

Phytochemical Analysis and Antihyperuricemic Activity of Ethanolic Extract of *Moringa oleifera* Seeds

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ABSTRACT

Background: *Moringa oleifera* is a popular plant that has been known to have several importance biological activities. **Objectives:** To perform phytochemical analysis, to in vivo evaluate the antihyperuricemic activity and to measure the inhibition of ethanolic extract of *Moringa oleifera* seed on xanthine oxidase enzyme. **Materials and Methods:** The seed were collected from Sigi regency, Central Sulawesi, Indonesia. Extraction was performed by maceration method with ethanol 96% as a solvent. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and spectrophotometric UV-Vis were used to determine the phytochemical contents. The antihyperuricemic activity was evaluated by using in vivo model of rat induced by potassium oxonate. The xanthine oxidase inhibitory activity was also determined by spectroscopic method by measuring the catalytic rate of xanthine oxidase enzyme. **Results:** Phytochemical analysis confirmed the presence of alkaloids, terpenoids, flavonoids, phenolics, saponin and tannin. Quantitative determination of total flavonoids and quercetin concentration found the values of 82.17 ± 0.684 mg QE/g and 0.5131 ± 0.0022 mg/g dried extract, respectively. Ethanolic extract of *Moringa oleifera* seed have potential antihyperuricemic activity in which it can significantly reduce the serum uric acid level on potassium oxonate-induced hyperuricemic rat model with the effective dose of 125 mg/kg BW. Xanthine oxidase inhibitory activity showed the moderate activity with IC_{50} of 88.39 μ g/ml. **Conclusion:** This study confirmed the potential of *Moringa oleifera* seed ethanolic extract, growing in Sigi, Central Sulawesi to be developed as herbal medicinal source for antihyperuricemic drug.

Key words: *Moringa oleifera*, Antihyperuricemic, Xanthine Oxidase, Total flavonoids, Quercetin.

INTRODUCTION

Uric acid is the end product of purine metabolism, a waste product that has no physiological role. Purines are natural substances which are one of the chemical structure groups that build up DNA and RNA. The limit of normal uric acid levels in men is 3.5-7 mg / dl and in women 2.6-6 mg / dL. Uric acid circulating in the human body is produced by the body (endogenous uric acid) and comes from food (exogenous uric acid) as well. Uric acid is formed every day through the digestive tract or kidney. If the body produces excess uric acid or low uric acid excretion, uric acid will be stored around the joint in crystal form. This stack is called a topos, and can be felt on the skin as a small and hard lump. It can cause inflammation and swelling as well.^{1,2}

Hyperuricemia is a condition where there is an increasing of uric acid level in the blood above normal. Hyperuricemia occurs because of an increasing in overproduction of metabolism, a decrease in gout or underexcretion, or a combination of both. Hyperuricemia can cause gout, a disease that arises when formed uric acid crystals in certain joints and tissues, so that cause inflammation. Gout is a disorder or term commonly used to describe a disease associated with hyperuricemia.³

In general, synthetic drug commonly consumed to treat gout is allopurinol that inhibits the activity of xanthine oxidase, the enzyme that converts hypoxanthine to xanthine, and subsequently becomes gout. Through a feedback mechanism, allopurinol inhibits purine synthesis which is a xanthine precursor. However, the use of allopurinol can cause detrimental effects such as disorders of the skin, stomach, intestine and blood disorders. Looking at the side effects caused by allopurinol, the screening of natural medicines that have antihyperuricemic activities are importantly needed.⁴

Moringa oleifera Lamk., known as drumstick, is a plant native to the foot of the Himalayan mountain in the northwestern part of India, Africa, Arabia, Southeast Asia, and the United States. *Moringa oleifera* is known throughout the world as a nutritious plant and the WHO (World Health Organization) has introduced *Moringa oleifera* as an alternative food to overcome malnutrition.⁵ Some studies, specifically on the benefits of *Moringa oleifera* seeds, state that *Moringa* seed has potency as antimicrobial, antitumor, anti-inflammatory, antiparasitic and antioxidant. However, there is still no report regarding its antihyperuricemic activity.⁶⁻⁹ Antioxidant and anti-inflammatory activities were always correlated with the antihyperuricemic activity. Therefore, In this study, *M. oleifera* seed ethanolic extract was tested as antihyperuricemia on oxonate induced rat and

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its inhibition on xanthine oxidase enzyme as well. Its phytochemical constituents were qualitatively and quantitatively determined including total flavonoids and quercetin content.

