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Total flavonoid and phenolic contents, xanthine oxidase inhibition, antioxidant and anti-inflammatory activities of ethanolic and aqueous extracts of the *Ya-Tom-Kae-Ka-Sai-Sen* remedy, a traditional Thai medicine

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ABSTRACT

Introduction: *Ya-Tom-Kae-Ka-Sai-Sen* (KS) is a traditional Thai decoction used for muscle and joint pain relief. This study evaluated the phenolic and flavonoid contents, xanthine oxidase inhibitory, antioxidant, and anti-inflammatory activities of KS extracts.

Methods: Five KS extracts were prepared by refluxing with 95% ethanol (KS95ER), 50% ethanol (KS50ER), and water (KSWR), and by maceration with 95% ethanol (KS95EM) and 50% ethanol (KS50EM). Total phenolic and total flavonoid contents were determined. *In vitro* assays of xanthine oxidase (XO) inhibition, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, and nitric oxide (NO) inhibition in lipopolysaccharide (LPS)-induced RAW 264.7 cells were performed.

Results: Total phenolic content ranged from 71.45 to 116.57 mg gallic acid equivalent (GAE)/g and total flavonoid content from 317.21 to 442.62 mg quercetin equivalent (QE)/g. The 95% ethanol extracts (KS95ER, KS95EM) showed the highest TFC ($P < 0.05$). At 1 mg/mL, ethanolic extracts showed superior *in vitro* XO inhibition (56.85–84.36%) compared to the aqueous extract (35.39%) ($P < 0.05$). KS95ER exhibited potent XO inhibition with an IC_{50} of 181.00 µg/mL. Its DPPH scavenging activity (IC_{50} 37.57 µg/mL) was comparable to ascorbic acid ($P > 0.05$). KS95ER and KS95EM showed the highest NO inhibition, with IC_{50} values (90.29 and 78.13 µg/mL, respectively) that were comparable to indomethacin ($P > 0.05$). Furthermore, all extracts were found to be non-cytotoxic at the tested concentrations.

Conclusion: Ethanolic KS extracts, especially KS95ER and KS95EM, show potent XO inhibitory and antioxidant activities and anti-inflammatory effects, supporting their traditional use and warranting further study for developing therapies for gout and inflammation.

Implication for health policy/practice/research/medical education:
This study offers the first scientific validation for the traditional use of the *Ya-Tom-Kae-Ka-Sai-Sen* (KS) remedy against gouty arthritis and other inflammatory, oxidative stress-related conditions. The findings provide a robust pharmacological basis to support the development of standardized KS extracts for clinical practice and guide future *in vivo* research by identifying the 95% ethanolic extracts as the most promising candidates to confirm therapeutic efficacy.

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Introduction

Gout, a prevalent inflammatory arthritis, results from hyperuricemia, a condition linked to metabolic syndrome and cardiovascular risk (1,2). The global burden of gout is substantial and escalating, projected to increase from 55.8 million cases in 2020 to 95.8 million by 2050 (1). This trend is mirrored in Thailand, where hyperuricemia prevalence in Bangkok markedly increased from 10.6% in 1999–2000 to 24.4% in 2009–2010 (2). Consistently, both global and local data confirm that the prevalence

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is significantly higher in males and increases with age, underscoring a growing public health challenge (1,2).

At the heart of these conditions is xanthine oxidase (XO), a key enzyme in purine metabolism that catalyzes the final steps of uric acid production (3). XO overactivity leads to the overproduction of uric acid and the concurrent generation of reactive oxygen species (ROS), contributing not only to hyperuricemia and gout but also to a wide array of oxidative stress-related diseases, including cardiovascular disorders, diabetes, and cancer (3). Current pharmacological treatments, such as allopurinol and febuxostat, are effective but are limited by significant adverse effects, including severe hypersensitivity reactions and liver dysfunction (4). These limitations underscore the urgent need for safer therapeutic alternatives.

Herbal medicines offer a promising approach, as many contain phenolics and flavonoids that are potent XO inhibitors and also exert antioxidant and anti-inflammatory effects (4,5). *Ya-Tom-Kae-Ka-Sai-Sen* (KS) is a polyherbal decoction listed in the National Thai Traditional Medicine Formulary for treating muscle and joint pain, conditions often associated with inflammatory processes like gout (6). The remedy consists of seven herbs: the rhizome of *Boesenbergia rotunda* (L.) Mansf., the rhizome of *Cyperus rotundus* L., the stem of *Maerua siamensis* (Kurz) Pax., the vine of *Ventilago denticulata* Willd., the stem of *Putranjiva roxburghii* Wall., the bark of *Diospyros rhodocalyx* Kurz, and the fruit of *Terminalia chebula* Retz. (6).

