Artikel 4

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Original Article

Phytochemical Analysis and Evaluation of Purified Extract of *Tinospora crispa* Stem for *In Vivo* Antihyperuricemic Effect

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Abstract

Background: *Tinospora crispa* is used in folk medicines for the treatment of gout, rheumatoid arthritis, and internal inflammation. The presence of flavonoids, polyphenols, glycosides, and alkaloids in *T. crispa* stem is supposed to contribute to these therapeutic effects. This study aimed to analyze qualitative and quantitative phytochemical of purified extract of *T. crispa* stem (PETS) and to evaluate the *in vivo* antihyperuricemic effect. **Materials and Methods:** First, total flavonoid and total alkaloid contents of PETS were determined by colorimetric and gravimetric methods. After that, potassium oxonate-induced hyperuricemic mice were treated with three doses of PETS at 50, 100, and 200 mg/kg, and hydroalcoholic extract at 500 mg/kg. Moreover, allopurinol at 10 mg/kg and sodium carboxymethylcellulose 0.5% were orally administered as positive and negative controls, respectively. Serum uric acid levels were measured by ultraviolet–visible spectrophotometry. **Results:** The high flavonoids content (31.08% ± 1.77% rutin equivalent) in *T. crispa* stem possesses a potential as uricostatic in the treatment of gout. The purified extract of *T. crispa* stem at a dose of 100 mg/kg revealed a significant uric acid–lowering effect compared with negative control ($P < 0.05$). **Conclusion:** This study indicates the potential of *T. crispa* purified extracts in the treatment of hyperuricemia and gout.

Keywords: *Antihyperuricemic, flavonoid, gout, Tinospora crispa, uric acid*

Introduction

In recent decades, the prevalence of gouty arthritis has risen as reported from several studies in the United States, United Kingdom, China, and New Zealand.[1] Similarly, in Indonesia, the prevalence of this type of joint disorder among the population aged 15–64 years and older reached 15.5% and 18.9%, respectively.[2] The pathogenesis of gout is closely related to hyperuricemia condition, which may elevate the risk of hypertension, cardiovascular disorders, kidney disease, and metabolic syndrome.[3] The long-term therapeutic agents for gout are uricostatic (allopurinol) and uricosuric (probenecid) through competitive inhibition of xanthine oxidase (XO) or blockade renal tubular reabsorption of urate, respectively.[4,5] However, urate-lowering agents are limited in availability, efficacy, and safety due to their potential adverse effects, drug interactions, and unsatisfied outcomes in clinical application.[6]

Recently, the search for new chemical substances from plants with potential therapeutic effects but fewer side effects has been increasing worldwide. *Tinospora crispa* (family Menispermaceae) is a well known traditional medicinal plant distributed in Indonesia (*Brotowali*), Malaysia (*Akar patawali*), and India (*Madhuparni*). Stem of *T. crispa* is an ingredient of Indonesian traditional medicine (*Jamu*), Thai folk remedies, and Ayurveda to treat gout, rheumatoid arthritis, and internal inflammation.[7,8] Bioactivity studies of *Tinospora* species showed antioxidant,[9,10] antinociceptive,[11] anti-inflammation,[11,12] antimicrobial,[13] anti-osteoporosis,[13] and immunostimulation[13] activities. Accordingly, exploration and development of non-purine-based drugs for antihyperuricemic therapy is a key strategy to provide the scientific evidences for medicinal plants, which are potential in the treatment of gout.

In the course of our screening for bioactive secondary metabolites, we reported that the 70% ethanol extract of *T. crispa* stem possessed high flavonoid content,[14] besides its potential as XO inhibitor.[15] The plant flavonoids and phenolics exhibited potential action to block the urate synthesis by inhibiting XO enzyme,[16,17] in addition to their antioxidant and anti-inflammatory properties.[18] Reportedly, *T. crispa* comprises a

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diversity of secondary metabolites such as terpenoids, steroids, lactones, lignans, saponins, tannins, flavonoids, glycosides (picoretoside, tinocrisposide, and tinosporine), and alkaloids (protoberberine, fluoroquinolone, and aporphine).[8,13,19-21] However, the scientific evidences of antihyperuricemic effect from *T. crispa* stem are limited. Accordingly, this study was conducted to evaluate antihyperuricemic activity of the purified extract of *T. crispa* stem (PETS) in potassium oxonate-induced acute hyperuricemic mice as well as to guide in screening for bioactive compounds, which could be developed as therapeutics for gout.

