ANTIHYPERURICEMIC ACTIVITY OF *Tinospora crispa* PURIFIED EXTRACT (PETC) ON POTASSIUM OXONATE INDUCED MICE

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ABSTRACT

Background: Brotowali (*Tinospora crispa*) has empirically used to treat gout that signed by hyperuricemia. This plant stem contains quaternary alkaloid, saponin, glycoside, and flavonoid.

Objective: to determine the *in vivo* antihyperuricemicactivity of purified extract of *T. crispa* (PETC) and to analyze its active compound.

Methods: The PETC was prepared from *T. crispa* ethanolic extract, then fractionated by n-hexane, chloroform, and ethyl acetate gradually, and its active substance was identified by TLC method. A total of 30 mice were divided into 6 equally groups including hyperuricemic (potassium oxonate 250 mg/kg intra peritoneal), and 5 treatment groups were orally administered to purified extract (PETC) at doses of 50; 100; 200 mg/kg, crude extract dose 500 mg/kg, and allopurinol 10 mg/kg. Serum uric acid level was determined by enzymatic-colorimetric method.

Outcome measured : the serum uric acid level

Results : Treatment with PETC at dose of 200 mg/kg and allopurinol significantly decreased uric acid level to 1.83±0.82 mg/dL and 3.51±0.72 mg/dL. While crude ethanolic extract dose 500 mg/kg was able to reduce uric acid level to 3.43±0.75 mg/dL.

Conclusion: The PETC at the dose of 200 mg/kg significantly reduced serum uric acid level compared to hyperuricemic mice. The PETC found to containflavonoid that might contribute to antihyperuricemic activity.

Keywords: *Tinospora crispa*, brotowali, antihyperuricemic activity, uric acid

INTRODUCTION

A number of epidemiological studies from a diverse range of countries suggest that gout has increased in prevalence and incidence in recent years (Roddy *et al*., 2007). The increasing prevalence of gout and hyperuricemia are highly correlated with the economic development as manifested by dietary and lifestyle changes (Miao *et al*., 2008). Hyperuricemia is the biochemical abnormalities in clinical practice signed by the serum uric acid greater than 7.0 mg/dL in male and over 6.0 mg/dL in female. Gout and hyperuricemia are associated with significant co-morbidity, including the metabolic syndrome and heart failure (Choi *et al*., 2007; Annemans *et al*., 2008).

Nowadays, the therapeutic agents for treatment of gout and hyperuricemia are still limited. Thus, the development of antihyperuricemic agents with more effectiveness and

safety is highly warranted.*Tinospora crispa* (brotowali) comes from Java and Malaya has been traditionally used by the Indonesian communities for gout, diuretic, cosmetic, renal inflammation and sometimes as abortifacient.

Tinospora crispa contains alcaloids, flavonoids, polyphenols, saponins, glycosides, tannins (Sudarsono *et al*., 2006; Handayani, 2010). In the development of pharmaceutical formulations, purification was needed to clean up some ballast substances such as tannin, chlorophyll, lipid, and other primary metabolites. However, the test preparation that was enriched with active compounds may increase therapeutic effect (Katno, 2008). In the present study, fractionationwas carried outgradually withn-hexane, chloroform, and ethyl acetate. The last solventis semi-polar and can dissolve some compounds, such asglycosides, flavonoids, alkaloids(Saputra, 2008; Koay and Amir, 2013).

Despite the facts that *T. crispa* stem have shown antioxidant, analgetic, and anti-inflammatory activities (Sulaiman *et al.*, 2008; Irianti *et al*., 2012; Hipol *et al*., 2012), but it is not clear whether *T. crispa* stem can reduce serum uric acid level in hyperuricemic model. The aim of this study was to evaluate the *in vivo*effects of ethyl acetate fraction of *T. crispa* ethanolic extract on reduction of serum uric acid level in hyperuricemic mice model that induced by potassium oxonate.