Previous studies report that most individual herbs in the KS formula possess XO inhibitory, antioxidant, and anti-inflammatory properties, largely attributed to their phenolic and flavonoid contents (7-25). However, the therapeutic efficacy of polyherbal remedies often arises from synergistic interactions among their components (26). To date, no scientific assessment has validated the bioactivity of the entire seven-herb KS formulation. This represents a critical research gap, as studying the complete remedy is essential to understanding its holistic therapeutic potential. Therefore, to scientifically validate the remedy's traditional use against muscle and joint pain, we selected three bioactivities that target the underlying mechanisms of such conditions. Since joint pain is often caused by inflammation and associated oxidative stress, we evaluated the direct anti-inflammatory (nitric oxide inhibition) and antioxidant (DPPH scavenging) activities. Furthermore, because gout is a severe form of inflammatory arthritis directly linked to XO activity, we also assessed the XO inhibitory potential of the extracts to investigate a specific mechanism relevant to joint disease. We evaluated the total phenolic and flavonoid contents of various ethanolic and aqueous extracts of the complete KS formulation and correlated these with the bioactivities. The findings aim to establish a scientific basis for the future development of the KS remedy as a therapeutic agent for gout, inflammation, and oxidative stress-related disorders.

Materials and Methods

Plant materials

Four dried herbs—*B. rotunda* (rhizome), *C. rotundus* (rhizome), *M. siamensis* (stem), and *T. chebula* (fruit)—were purchased from an herbal store in Bangkok, while *V. denticulate* (vine), *P. roxburghii* (stem), and *D. rhodocalyx* (bark) were collected from Ubon Ratchathani. Dr. Nuttapong Wichai identified all the plant species. Fresh samples were sliced, air-dried, and ground into fine powders. Equal portions of each herb were mixed for extraction. Herbarium codes were assigned at the Faculty of Pharmacy, Mahasarakham University, as follows: MSU.PH-ZIN-B1 (*B. rotunda*), MSU.PH-CYP-C1 (*C. rotundus*), MSU.PH-CAP-M1 (*M. siamensis*), MSU.PH-COM-T1 (*T. chebula*), MSU.PH-RHA-V1 (*V. denticulata*), MSU.PH-PUT-P1 (*P. roxburghii*), and MSU.PH-EBE-D1 (*D. rhodocalyx*).

Chemical reagents

Chemical reagents were obtained from the following suppliers: xanthine and allopurinol (Wako, Japan); xanthine oxidase (Calbiochem®, Merck, USA); uric acid, quercetin, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Merck, USA); methanol (Labscan Asia, Thailand); Folin-Ciocalteu reagent (LOBA CHEMIE, India); sodium dihydrogen phosphate (Merck, Germany); ethanol, sodium nitrite, sodium hydroxide, sodium carbonate, formic acid, N-(1-Naphtyl)ethylenediamine dihydrochloride, sulphanilamide, and orthophosphoric acid (CARLO ERBA, France); gallic acid (ACROS ORGANICS, Belgium); aluminum chloride (Ajax Finechem, Australia); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, Thermo Fisher Scientific, USA); dimethyl sulfoxide (DMSO) (PanReac AppliChem, Spain); Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic (Gibco, Thermo Fisher Scientific, USA).

Preparation of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts

The extraction protocol was designed to systematically evaluate the effects of two key parameters: temperature and solvent polarity. To assess the influence of temperature on the extraction of bioactive compounds, two methods were employed: maceration (extraction at room temperature) and reflux (heat-assisted extraction) (27). Concurrently, to ensure comprehensive phytochemical extraction, a solvent polarity gradient was established utilizing water and ethanol, a solvent favored for its low toxicity and established safety for human consumption, making it suitable for developing therapeutic products (28). The specific solvent systems included water, to simulate

traditional decoction methods and extract highly polar compounds (6), 50% ethanol, a hydroalcoholic system known for its efficacy in extracting a broad spectrum of polyphenols, and 95% ethanol, to target less polar constituents such as flavonoid aglycones (29,30).

The powdered herbal mixture (seven herbs in equal proportions) was extracted using reflux and maceration. Reflux was performed using 95% ethanol, 50% ethanol, and water to obtain KS95ER, KS50ER, and KSWR, respectively (30 g powder in 300 mL solvent, 3 h per cycle, 3 cycles). Maceration with 95% and 50% ethanol yielded KS95EM and KS50EM (80 g powder in 800 mL solvent, 24 h, thrice). The extracts were filtered, the water extract was freeze-dried, and the ethanol extracts were evaporated at 70–80 °C using a rotary evaporator (R-100 Rotavapor®, Buchi, USA). The crude extract weight and percent yield were recorded.