MATERIAL AND METHODS

Materials

Moringa oleifera plant was collected from Sigi Regency, Central Sulawesi, Indonesia on January 2017. Ethanol 96%, methanol, potassium oxonate, aluminium chloride, allupurinol, sodium chloride 0.9%, betadine, aquadest and other chemicals (analytical grade) were obtained from Sigma Aldrich, uric acid kit reagent was from Rajawali Nusindo Indonesia and chicken heart.

Extractions

The seed of *Moringa oleifera* was separated from the mature fruit and then dried at room temperature. After drying, about 1.025 g sample was extracted by 5 L ethanol 96% in maceration vessel for 5 x 24 hours. The filtrate obtained is then evaporated with a rotary evaporator until obtained the ethanolic extract. The procedure was repeated 3 times until reached the proper amount of extract (45.73 g) for further analysis.

Phytochemical analysis

Phytochemical analysis was performed on the ethanolic extract of *M. oleifera* seed. For qualitative analysis, thin layer chromatography (TLC) method with spraying reagents of dragendorff, aluminum chloride, ferric chloride, 10% sulfuric acid, and Liebermann Burchard was used to identify the presence of alkaloids, flavonoids, phenolics, steroids, terpenoids and tannin, respectively. For quantitative analysis, total flavonoids and quercetin concentration were determined as our previous research.¹⁴ The analysis of quercetin as a marker compound on ethanolic extract (after confirmation of its presence) was done by Reverse-Phase-High Performance Liquid Chromatography (RP-HPLC). Briefly, the series concentration of standard quercetin (1.2, 2.4 and 4.8 µg/mL) in methanol was prepared. Meanwhile, 25 mg dried ethanolic extract was dissolved in 10 mL of methanol and sonicated for 15 min as sample solution. 0.45 µm Millipore filter was used to filter the standards and samples solutions. HPLC Cecil CE4201 (UV visible detector) installed by Data Stream software system was used for analysis with the parameters as follow: Column was C18 size 250 mm x 4.6 mm (inside diameter), solvent was combination of methanol: water (59:41, v/v), flow rate was 1 mL/min, injection volume was 20 µL and detection wavelength was 370 nm.

Antihyperuricemic activity

Animals

The antihyperuricemic test used male white rats weighing 150-250 g and approximately 2-3 months old. All animals were maintained in accordance with the ethical standards of laboratory animals.¹⁰ They were fed with regular diet contain chicken hearth and water supplied *ad libitum* during 7 days preceding the experiments. The ethical protocol was approved by the Ethical Research Committee of Medicine Faculty, Tadulako University with the number 6829/UN28.1.30/KL/2020.

Rats model of hyperuricemia

Rats were intraperitoneally injected by potassium oxonate (PO) using single dose of 250 mg/kg to induce hyperuricemia in one hour before administrating the tested extract according to previous study.^{11,12}

Animal experimental protocol

Rats were randomly divided into five groups with each group contains five animals. Before the treatment, all animals were fasted for 18 hours. The first group was negative control (hyperuricemic rats which injected

by potassium oxonate and 0.5% carboxymethyl cellulose (CMC)) and second group was positive control (rats that orally injected by allopurinol using single dose of 27 mg/kg). The third, fourth and fifth groups were injected by tested sample (ethanolic extract of *M. oleifera* seed) using three dose variations of 125, 250 and 375 mg/kg, respectively. During 7 days, all rats were treated orally once daily. At the 8th day after one hour of final administration, from the tail vein of the rats, the blood samples were taken and allowed to clot at room temperature for one hour then centrifuged at 7000 rpm for 5 min to separate the serum. Assay kits were used to determine the serum uric acid levels. The animals were anaesthetized with isoflurane and then sacrificed by cervical dislocation at the end of the treatment period.