Materials and Methods

Purified extract preparation

T. crispa stem, whose local name is *Brotowali*, was collected in North Purwokerto, Banyumas, Central Java, Indonesia, then authenticated by a taxonomist at Universitas Jenderal Soedirman, Indonesia. The voucher specimen was deposited at the corresponding authors' laboratory (H.H.) with the herbarium code 657/FB-Unsoed. The dried stems (500 g) were powdered and macerated by 96% ethanol for 3 × 24 h. This hydroalcoholic extract (78 g) was concentrated using a rotary evaporator at 37°C, then was fractionated with *n*-hexane by using liquid–liquid extraction method to afford *n*-hexane soluble (28 g) and *n*-hexane insoluble (50 g) fractions. The latter fraction, after this referred to as the purified extract of *T. crispa* stem (abbreviated as PETS), was subsequently dissolved in 0.5% sodium carboxymethylcellulose (Na-CMC) to obtain suspension form.

Phytochemical analysis

Preliminary qualitative phytochemical analysis was conducted to identify flavonoids and alkaloids present in the purified extracts of *T. crispa* stem using thin-layer chromatography (TLC) method. The PETS (sample) and rutin (reference substance) were spotted on silica gel 60 F_{254} plates and developed in *n*-butanol:glacial acetic acid:water (4:1:5 v/v) and then sprayed with citroboric reagent. Meanwhile, for alkaloid identification, PETS was spotted on silica gel 60 F_{254} plates and developed in chloroform:methanol (9:1 v/v) and then sprayed by Dragendorff's reagent.[14,22] Those spots were observed under visible, ultraviolet $(UV)_{254}$, and UV_{366} lights before and after spraying, then their homologous retardation factor (hRf) values were calculated.[23] Subsequently, quantitative estimation of both secondary metabolites was performed by following standard procedures.

Total flavonoid content

The total flavonoid content (TFC) was estimated using the colorimetric method as described in the previous studies.[24,25] A total of 0.1 mL of aluminum chloride (10%) was added to 1 mL diluted fraction solution and vortexed, and then incubated for 30 min in the dark. The absorbance was measured at 415 nm, whereas the appearance of pink color showed the presence of flavonoid content. The TFC was expressed as rutin equivalent (RE) mg/g extract on a dry weight basis using the standard curve [Figure 1].

Total alkaloid content

The total alkaloid content (TAC) was determined using the gravimetric method adopted from Harborne.[26] A total of 50 mL of acetic acid (10%) was added to 5 g of PETS taken in a separate 250-mL beaker and covered to stand for 4 h. This mixture-containing solution was filtered, and the volume was reduced to one-quarter using water bath. To this sample, concentrated ammonium hydroxide was added dropwise until the precipitate was complete. The whole solution was allowed to settle, and the precipitate was collected by filtration and weighed. The percentage of TAC was calculated as: Weight of residue × 100/Weight of sample taken.[27]

Animals and experimental design

Male Balb/C mice weighing 30–40 g were obtained from the Laboratory of Pharmacology, Faculty of Pharmacy, Universitas Muhammadiyah Purwokerto, Indonesia. The animal handling protocols of this study were following the guidelines for laboratory animal care and were approved by the Research Ethics Committee at Faculty of Medicine and Health Sciences, Universitas Jenderal Soedirman, Indonesia (certificate number of ethical approval: No.082/KEPK/IV/2014). All experimental animals were administered intraperitoneal injection of potassium oxonate (250 mg/kg) at 1 h after the administration of a single dose of each test sample adapted from the previous study.[28] In our preliminary study, the optimal time for blood sampling was 2 h after inducing by potassium oxonate, which is linear to pharmacokinetic data of potassium oxonate with T_{max}-time 2.8 ± 1.7 h and T_{max} 3.0 ± 1.1 h.[29] Subsequently, blood samples were centrifuged at 3000 rpm for 10 min to obtain serum and then measured after 2 h and recorded by enzymatic-colorimetric method using UV-visible spectrophotometer at 520 nm of wavelength to determine uric acid levels. Potassium oxonate-induced acute hyperuricemic mice were randomly divided into seven groups with five mice in each group as the following description:

Group I-III: Hyperuricemic mice; given single doses of PETS at 50, 100, and 200 mg/kg per oral