Total phenolic content assay

The assay protocol was performed following a protocol adapted from a previous study (31). KS extracts were dissolved in DMSO to prepare stock solutions with a concentration of 500 µg/mL. For the assay, 20 µL of extract was mixed with 100 µL of diluted Folin-Ciocalteu reagent (1:10) and 80 µL of 1 M sodium carbonate in a 96-well plate. After 10 minutes of incubation at room temperature, the absorbance was measured at 630 nm using a UV-visible spectrometer (Varioskan LUX, Thermo Fisher Scientific, USA). The total phenolic content was calculated as mg gallic acid equivalent (GAE)/g extract using a gallic acid standard curve (0–500 µg/mL).

Total flavonoid content assay

The assay was adapted from a previous study (31). In a 96-well plate, 50 µL of KS extract was mixed with 20 µL of 5% sodium nitrite, 20 µL of 10% aluminum chloride, and 100 µL of 1 M NaOH. After gentle shaking and 10 min of incubation at room temperature, the absorbance was measured at 510 nm using a UV-visible spectrometer. The total flavonoid content was calculated as mg quercetin equivalent (QE)/g extract using a quercetin standard curve (0–800 µg/mL).

Xanthine oxidase activity assay

This method was adapted from Dhammaraj et al (32). XO was diluted 1:20 in 100 mM sodium dihydrogen phosphate buffer (pH 7.5). In a 96-well plate, 20 µL of 1 mM xanthine and 170 µL of buffer were mixed, and the baseline absorbance at 295 nm was recorded for 1 min. Then, 10 µL of the enzyme solution was added, and the absorbance was measured for 5 minutes. Enzyme activity (units/mL) was calculated from the change in absorbance per minute using a uric acid standard curve (20–60 µM) (Figure S1), where 1 unit = 1 µmol of substrate converted per minute.

Xanthine oxidase inhibitory assay

The assay was adapted from the method described by Dhammaraj et al (32). Enzyme inhibition was assessed in a 500 µL reaction mixture alongside a negative control (DMSO). A master mix containing XO (final concentration: 0.60 mU/mL) was combined with the test sample (final concentration: 1 mg/mL) and incubated at 25 °C for 10 minutes. Xanthine was added (final concentration: 100 µM), followed by an additional 10-minute incubation at 25 °C. The reaction was quenched by adding an equal volume of 0.2 M HCl. The mixture was filtered using a 10 kDa cut-off filter (Amicon® Ultra, Germany). Uric acid levels were quantified using HPLC-UV-DAD (Agilent 1260 Infinity II) with a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) and a gradient mobile phase of 0.1% formic acid–methanol (MeOH). The flow rate was 0.5 mL/min at ambient temperature, with detection at 270 nm and 295 nm. The percent inhibition was calculated using equation 1 (Eq. 1), and IC_{50} values were determined from inhibition-concentration curves using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Each sample was tested in triplicate across three independent experiments.

$$\%\,Xanthine\ oxidase\ inhibition = \frac{peak\ area\ of\ uric\ acid_{control} - peak\ area\ of\ uric\ acid_{sample}}{peak\ area\ of\ uric\ acid_{control}} \times 100 \quad \text{Eq. (1)}$$

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH assay was performed as previously described (32). The crude extracts were dissolved in DMSO. In a 96-well plate, 180 µL of 0.1 mM DPPH in ethanol was mixed with 20 µL of the sample at the desired concentration. After shaking and incubating in the dark at room temperature for 30 minutes, the absorbance was measured at 517 nm. DMSO was used as the negative control. All tests were performed in triplicate. The percentage of radical scavenging was calculated (Eq. 2), and IC_{50} values were determined from the inhibition-concentration plots.

$$\%\,DPPH\ radical\ scavenging = \frac{Absorbance\,517\,nm_{control} - Absorbance\,517\,nm_{sample}}{Absorbance\,517\,nm_{control}} \times 100 \quad \text{Eq. 2}$$

Nitric oxide (NO) inhibition analysis in RAW264.7 cells and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