Xanthine oxidase inhibitory activity

Xanthine oxidase inhibitory activity was measured by using spectrophotometric UV-Vis at 290 nm as previous study.¹³ Firstly, Dimethyl sulfoxide (DMSO) was used to dissolve the tested samples and diluted by phosphate buffer with pH 7.5 until reached the desired final concentrations (50, 100, 150 and 200 µg/mL). About 50 µL of this tested sample solution were mixed with 35 µL of phosphate buffer and 30 µL of XO solution (0.1 U/mL) and then pre-incubated at 25°C for 15 min. 60 µL of xanthine solution (150 µM) was then added to the mixture to initiate the reactions and incubated again at 25°C for 30 min. 25 µL of HCl solution (1 N) was added to stop the reaction and the absorbances were then measured. Negative control (blank) was prepared similarly without the adding of tested sample solution, meanwhile the positive control is using allopurinol. The inhibition percentages were calculated using the equation after experiment run 3 times:

$$\% \text{ XO inhibition} = \frac{C - (S_1 - S_0)}{C} \times 100$$

In which C is absorbance of control, S_1 is absorbance of tested sample with adding XO enzyme and S_0 is absorbance of tested sample without adding XO enzyme

Statistical analysis

One-way analyses of variance (ANOVA) followed by a Duncan post hoc test on SPSS 17.0 (SPSS, Inc, Chicago IL, USA) software was used to analyze the obtained data. The results were reported as mean ± SEM (standard error of the mean). The significance was obtained if p-value less than 0.05. The correlation of concentration (µg/mL) and inhibition percentage (%) was used to calculate the IC_{50} values of XO inhibition.

RESULTS

Phytochemical analysis

TLC silica gel 60 F254 was used to identify the phytochemical contents of ethanolic extract of *M. oleifera* seed with two different mobile phases; n-hexane-ethyl acetate (3:7) and n-hexane: ethyl acetate: butanol (1:2:5). The results showed that the extract contains alkaloids (one spot with R_f 0.65 after spraying dragendorff), flavonoids (one spot with R_f 0.12 after spraying aluminum chloride), saponin (one spot with R_f 0.4 after spraying 10% sulfuric acid), tannin (one spot with R_f 0.81 after spraying with ferric chloride), terpenoids (one spot with R_f 0.34 after spraying with Liebermann Burchard) and phenolic (one spot with R_f 0.81 after spraying ferric chloride). There is no steroid identified in the extracts.

A linear calibration curve of quercetin was used to determine the total flavonoid of *M. oleifera* seed ethanolic extract. The series concentrations used (2, 4, 6, 8 and 10 µg/mL) were correlated with the absorbances producing a linear regression with R^2 value of 0.948 (Figure 1). The total flavonoid content of *M. oleifera* seed ethanolic extract was found of 82.17 ± 0.684 mg QE/g dried extract. The quercetin concentration in *M. oleifera* seed ethanolic extract was measured by reverse phase-

high performance liquid chromatography (RP-HPLC) method. Figure 2 showed the peak of quercetin standard was obtained at retention time of 2.45 minutes. The wider of areas obtained were used for the quantification of quercetin concentration on extract by correlating with the concentrations. Figure 3 showed this calibration curve with a good coefficient correlation (R^2) of 0.993. It was found that the quercetin content was 0.5131 ± 0.0022 mg/g.

In vivo antihyperuricemic activity

The result of in vivo antihyperuricemic activity of *M. oleifera* seed ethanolic extract was given in Table 1 and Figure 4. The uric acid levels obtained from each treatment group showed that all treatment doses can reduce the uric acid levels on oxonate induce rats with the uric acid levels range from 3.04 to 3.21 mg/dL. Further statistical analysis by One Way Anova and continued with PostHoc Duncan test showed that there

is no significant differences on each treatment dose (125 mg/kg BW, 250 mg/kg BW and 375 mg/kg BW). However, they were significantly difference to positive control Allopurinol 27 mg/kg BW and negative control. Therefore, the effective dose of *M. oleifera* seed ethanolic extract to reduce the uric acid concentration in rat blood plasma was 125 mg/kg BW.

