant compounds, could enhance the antioxidant activity of YKSS.

Anti-inflammatory activity was evaluated using two assays: NO production inhibition and anti-LOX activity. The results revealed that YKSS extract exhibited low activity in inhibiting NO production, a key inflammatory mediator, as evidenced by the high $IC_{50}$ values. Interestingly, the YKSS extract demonstrated strong ($IC_{50}$ of 50 - 100 μg/mL) to very strong ($IC_{50}$ < 50 μg/mL) anti-LOX activity, indicating its potential effectiveness in inhibiting LOX [79,80]. These findings suggest that the anti-inflammatory activity of YKSS extract is primarily mediated through LOX inhibition. However, further investigations are warranted to explore additional mechanisms, such as the inhibition of cyclooxygenase (COX)-1, COX-2, leukotrienes, prostaglandins and proinflammatory cytokines, etc. [152].

Several plants, including *S. chinensis* [153], *D. scandens* [88,154-156], *G. macrostachym* [91], *G. Montanum* [157,158], *L. cubeba* [101,102,159-165], *A. flava* [166-168], *C. fenestratum* [169,170], *F. Foveolata* [171], *V. denticulata* [172], *T. asiatica* [117,173-176] and *S. pentandrum* [177], exhibited anti-inflammatory activity. Furthermore, several specific compounds based on LC-MS profiles, have been reported to exhibit anti-inflammatory activity, including toddaculin [176], xanthoplanine [178], reticuline [164,179], *N*-trans-feruloyltyramine [120,180], citric acid [123], vanillic acid [124-126,181-184], isopimpinellin [185], (-)-epicatechin [186,187], phthalic acid [188], vanillin [129-131,189-191], *p*-hydroxybenzaldehyde [132,192,193], gnetol [194], isocorydine [195], (+)-isoboldine [196], *cis*-syringin [136,197,198], 20-hydroxyecdysone [137,199-201], hesperidin [140-142,202,203], pinosylvin [143,204], isorhapontigenin [205], bergapten [147,148,206-210], citronellol [149, 211-215], menthol [150, 216-218] and citral [219-222].

Among all the plant components, *D. scandens* has the most reports on anti-inflammatory activity. The anti-inflammatory effect of *D. scandens* extract was not attributed to its antioxidant or anti-oxidative stress properties but likely stemmed from its ability to inhibit multiple pro-inflammatory targets, including cytokines like interleukin (IL)-1α, IL-1β and IL-6, as well as COX-2 and matrix metalloproteinases (MMP) such as MMP-1 and MMP-9 [154]. Other study found that *D. scandens* extract demonstrated the ability to inhibit NO production and reduce the expression of inducible nitric oxide synthase (iNOS), COX-2, IL-6 and 5-LOX [155]. The anti-inflammatory biomarkers identified in *D. scandens* include genistein-7-O-[α-rhamnopyranosyl-(1 → 6)]-β-glucopyranoside, genistein, derrisisoflavone A, lupalbigenin and 6, 8-diprenylgenistein [155]. The analgesic activity of *D. scandens* in humans has been demonstrated in several studies [223-226], as well as in a meta-analysis of randomized controlled trials [227].

This work used HepG2 cells for evaluation of safety of YKSS. According to ISO 10993-5:2009, a sample is considered toxic if cell viability is less than 70% compared to the control group [228]. The concentrations at which toxicity (viability < 70%) was observed were relatively high: 1 mg/mL for YKSS 1-3; 5 mg/mL for YKSS 1-2, 1-4 and 2-2; and 10 mg/mL for YKSS 1-1, 2-1, 2-3 and 2-4.

Based on the typical blood volume of an adult (approximately 5 L) [229], if the entire extract were absorbed, the total concentration of the extract in the blood would be approximately 3,260 mg/5 L, equivalent to 0.652 mg/mL. This concentration is below the toxic threshold. However, in practical use, not all of the extract is absorbed. Additionally, as one pot of the formula is re-boiled with the addition of more water and consumed over four to five days, the extract becomes more diluted by the final day of use compared to the first day of preparation. Therefore, consumption of this herbal formula is considered safe.