RAW264.7 cell line (ATCC, USA) was maintained in DMEM supplemented with 10% FBS and 1% Antibiotic-Antimycotic prior to use. The protocol was modified from that of previous studies (33). RAW264.7 cells were seeded in 96-well plates at a density of 1,000 cells/µL and incubated for 24 hours. The cells were then separately treated with five different extracts (KS95ER, KS95EM, KS50ER, KS50EM, and KSWR) at a final concentration of 100 µg/mL, along with 1 µg/mL lipopolysaccharide (LPS)

in DMEM, and incubated for an additional 24 hours. Untreated control cells underwent the same protocol as the treated cells, except that the extract was replaced with 0.2% DMSO in DMEM. Nitric oxide (NO) levels in the culture medium were determined using Griess reagent, and the absorbance was measured at 540 nm. NO inhibition is expressed as a percentage relative to that of the untreated control (Eq. 3). All experiments were performed in triplicate.

$$\%\,Nitric\,oxide\,(NO)\,inhibition = \frac{Absorbance\,540\,nm_{control} - Absorbance\,540\,nm_{sample}}{Absorbance\,540\,nm_{control}} \times 100 \quad \text{Eq. 3}$$

For cytotoxicity evaluation, MTT was added to the remaining cells and incubated for 3 hours. After formazan formation, the medium was discarded, and the formazan was dissolved in DMSO, and the absorbance was recorded at 570 nm. Cell viability was calculated as a percentage relative to the untreated control (Eq. 4).

$$\%\,Cytotoxicity = \frac{Absorbance\,570\,nm_{control} - Absorbance\,570\,nm_{sample}}{Absorbance\,570\,nm_{control}} \times 100 \quad \text{Eq. 4}$$

Extracts showing significant NO inhibition were selected for IC_{50} determination. Indomethacin was used as the positive control. IC_{50} values were calculated from inhibition-concentration curves using GraphPad Prism 6.0. All experiments were conducted independently in triplicate.

Statistical analysis:

Data are presented as mean ± standard deviation (SD). Statistical analyses were performed using IBM SPSS Statistics v29 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) followed by the Scheffé's post hoc test was used for normally distributed data, whereas the Kruskal-Wallis test with the Mann-Whitney U test was used for non-normally distributed data. Statistical significance was set at $P < 0.05$.

Results

Preparation and yields of *Ya-Tom-Kae-Ka-Sai-Sen* extracts

In this study, KS remedy extracts were prepared using different solvents and extraction methods, as described in the Materials and Methods section. KS95ER, KS50ER, and KSWR were obtained via reflux extraction using 95% ethanol, 50% ethanol, and water, respectively. KS95EM and KS50EM were prepared using a maceration method with 95% and 50% ethanol, respectively. Figure S2 (Supplementary file 1) illustrates the physical characteristics of the KS extracts. The yields of the KS extracts are listed in Table 1.

Total phenolic content and total flavonoid content of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts

The total phenolic and flavonoid contents of the KS extracts were determined and expressed as milligrams of gallic acid equivalent (mg GAE/g dry weight) and milligrams of quercetin equivalent (mg QE/g dry weight), respectively. Each extract was analyzed in triplicate. The results are presented in Table 1. Standard calibration curves for gallic acid and quercetin are provided in the Supplementary Information (Figures S3 and S4).

Although KS95ER showed the highest mean value for total phenolic content (116.57 ± 4.03 mg GAE/g), there were no statistically significant differences among the ethanolic extracts (KS95ER, KS50ER, KS95EM, and KS50EM). In contrast, the aqueous extract (KSWR) yielded the lowest phenolic content (71.45 ± 3.67 mg GAE/g), which was significantly lower than all of the ethanolic extracts ($P < 0.05$) (Table 1). These results suggest that the ethanol-based extracts (95% and 50%) yielded higher phenolic content than the water extract.

The 95% ethanol extracts (KS95ER and KS95EM) produced the highest total flavonoid contents, and the two extracts were not significantly different from each other. These contents were, however, significantly higher than those found in the 50% ethanol and aqueous extracts ($P < 0.05$) (Table 1). These findings highlight that ethanol extraction, particularly at a 95% concentration, yielded

Table 1. Percentage yield, total phenolic content, and total flavonoid content of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts

***Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts**	**%Yield of crude extracts**	**Total phenolic content (mg GAE/g of dry weight)**	**Total flavonoid content (mg QE/g of dry weight)**
KS95ER	11.21 ± 0.01	116.57 ± 4.03[a]	442.62 ± 12.52[c]
KS95EM	13.75 ± 0.13	110.30 ± 7.58[a]	433.13 ± 12.82[c]
KS50ER	15.91 ± 0.02	113.21 ± 17.04[a]	340.54 ± 31.65[d]
KS50EM	17.56 ± 0.77	102.54 ± 5.18[a]	317.21 ± 16.4[d]
KSWR	15.02 ± 0.02	71.45 ± 3.67[b]	318.61 ± 11.09[d]