Xanthine oxidase inhibitory activity

The inhibition of xanthine oxidase enzyme of *M. oleifera* seed ethanolic extract can be seen in Figure 5. It exhibited the inhibition effect in a concentration-dependent manner where at concentration 50-200 μ g/mL, the inhibition percentage ranged from 40.67 to 71.49%, respectively. The IC_{50} was found moderate with the IC_{50} of 88.39 μ g/mL, meanwhile allopurinol showed an IC_{50} value of 9.23 μ g/mL.

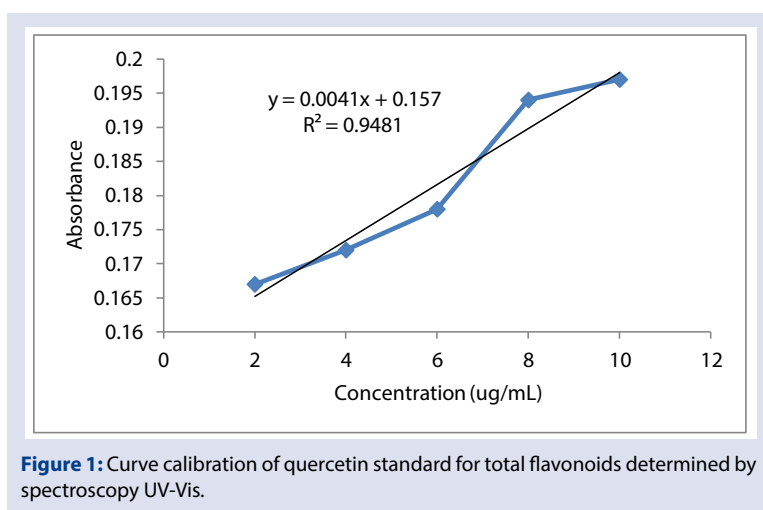


Figure 1: Curve calibration of quercetin standard for total flavonoids determined by spectroscopy UV-Vis.