METHODS

Materials

The materials that were used as follows: ethanol 70% (PG. Madukismo, Indonesia), *n*-heksan, kloroform, ethyl acetate was purchased from Sigma Chemical Co. (St.Louis, MO, USA), potassium oxonate (Aldrich Inc., Germany), allopurinol (PT. Kimia Farma, Indonesia), FS*TBHBA (*2,4,6-tribromo-3hidroksi benzoat)* [Diagnostic System Internasional (Diasys), and CMC-Na [E. Merck, Germany].

Animals

Male Balb/C mice weighing 30-40 g were obtained from Pharmacology Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Purwokerto, Indonesia. The animal handling protocols of this study were in accordance with the guidelines for laboratory animal care. All experimental procedures were approved by ethics committee from Faculty of Medicine and Health Sciences Universitas Jenderal Soedirman.

Extraction and Purification

Tinospora crispa were collected from Purwosari village, Purwokerto, Indonesia and had been identified by a botanist. These stem were then dried and powdered. One kg of powder was macerated by ethanol 70% for 24 hours. Subsequently, the residue was remacerated three times by the same solvent, and the extract was mixed into the previous ones. The collected extract was then evaporated under reduced pressure to give viscous ethanolic extract (EETC), then fractionated gradually by n-hexane, chloroform, and ethyl acetate,then were concentrated by rotary vacuum evaporator. The last fraction that yielded was ethyl acetate fraction and so called the purified extract of *T. crispa* (PETC).

In vivo antihyperuricemic assay

As many as 30 male Balb/C mice were grouped into 6 treatment groups, each group consisted of 5 rats. Group 1 was negative control (potassium oxonate 250 mg/kg intra peritoneal), group 2 was positive control (10 mg/kg per oral allopurinol). Groups 3 to 6 were given per oral PETC with respective doses 50; 100; 200 mg/kg; and crude extract

500 mg/kg. All groups were given intra peritoneal injection of potassium oxonate dose of 250 mg/kg at 60 minutes after the administration of single dose per oral treatment. The serum uric acid level were measured and recorded by enzymatic-colorimetric method using UV-Vis spectrophotometer at 520 nm of wave length.

Identification of flavonoid and alkaloid in PETC

Purified extract of *T. crispa* (PETC) was identified the chemical constituents such as flavonoid and alkaloid by TLC method. The PETC as sample and standard used (rutin and quercetin) were spotted on silica gel 60 F_{254} plate and developed in n-butanol : acetic acid : water (4:1:5 v/v), then sprayed with citroboric reagent. These spots were observed under UV_{366} light before and after sprayed, then its hRf values were determined. For alkaloid identification, PETC were spotted on silica gel 60 F_{254} plate and developed in methanol : ammonium (200:3 v/v), then sprayed with Dragendorff reagent (Harborne, 1998).

Data analysis and statistical

The datas were presented as mean±the standard error of mean (SEM). Then datas were analyzed statistically using one-way analysis of variance (ANOVA) followed by Bonferroni test. The P-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Preparation of purified extract

Brotowali extract was made by maceration using ethanol 70%. The principle of this method was the chemical compound in crude drugs will move then diffuses into the solvent (Harborne, 1998). Ethanol 70% was semi-polar solvent so could dissolve both polar and non-polar compounds in brotowali, such as alkaloids, phenols, flavonoids, glycosides, saponins, and tannins (James *et al*., 2011). The extraction process produced 352.2 grams viscous extract from 3,150 grams of brotowali powder and the rendement was 11.18%

Fractionation was done by liquid-liquid extraction based on the polarity grade of solvent from non-polar to polar (n-hexane, chloroform, and ethyl acetate). The n-hexane solvent cleaned extracts from impurities (Nurulita *et al*., 2011), while the chloroform dissolved terpenoids and phenolic compounds (Harborne, 1998). Active compounds were dissolved in ethyl acetate fraction, among others, glycosides, flavonoids, alkaloids, N-trans-feruloyl tyramine and N-cis-feruloyl tyramine (Koay and Amir, 2013). The rendement was 0.16% from 352.2 g ethanolic extract.