Abbreviation: KS95ER: 95% ethanol reflux extract; KS95EM: 95% ethanol maceration extract; KS50ER: 50% ethanol reflux extract; KS50EM: 50% ethanol maceration extract; KSWR: water reflux extract; GAE: gallic acid equivalent; QE: quercetin equivalent.
All data are represented as mean ± SD (n = 3). Within the same column, values marked with different superscript letters (a, b, c, d) are significantly different from each other ($P < 0.05$), as determined by the Kruskal-Wallis test followed by the Mann-Whitney U test.

higher flavonoid levels than 50% ethanol and water extraction.

In vitro xanthine oxidase inhibitory assay of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts

The *in vitro* XO inhibitory activities of the KS extracts were evaluated as described in the Materials and Methods section. Each extract was tested in triplicate. HPLC chromatograms of the reactions between XO and xanthine with or without KSWR are shown in Figure S5, along with the xanthine and uric acid chromatograms. The retention times of xanthine and uric acid in the reactions were 14.93 and 12.90 minutes, respectively, matching those of authentic compounds.

The inhibitory activities of the KS extracts (1 mg/mL) were determined by comparing the peak areas of uric acid in the control and sample reactions (Table 2). Allopurinol (1 μg/mL) was used as a positive control and exhibited 77.62 ± 23.86% inhibition. The XO inhibitory activities of the ethanolic extracts (KS95ER, KS95EM, KS50ER, and KS50EM) were statistically comparable to the positive control, allopurinol. In contrast, the aqueous extract (KSWR) demonstrated significantly lower activity than all other tested groups ($P < 0.05$).

As all ethanolic extracts demonstrated potent and statistically comparable inhibitory activity, KS95ER was selected as a representative candidate for IC_{50} determination based on its high mean inhibition value. Inhibition-concentration curves from three independent experiments were plotted using GraphPad Prism 6.0, and the IC_{50} value of KS95ER was calculated to be 181.00 ± 7.55 μg/mL (Figure 1 and Table 3).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts

The antioxidant activities of the KS extracts were assessed using a DPPH radical scavenging assay. IC_{50} values derived from the inhibition–concentration curves of three independent experiments are summarized in Table 3. Although KS95ER had the lowest mean IC_{50} value (37.57 ± 9.24 μg/mL), there were no significant differences in DPPH radical scavenging activity among any of the KS extracts ($P > 0.05$). The activity of the positive control, ascorbic acid (IC_{50} = 5.33 ± 0.52 μg/mL), was not significantly different from that of KS95ER but was significantly more potent than all other KS extracts (KS95EM, KS50ER, KS50EM, and KSWR) ($P < 0.05$).

Table 2. Xanthine oxidase (XO) inhibitory activity of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts at 1 mg/mL

Sample	XO inhibition (%)
KS95ER	84.36 ± 13.52 [a]
KS95EM	63.52 ± 7.36 [a]
KS50ER	59.82 ± 2.92 [a]
KS50EM	56.85 ± 12.23 [a]
KSWR	35.39 ± 6.21 [b]
Allopurinol (1 μg/mL)	77.62 ± 23.86 [a]

Abbreviation: KS95ER: 95% ethanol reflux extract; KS95EM: 95% ethanol maceration extract; KS50ER: 50% ethanol reflux extract; KS50EM: 50% ethanol maceration extract; KSWR: water reflux extract; XO: xanthine oxidase.
All data are represented as mean ± SD (n = 3). Within the same column, values marked with different superscript letters (a, b) are significantly different from each other ($P < 0.05$), as determined by one-way ANOVA followed by Scheffé's post hoc test.

Inhibition of nitric oxide production and cytotoxicity of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts in LPS-induced RAW264.7 cells

The anti-inflammatory activity of KS extracts (100 μg/mL) was evaluated using a NO inhibition assay in LPS-induced RAW264.7 cells. As shown in Table 4, the 95% ethanol extracts (KS95ER and KS95EM) demonstrated