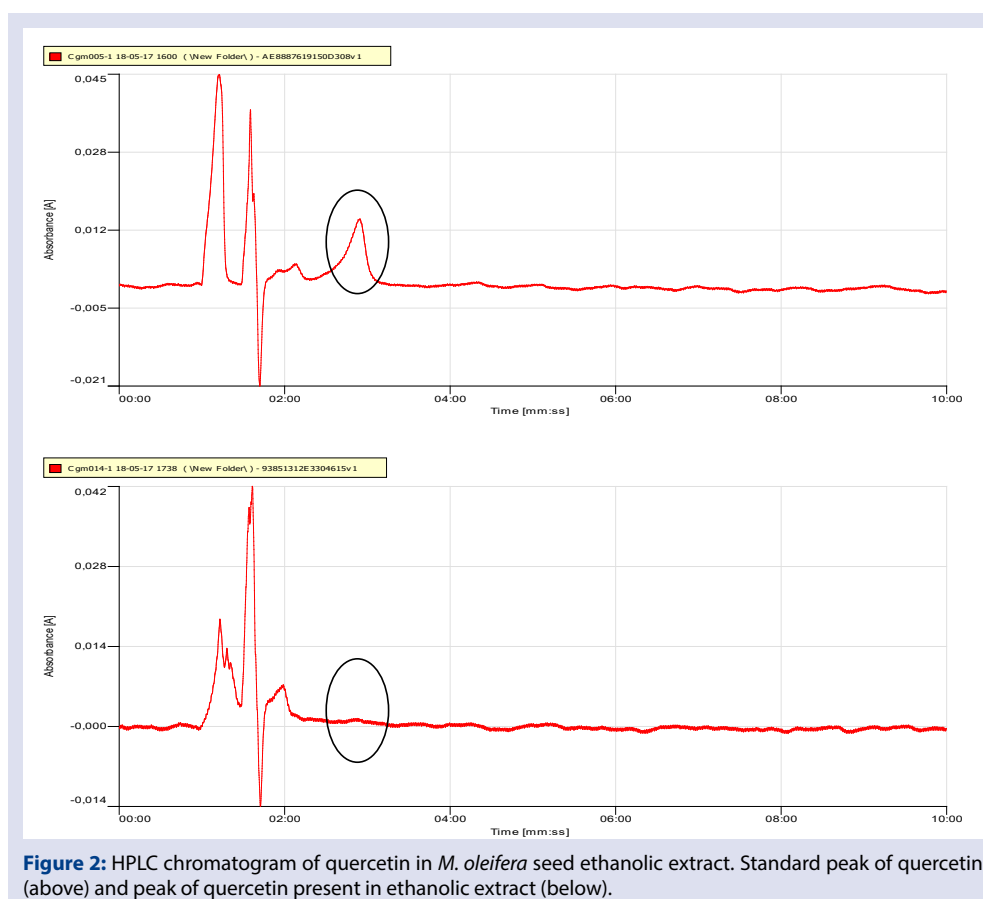


Figure 2: HPLC chromatogram of quercetin in *M. oleifera* seed ethanolic extract. Standard peak of quercetin (above) and peak of quercetin present in ethanolic extract (below).

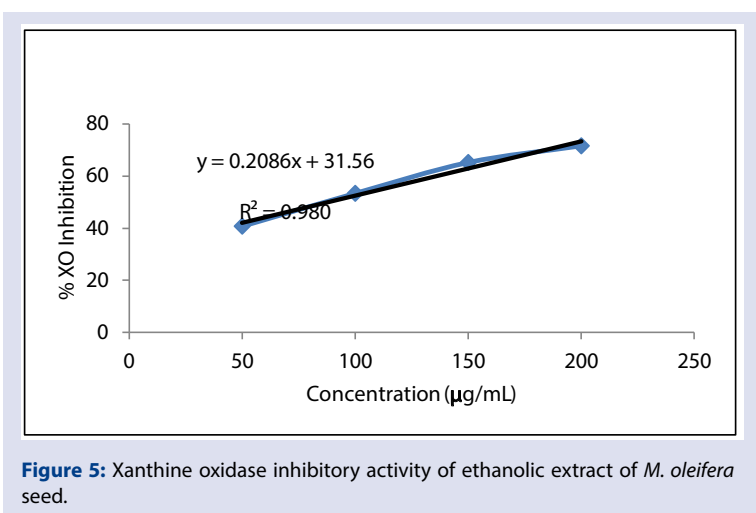
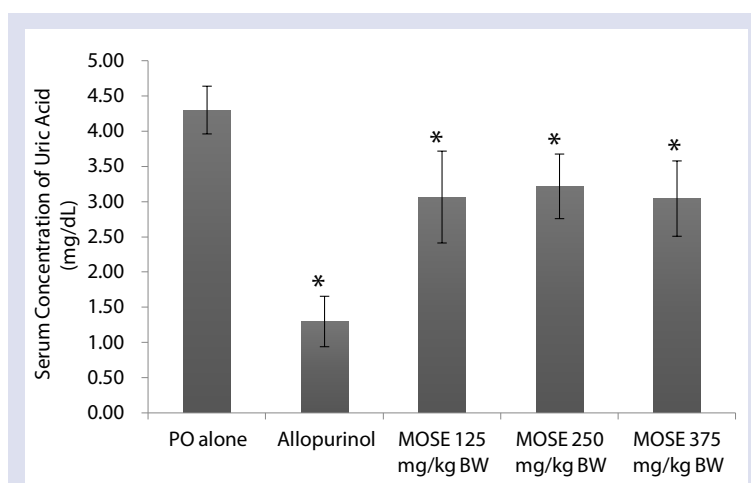
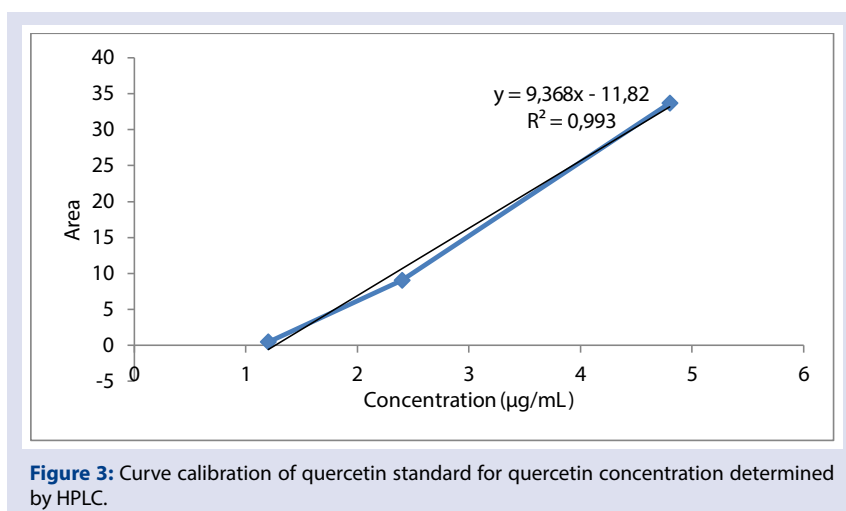