Stability data revealed that TPC and TFC appeared to be unstable in some samples. However, the activity of the samples after the stability test was not assessed in this study. The authors noted that a biomarker with a higher quantity should be selected based on LC-MS profiles. In this case, *N*-trans-feruloyltyramine is a potential candidate biomarker. However, further investigation of its properties and stability is required before it can be confirmed as the selected biomarker.

The *in vivo* study was conducted to evaluate both the safety and effectiveness of the YKSS formula. The assessment of acute oral toxicity was in compliance with the protocols outlined by the OECD guideline 420 [23]. The results showed that YKSS did not cause death or other notable alterations in animal behavior. The

findings indicate that YKSS is a safe and non-toxic herbal product. YKSS has been traditionally used in folk medicine for pain relief, yet there has been a lack of scientific validation of its effectiveness. Therefore, this study focuses on assessing the pain-relieving of YKSS through experiments with animal models. The study employed both the acetic acid-induced writhing and hot plate tests to measure the analgesic effects, which are indicative of peripheral and central nervous system actions, respectively.

In the acetic acid-induced writhing test, the administration of acetic acid prompts a series of reactions in mice, manifesting as abdominal pain. This pain stimulates various physical responses, including abdominal contractions, body distortions, back arching, and uncoordinated movements. These symptoms serve as indicators of the pain and discomfort experienced by the animal due to the irritation of the peritoneal membrane [230,231].

This study demonstrated that YKSS at doses of 400, 800 and 1200 mg/kg significantly reduced abdominal constrictions in a dose-dependent manner. The reduction in writhing episodes after YKSS administration suggests its potential to mitigate pain and inflammation. Notably, YKSS at the highest dose (1200 mg/kg) was as effective in inhibiting acetic acid-induced writhing as the standard drug, indomethacin, highlighting its antinociceptive properties. The pain associated with acetic acid is likely due to nociceptive nerve fiber activation and a reduction in abdominal pH, which triggers the release of inflammatory mediators such as serotonin, histamine, bradykinin and prostaglandins [232,233]. Moreover, enhances the synthesis of prostaglandins ($PGE_2$ and $PGF_{2\alpha}$) via the COX pathway, which sensitizes nociceptors and amplifies pain perception [234]. The decrease in writhing behavior observed with the administration of indomethacin in this study supports the pain-relieving properties of NSAIDs. A reduction in the writhing response suggests that the YKSS formula may contain a COX inhibitor similar to NSAIDs, which suppresses prostaglandin production and reduces inflammation. In addition, phenolic and flavonoid compounds found in YKSS have been reported to exhibit COX-2 inhibitory effects, providing natural anti-inflammatory properties [235-237]. Acetic acid also triggers the formation of reactive oxygen species (ROS), which contribute to oxidative stress, mitochondrial dysfunction and increased inflammation [238]. Oxidative stress enhances nociceptive signaling by activating NF-κB, leading to an increased release of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), which further intensify pain pathways [239]. The presence of antioxidant compounds in the YKSS formula suggests that it scavenges ROS and inhibits NF-κB activation, thereby reducing pain-related inflammation. The ability of the YKSS formula to reduce writhing responses may be attributed not only to COX inhibition but also to its antioxidant activity.

In this study, YKSS markedly reduced the writhing response induced by acetic acid, both after a single administration and following seven days of repeated dosing. A comparative analysis of the analgesic effects showed that repeated doses of YKSS were more effective than a single dose, as evidenced by the results presented in **Table 4**. These findings indicate that a regimen involving multiple doses of YKSS is more efficacious in alleviating pain than a one-time dose. This supports the traditional practice of consuming YKSS throughout the day instead of water, highlighting its enhanced effectiveness in pain management when taken repeatedly.