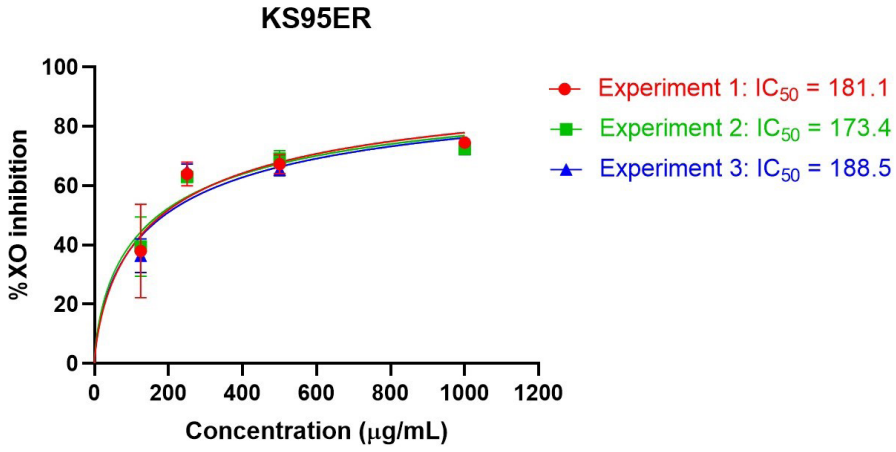

Figure 1. %Xanthine oxidase (XO) inhibition-concentration (μg/mL) curves and IC_{50} values of KS95ER (95% ethanol, reflux) extract, obtained from three independent experiments using an *in vitro* XO inhibitory assay.

Table 3. IC_{50} values of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts for xanthine oxidase (XO) inhibition, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and nitric oxide (NO) inhibition

Sample	IC_{50} (μg/mL)		
	XO inhibition	DPPH scavenging	NO inhibition
KS95ER	181.00 ± 7.55	37.57 ± 9.24[a,b]	90.29 ± 7.69[d]
KS95EM	N/A	57.09 ± 10.11[b]	78.13 ± 14.29[d]
KS50ER	N/A	48.54 ± 9.16[b]	N/A
KS50EM	N/A	68.69 ± 13.85[b]	N/A
KSWR	N/A	64.76 ± 25.34[b]	N/A
Allopurinol (1 μg/mL)	N/A	N/A	N/A
Ascorbic acid	N/A	5.33 ± 0.52[a]	N/A
Indomethacin	N/A	N/A	78.50 ± 3.73[d]

Abbreviation: KS95ER: 95% ethanol reflux extract; KS95EM: 95% ethanol maceration extract; KS50ER: 50% ethanol reflux extract; KS50EM: 50% ethanol maceration extract; KSWR: water reflux extract; XO: xanthine oxidase; DPPH: 2,2-diphenyl-1-picrylhydrazyl; NO: nitric oxide; N/A: not analyzed.
All data are represented as mean ± SD (n = 3). Within the same column, values marked with different superscript letters (*a, b, c, d*) are significantly different from each other ($p < 0.05$), as determined by one-way ANOVA followed by Scheffé's post hoc test.

Table 4. Nitric oxide (NO) inhibition and cytotoxicity of *Ya-Tom-Kae-Ka-Sai-Sen* (KS) extracts in lipopolysaccharide (LPS)-induced RAW264.7 cells at 100 μg/mL

Sample	NO inhibition (%)	Cytotoxicity (%)
KS95ER	77.49 ± 6.40[a]	17.04 ± 12.77[f]
KS95EM	73.14 ± 1.22[a]	13.51 ± 5.42[g]
KS50ER	53.96 ± 4.70[b]	6.16 ± 2.17[h]
KS50EM	34.44 ± 11.06[c]	4.20 ± 3.44[h]
KSWR	5.37 ± 1.41[d]	5.69 ± 3.19[h]
Untreated (0.2% DMSO)	0.00 ± 1.15[e]	0.00 ± 2.71[i]

Abbreviation: KS95ER: 95% ethanol reflux extract; KS95EM: 95% ethanol maceration extract; KS50ER: 50% ethanol reflux extract; KS50EM: 50% ethanol maceration extract; KSWR: water reflux extract; NO: nitric oxide; LPS: lipopolysaccharide; DMSO: dimethylsulfoxide.
All data are represented as mean ± SD (n = 3). Within the same column, values marked with different superscript letters (a, b, c, d, e, f, g, h, i) are significantly different from each other ($P < 0.05$), as determined by the Kruskal-Wallis test followed by the Mann-Whitney U test.

the most potent NO inhibitory activity. Their effects were statistically similar to each other and significantly greater than the inhibition shown by KS50ER, KS50EM, and KSWR ($P < 0.05$).