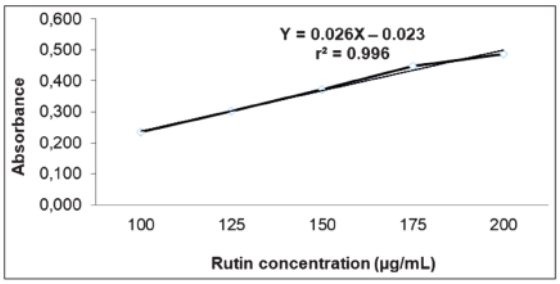

Figure 1: Linear curve of rutin concentration (µg/mL) versus absorbance for determination of total flavonoid content in the purified extract of *Tinospora crispa* stem

Group IV: Hyperuricemic mice; given single dose of hydroalcoholic extract at 500 mg/kg per oral
Group V: Hyperuricemic mice; given single dose of allopurinol 10 mg/kg per oral (positive control)
Group VI: Hyperuricemic mice; only induced with potassium oxonate (250 mg/kg) intraperitoneal (negative control)
Group VII: Normal control (placebo); only given Na-CMC (0.5%, w/v)

Data analysis

The serum uric acid (SUA) levels were expressed in mg/dL, whereas the response was stated as the percentage of decrease in uric acid level, which formulated as $([UA_{po} - UA_{tre}]/[UA_{po} - UA_{nor}]) \times 100$, where, UA_{po}, UA_{tre}, and UA_{nor} are SUA levels in the negative control, treatment, and normal (placebo) groups, respectively. Data were presented as mean ± standard error of mean (SEM), then analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. Statistical significance was considered as $P \leq 0.05$.

Results

Phytochemical analysis

The TLC profiles in Figure 2 revealed the presence of alkaloids and flavonoids in the PETS as previously observed in the 70% ethanol extract.[14] Flavonoids detected in PETS were indicated by yellowish spots on TLC plates. Flavonoid spots would be extinguished as dark blue fluorescence under UV_{254} light. In addition, these spots showed yellow fluorescence after spraying with citroboric and looked brighter under UV_{366} light [Figure 2A and B]. The retardation factors compared with rutin for hydroalcoholic extract and PETS were 96% and 98%, respectively. Moreover, alkaloids were marked by yellowish spots on visible light and showed orange fluorescence under UV_{366} light after spraying with Dragendorff's reagent [Figure 2C].

The TFC of purified extracts of *T. crispa* was expressed in gram rutin per 100 g samples as shown in Table 1. Rutin was used as a reference standard since it was previously reported as the second highest flavonoids in *T.crispa* stem.[30] The TFC of PETS was 31.08% ± 1.77% RE, which means that each gram of the purified extract contained total flavonoid equivalent to 310 mg of rutin [Table 1]. Meanwhile, the percentage of precipitate mass was a parameter derived from gravimetric method, which was performed to quantify the TAC in the purified extract.[27] In this study, the TAC of PETS was 5.76% ± 1.29%, which means that each gram of the purified extract contained total alkaloid, which was around 60 mg [Table 2].

Antihyperuricemic activity

Serum uric acid levels in the negative control exceed 4 mg/dL as shown in Figure 3A, indicating that potassium oxonate at 250 mg/kg was able to induce acute hyperuricemia in mice. The SUA levels were decreased in all treatment groups, but only the purified extract of *T. crispa* at a single dose of 100 mg/kg showed the significant uric acid–lowering effect respect to allopurinol (10 mg/kg). However, the highest dose of PETS (200 mg/kg) showed no enhancement in hypouricemic effect, even less potent than that of hydroalcoholic extract (500 mg/kg). The uricostatic effects of PETS at 100 mg/kg of allopurinol 10 mg/kg exhibited significantly differences compared with the negative control ($p \leq 0.05$) as shown in Figure 3A. Treatment of hyperuricemic mice with PETS at doses 50, 100, and 200 mg/kg revealed the uric acid–lowering effects ranging from 49% to 78% [Figure 3B].