Preliminary study

Preliminary study was conducted to determine the exact time of blood sampling so that it can be shown when the serum uric acid reached a peak level after induction by potassium oxonate. The highest uric acid level was 9.37 mg/dL when blood sampling at the second hour after mice induced by potassium oxonate at dose of 250 mg/kg. Then it can be determined the optimal time for blood sampling was 2 hours after potassium oxonate induction. Potasium oxonate was a competitive inhibitor of the uricase enzyme which can increase uric acid level by inhibiting the formation of allantoin. Inhibiting uricase enzyme by potassium oxonate caused an increase of uric acid because it can not be eliminated through the urine. Thus, it can be used as an inducer compound of hyperuricemic condition (Martin, 1987).

Antihyperuricemic activity

The animals used was Balb/C mice because it had uricase enzyme which used as hyperuricemic model by potassium oxonate induction (Hall *et al.*, 1990). Uric acid levels were determined by the enzymatic method with the mechanism of uric acid is oxidized by the uricase enzyme with the help of H_2O and O_2 into allantoin, carbon dioxide and hydrogen peroxide. Hydrogen peroxide formed would react with 2,4,6-tribromo-3hydroxy-benzoic acid (TBHBA) and 4-amino antipirin and the result was quinonimin whose pink colour. This reaction was catalyzed by peroxidase (POD) and the intensity of the colour produced by quinonimin equivalent to the levels of uric acid in the blood.

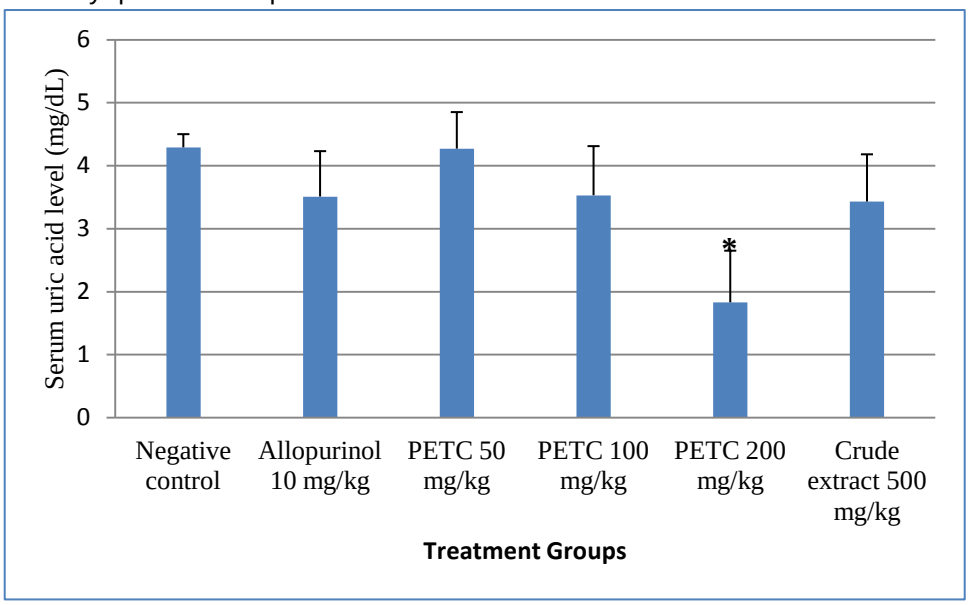

Figure 1. Serum uric acid levels in all treatment groups

Explanation: PETC = Purified extract of *Tinospora crispa*; * = significant difference compared to negative control ($p < 0.05$)

Based on the serum uric acid levels, the negative control group have shown hyperuricemia condition with uric acid levels more than 4 mg / dL (Figure 1). Treatment with allopurinol 10 mg/kg, PETC at the dose of 100 and 200 mg/kg, and the ethanolic extract 500 mg/kg were able to lower serum uric acid levels of mice. Statistical analysis showed that the PETC dose of 200 mg/kg showed significant difference in uric acid levels when compared to the negative control group ($p<0.05$). In addition, the PETC dose of 200 mg/kg have antihyperuricemic activity better than the positive control group (allopurinol).