KS95ER and KS95EM were selected for IC_{50} determination and compared with indomethacin. Based on three independent experiments (Figure 2 and Table 3), their IC_{50} values were 90.29 ± 7.69 μg/mL and 78.13 ± 14.29 μg/mL, respectively, while indomethacin showed an IC_{50} of 78.50 ± 3.73 μg/mL. No significant differences were observed among the three groups ($P > 0.05$), indicating comparable anti-inflammatory effects.

At a concentration of 100 μg/mL, all KS extracts were determined to be non-cytotoxic, as they induced a reduction in cell viability of less than 30% (34). KS95ER extract exhibited the highest cytotoxic value at 17.04 ± 12.77%, which was significantly greater than that of the other extracts and the control ($P < 0.05$) (Table 4).

Discussion

In this study, the traditional Thai polyherbal remedy *Ya-Tom-Kae-Ka-Sai-Sen* (KS) was systematically evaluated to validate its therapeutic potential for managing conditions like gouty arthritis and inflammation. To achieve this, various extracts were prepared using maceration and reflux methods with solvents of differing polarities (95% ethanol, 50% ethanol and water), and then assessed for their phytochemical contents and bioactivities.

A key finding was that solvent polarity, rather than extraction temperature, was the dominant factor influencing phytochemical yield and bioactivity. Both maceration (room temperature) and reflux (heat-assisted) methods yielded comparable results for a given solvent, suggesting that the key bioactive compounds in the KS remedy were largely thermostable.

The yields of the KS crude extracts ranged from 11.21% to 17.56% (Table 1). Regarding total phenolic contents, all ethanolic extracts (KS95ER, KS95EM, KS50ER, and KS50EM) yielded high and statistically comparable levels. These levels were, however, significantly higher (approx. 1.6-fold, $P<0.05$) than that of the aqueous extract (KSWR) (Table 1). A clearer distinction was observed with flavonoid content. The 95% ethanolic extracts (KS95ER and KS95EM) yielded the highest flavonoid levels, which

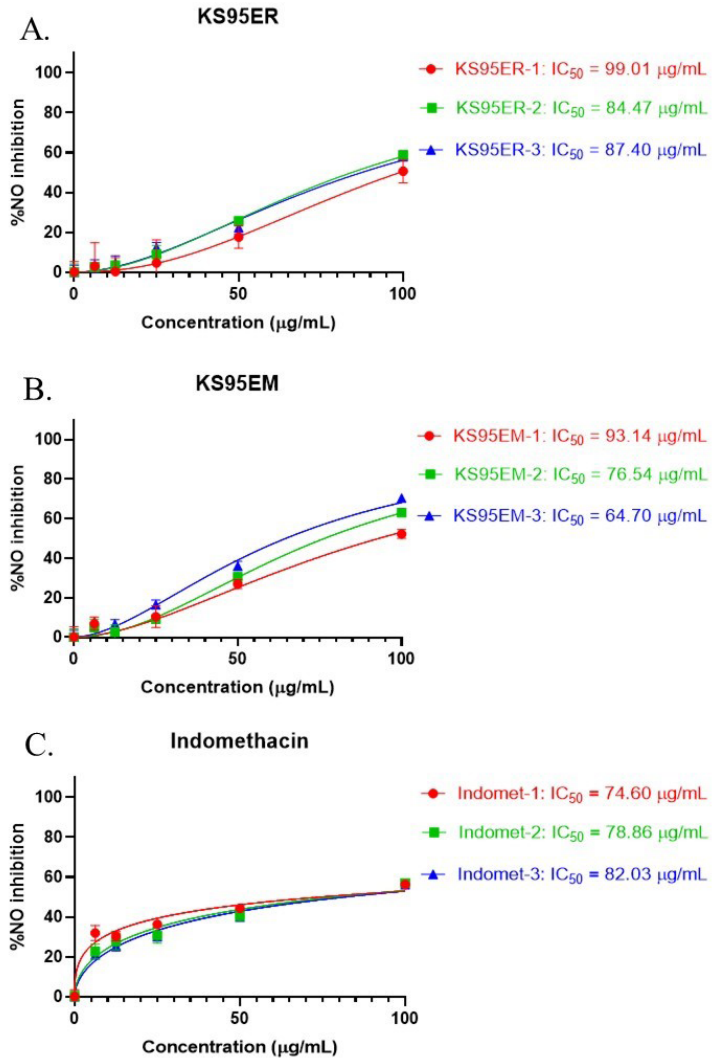

Figure 2. Nitric oxide (NO) inhibition curves of KS95ER (95% ethanol, reflux) (A), KS95EM (95% ethanol, maceration) (B), and indomethacin (C) in lipopolysaccharide (LPS)-induced RAW264.7 cells.