Discussion

In this study, the purified extracts were provided by fractionation with nonpolar organic solvents such as hexane or chloroform in order to clean up the ballast substances in the crude extract, such as chlorophyll, resin, and lipids.[10] The purified extract, which is rich in bioactive compounds,

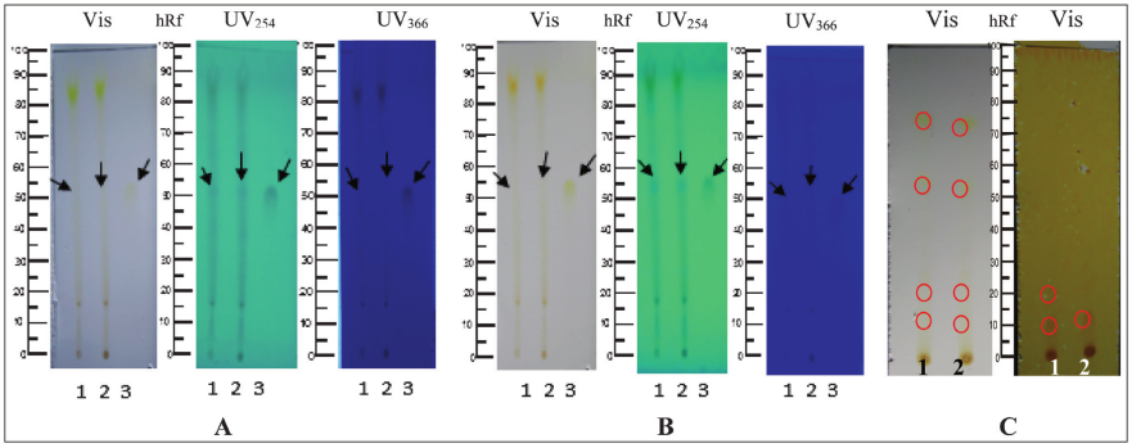

Figure 2: Thin-layer chromatography profiles of hydroalcoholic extract (1), purified extract of *Tinospora crispa* stem (2), and rutin (3) under ultraviolet and visible lights. Stationary phase: silica gel 60 F_{254}, mobile phases: (A and B) *n*-buthanol:glacial acetic acid:water (4:1:5 v/v) and (C) chloroform:methanol (9:1 v/v), reagents: Dragendorff's, before (A) and after (B) spraying, and citroboric (C)

Table 1: Percentage of total flavonoid content in the purified extract of *Tinospora crispa*

PETS concentration (*a*) (µg/mL)	Absorbance (*b*) (*n*) (λ 415 nm)	Total flavonoid in each sample (*c*) (µg/mL)	TFC (*d*) (RE% w/w)
400	0.280	116.5	29.12
400	0.337	138.5	34.62
400	0.284	118.0	29.50
	Mean ± SEM	124.3 ± 0.71	31.08 ± 1.77

PETS = purified extract of *Tinospora crispa* stem, TFC = total flavonoid content, RE = rutin equivalent, SEM = standard error of mean

$b = 0.026.c - 0.023$. $d = ([c/a]) \times 100$

Table 2: Percentage of total alkaloid content in the purified extract of *Tinospora crispa*

PETS early weight (*a*) (g)	Precipitate weight (*b*) (g)	TAC (*c*) (% w/w)
5	0.299	5.98
5	0.273	5.46
5	0.268	5.36
	Mean ± SEM	5.60 ± 0.19

PETS = purified extract of *Tinospora crispa* stem, TAC = total alkaloid content, SEM = standard error of mean

$c = ([b/a]) \times 100$

was produced by purification steps to enhance its therapeutic effect.[31] The phytochemical analysis showed high flavonoid content in the purified extract, which was also observed in our former report on the 70% ethanol extract of *T. crispa* stem.[14] Surprisingly, TFC in this study was 30-fold higher than that reported from a previous study using quercetin as reference standard.[9] Hence, the presence of flavonoids in the purified extract of *T. crispa* stem was more dominant than that of alkaloids, indicating the bioactive constituents for antihyperuricemic activity. The treatment of purified extracts of *T. crispa* stem increas uric acid–lowering effects in hyperuricemic mice until the dose of 100 mg/kg. Since the PETS treatment at the highest dose of 200 mg/kg displayed no enhancement effects, thus antihyperuricemic effect of PETS might be not in dose dependent manner. In contrast, the mild uric acid–lowering effect was shown in the treatment of PETS at 50 mg/kg, which was estimated to be containing the lowest bioactive compounds such as flavonoids. Meanwhile, the crude extract at 500 mg/kg possessed equal antihyperuricemic effect with the purified extract at the effective dose of 100 mg/kg. Similarly, treatment over seven days with e extracts of *T. cordifolia* stem was able to decrease SUA levels in potassium oxonate-induced hyperuricemic mice and revealed uricosuric effect.[32]