The hot plate test, which employs thermal stimulation, was used to evaluate the antinociceptive activity of YKSS through central pathways [240]. This test involves observing licking or jumping reflexes resulting from supraspinal sensory integration [240,241]. The findings revealed that YKSS did not significantly prolong the latency period to thermal stimuli, indicating that central mechanisms do not mediate its antinociceptive effects. In contrast, tramadol, the reference drug used in the study, demonstrated the most pronounced analgesic effect at all observed times, confirming its effectiveness in modulating pain transmission within the central nervous system [242].

The YKSS formula demonstrated significant analgesic effects in the acetic acid-induced writhing test, suggesting its effectiveness in inhibiting peripheral pain signals. However, it did not show notable analgesic activity in the hot plate test, which evaluates central pain pathways. This suggests that the analgesic properties of YKSS are primarily peripheral and do not extend to central nervous system mechanisms. This pattern of

activity is similar to that observed with another Thai folk medicine, the Yafon formula [243]. As mentioned earlier, YKSS contains 18 herbs, and the formula is used for pain treatment. Several plants, including *S. chinensis* [244], *D. scandens* [223-226], *G. montanum* [157], *L. cubeba* [245], *C. fenestratum* [170], *T. asiatica* [173,175] and *S. pentandrum* [246,247], the component of YKSS, has analgesic activity. Furthermore, several specific compounds based on LC-MS profiles, have been reported to exhibit analgesic activity, including vanillic acid [124,182], (-)-epicatechin [187], vanillin [184,189], *p*-hydroxybenzaldehyde [193], gnetol [194], hesperidin [202,203], bergapten [206], citronellol [212], menthol [218,248,249] and citral [151,220-222].

The YKSS extract was preliminarily prepared in capsule form and was easily filled into capsule shells. However, the authors noted that the extract was prepared using a freeze-drying technique due to the availability of equipment in the laboratory. This method may not be economical for industrial-scale production. Spray drying could be a more suitable technique for this formula, as it involves heat, which aligns with the decoction extraction process. Additionally, the powder obtained from spray drying is likely to have better flowability, which would improve the formulation process [250].

## Conclusions

This study provides scientific evidence supporting the biological and pharmacological activities of YKSS, a traditional Thai folk analgesic herbal formula used in rural areas of Thailand. The formula exhibited significant antioxidant, anti-inflammatory and analgesic properties, demonstrated through *in vitro* assays, including DPPH radical scavenging and FRAP assays, which highlighted its antioxidant potential. The anti-inflammatory effects were further validated by the inhibition of NO production and anti-LOX activity. Cytotoxicity studies confirmed the safety of YKSS on HepG2 cells, suggesting its potential for therapeutic applications. The chemical profile, identified through LC-MS, revealed active compounds such as *N*-trans-feruloyltyramine and maltose, contributing to its bioactivity. Stability testing confirmed that some extracts maintained their viability under ambient conditions for up to one year, supporting practical use. *In vivo* studies revealed a favorable safety profile, with no acute toxicity observed at 2,000 mg/kg body weight. Analgesic activity was supported by a significant reduction in acetic acid-induced writhing episodes, indicating peripheral nociception inhibition as the primary pain relief mechanism. However, no significant effects were noted in the hot plate test, suggesting limited central analgesic activity. The successful development of a capsule formulation highlights the potential for standardizing this traditional remedy.

Future research should focus on investigating the molecular mechanisms underlying YKSS's bioactivities, evaluating its efficacy in clinical conditions, and assessing its potential for integration into evidence-based medicine. Additionally, refining its formulation and establishing standardized dosages will be essential to maximize its therapeutic potential. This study not only reinforces the scientific basis for the therapeutic use of traditional herbal medicines but also highlights the importance of preserving and validating indigenous knowledge for broader clinical applications.

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## Declaration of Generative AI in Scientific Writing

During the preparation of this work, the authors used ChatGPT 4o-mini in order to proofread and correct grammatical errors during the manuscript writing process. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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