were significantly greater by approximately 1.3-1.4-fold than those from the 50% ethanolic and aqueous extracts ($P < 0.05$) (Table 1). This indicates that 95% ethanol is the most effective solvent for extracting flavonoids from this formulation. These findings are consistent with previous reports showing that hydro-alcoholic solvents often outperform water in extracting phenolics and flavonoids due to their ability to dissolve a wider range of compounds (29,30,35)

The potent phytochemical profile of the ethanolic extracts translated directly to their biological activities. In the *in vitro* XO inhibition assay, all ethanolic extracts demonstrated strong inhibitory activity comparable to the positive control, allopurinol, whereas the aqueous extract (KSWR) was significantly less effective. KS95ER, which had the highest phytochemical content, was selected for further analysis and exhibited an IC_{50} value of 181.00 ± 7.55 µg/mL (Table 3, Figure 1). Similarly, in the DPPH radical scavenging assay, KS95ER demonstrated the most potent antioxidant activity, with an IC_{50} value of 37.57 ± 9.24 µg/mL. While the antioxidant capacities of the other extracts were lower, the activity of KS95ER was statistically comparable to that of the potent standard, ascorbic acid (IC_{50} = 5.33 ± 0.52 µg/mL) (Table 3). This antioxidant potential is crucial, as it may work synergistically with XO inhibition to mitigate the oxidative stress associated with gout and other ROS-related diseases (32,36,37).

The anti-inflammatory potential of the extracts was confirmed via an NO inhibition assay in LPS-induced RAW264.7 cells. The 95% ethanolic extracts, KS95ER and KS95EM, showed the highest NO inhibition (77.49% and 73.14%, respectively) (Table 4). Their calculated IC_{50} values (90.29 ± 7.69 µg/mL for KS95ER and 78.13 ± 14.29 µg/mL for KS95EM) were statistically comparable to the anti-inflammatory drug indomethacin (78.50 ± 3.73 µg/mL), highlighting their significant therapeutic potential (Table 3, Figure 2). Importantly, the MTT assay confirmed that these effects were not due to cytotoxicity, as they induced

a reduction in cell viability of less than 30% (Table 4) (34).

The strong correlation between the high flavonoid content of KS95ER and KS95EM and their superior bioactivities suggests that flavonoids are the primary drivers of the remedy's efficacy. The link between phenolic/flavonoid content and XO inhibition, antioxidant, and anti-inflammatory activities is well-established (4,5,31,38-40). The observed activities are further substantiated by literature on the remedy's individual herbs, all of which are reported to possess NO-inhibitory properties, showing significant XO-inhibitory and antioxidant effects (7,14-23,41,42).

Mechanistically, the potent bioactivity of the KS extracts likely stems from a multi-pronged approach. The anti-inflammatory effect is plausibly achieved by suppressing the expression of inducible nitric oxide synthase (iNOS). This is often mediated through the inhibition of the nuclear factor-kappa B (NF-kB) signaling pathway, a primary regulator of inflammation (43). Flavonoids like quercetin and kaempferol are well-known inhibitors of this pathway (39,43,44). This action is synergistic with the observed antioxidant and XO-inhibitory activities. By scavenging ROS, the extracts can prevent the activation of pro-inflammatory pathways like NF-kB (45), while XO inhibition directly reduces a major source of ROS and uric acid production (46).

Conclusion

This study provides the first scientific validation for the pharmacological potential of the KS remedy. Extracts prepared with 95% ethanol, particularly KS95ER and KS95EM, exhibited the most potent XO inhibition, antioxidant activity, and anti-inflammatory effects, the latter being comparable to the standard drug indomethacin. These bioactivities strongly correlate with the high phenolic and flavonoid content found in these extracts, offering a scientific basis for the remedy's traditional use in alleviating joint and muscle pain. The promising bioactivities demonstrated in this *in vitro* study warrant further investigation in *in vivo* models to establish their therapeutic potential and safety. These results support the further development of a standardized KS extract as a novel natural therapeutic agent for managing gout, inflammation, and other oxidative stress-related disorders.

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Authors' contribution

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Conflict of interests

The authors declare that they have no conflicts of interest.

Data availability statement

The data included in this manuscript and Supplementary Information are openly accessible through the corresponding author.

Ethical considerations

Animal and human models were not used in this study. The authors considered ethical issues, including plagiarism, misconduct, data fabrication, falsification, duplicate publication, and redundancy.

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Supplementary files

Supplementary file 1 contains Figures S1-S5.

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