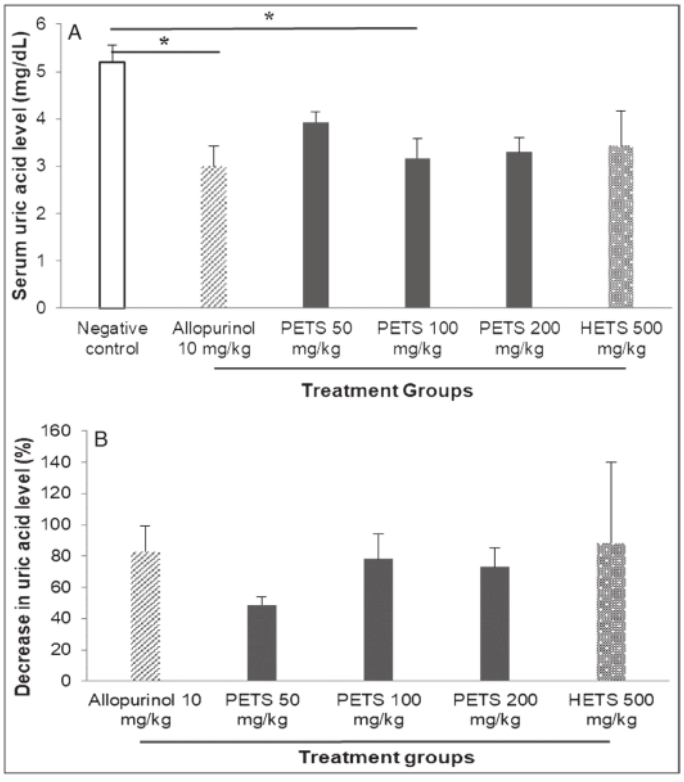

Figure 3: Serum uric acid levels (A) and the percentage of decrease in uric acid level (B) in the treatment groups (PETS = purified extract of *Tinospora crispa*, HETS = hydroalcoholic extract of *T. crispa* stem). The symbol (*) indicates significant difference at $P \leq 0.05$

The major compounds in these purified extracts, flavonoids, were previously reported as flavon-*O*-glycosides, including apigenin, luteolin, morin, and rutin.[30,33] Several *in vivo* and *in vitro* studies proved that flavonoids contributed to antihyperuricemic activities by reducing SUA levels and/or by inhibiting XO enzyme.[16,17,33-35] Meanwhile, the putative metabolites in the lowest detected spots were alkaloids in the salt form, probably quaternary alkaloids, for example, protoberberine, columbamine, and magnoflorine, as mostly found in *T. crispa*.[8,13,20] Few studies reported that alkaloids in *T. crispa* stem showed antioxidant, antimicrobial, antiparasitic, and antidiabetic activities.[20,36,37] On the basis of these reported data, we indicated that antioxidant and anti-inflammatory properties of *T. crispa* stem strongly contribute to antihyperuricemic effect.[9-12]

Furthermore, we postulate that the high flavonoid content in *T. crispa* stem is potential for either *in vivo* or *in vitro* antihyperuricemic effects. Unexpectedly, a recent study reported antihyperuricemic effect of polysaccharide-rich extracts derived from *T. cordifolia* stem.[32] In addition, the endophytic fungi isolated from this plant species revealed potent XO inhibitory activity.[38] Thereby, it will be interesting to investigate XO inhibitory effect by performing the next enzyme assay. On the contrary, the appropriate dosage forms and toxicity assessments should be addressed in further studies aimed to develop a standardized herbal medicine. The comprehensive data are warranted for development of the herbal remedies, which meet pharmaceutical qualifications, including efficacy, safety, and acceptability.

Conclusion

This study showed that the purified extract of *T. crispa* stem contained total flavonoids (31.08% ± 1.77% RE) as the major constituent, instead of the alkaloid content (5.60% ± 0.19%). This purified extract possessed *in vivo* antihyperuricemic effect in hyperuricemic mice pretreated with potassium oxonate; thereby, it might be a potential therapeutic agent for the treatment of gout.

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Conflicts of interest

There are no conflicts of interest.

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