Chemical identification of *T. crispa* purified extract

The TLC profiles in Figure 2 shows the presence of yellowish spots on UV_{366} and after being sprayed with citroboric, yellow fluorescence look brighter. It means that PETC containing flavonoids that marked by bright yellow spots. The hRf value of PETC was 88, while quercetin was 53 and rutin was 90. Thus, the hRf value of of PETC nearing the hRf value of rutin, so that flavonoids group contained in PETC possible was rutin.

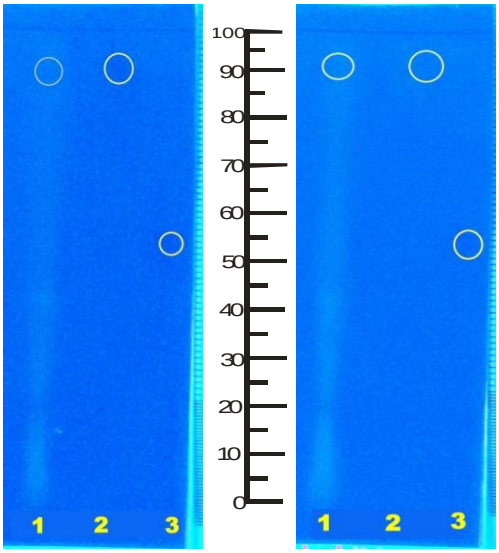

Figure 2. TLC profile on UV_{366} light before (I) and after (II) sprayed
(1: purified extract of *T.crispa*, 2: rutin, 3: quercetin)

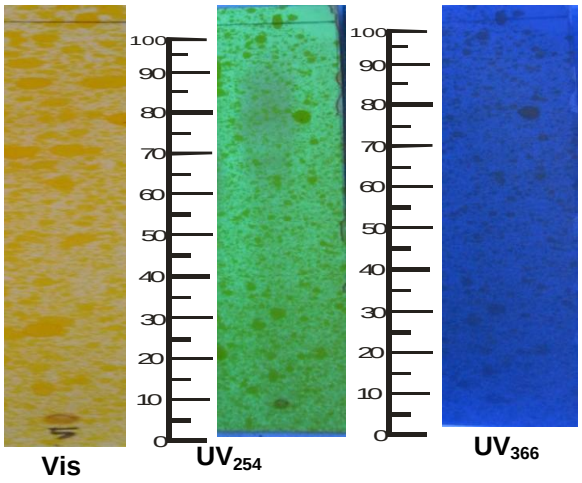

Figure 3. TLC profile of PETC for alkaloid identification

Identification of the chemical constituents of PETC does not contain alkaloids caused by the absence of orange spots after being sprayed with Dragendorff reagent (Figure 3). Therefore, PETC more dominant containing flavonoids. The flavonoid type suspected in PETC was flavonols without free 5-OH group or flavonols with unsubstitued 5-OH group (Wagner and Bladt, 1996). Irianti *et al.* (2012) reported that PETC contained flavonoid and coumarin (glycoside) which responsible for antioxidant activity.

According to the study of Amom *et al.* (2009) proved that brotowali contains flavone-O-glycosides (apigenin) and flavone glycosides such as catechin, luteolin, morin, and rutin. De Souza *et al.* (2012) reported that apigenin and luteolin have potential effect in lowering uric acid levels and this effect was significant at dose of 25 mg/kg. However, the presence of other chemical compounds in PETC are also potential as antihyperuricemic.

CONCLUSION

Purified extract of *T. crispa* (PETC) has activity in lowering uric acid levels in dose-dependent manner. The most significant antihyperuricemic activity of PETC shown at a dose of 200 mg/kg.

DISCLOSURE: -

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