Table 1: Uric acid levels of rats after giving *M. oleifera* seed ethanolic extract at concentrations of 125, 250 and 375 mg/kg.

Treatment	Dose (mg/kg BW)	Uric Acid Level (mg/dL)
PO alone	-	4.30 ± 0.339 ^a
Allopurinol	27	1.29 ± 0.358 ^b
<i>M. oleifera</i> seed	125	3.06 ± 0.652 ^c
	250	3.21 ± 0.458 ^c
	375	3.04 ± 0.535 ^c

DISCUSSION

In this study, *M. oleifera* seed ethanolic extract was tested on the activity of decreasing the serum uric acid level in oxonate induced rats. The mechanism of the *M. oleifera* seed ethanolic extract was also determined by using in vitro methods with the target of xanthin oxidase enzyme. *M. oleifera* seed was extracted by ethanol to obtain the crude extract as an viscous ethanolic extract material. The seed of *M. oleifera* was collected from the cultivation area at the morning. The mature fruit was taken and the seed was directly excluded from the fruit. The seed was then dried on the shade room until ready for extraction. Ethanol 96% was chosen as solvent for extraction based on the non toxic solvent and extraction effectivity with high rendamen.¹⁵

The decreasing of uric acid levels of *M. oleifera* seed ethanolic extract was compared to standard drug allopurinol, the most common synthetic drug used in medicine to reduce uric acid levels. Potassium oxonate was used to induce gout in rats by inhibiting uricase, enzymes which convert uric acid to allantoin which is more water soluble. The highest uric acid levels can be obtained in two hours after administration and decreased again to normal levels in eight hours after administration.¹⁶ It is found that 125 mg/kg BW was the effective dose of *M. oleifera* seed ethanolic extract to reduce the uric acid concentration in rat blood plasma. Further study on the mechanism of this activity, xanthin oxidase inhibition assay was performed. It is found that *M. oleifera* seed ethanolic extract have moderate inhibition on XO comparing to allopurinol.

Biological activity of plant extracts was determined by their secondary metabolite contents. Therefore, qualitative phytochemical screening on *M. oleifera* seed ethanolic extract was performed by TLC using particular spraying reagents. It was successfully identified the presence of alkaloids, phenolics, flavonoids, terpenoids, saponin and tannin on the extracts. *M. oleifera* seed was reported to contain moringine (alkaloid), catechin, epicatechin, quercetin and kaempferol (flavonoid), gallic acid, p-coumaric acid, ferulic acid, caffeic acid (phenolic acid) and glycosides beside sterol, tokoferol and fatty acids.¹⁷ Among flavonoids, quercetin was considered as major compound in *M. oleifera* seed.¹⁸ It is suggested that flavonoid is the most potential substance that can inhibit xanthin oxidase leading to the decreasing of serum uric acid level in the body. Some studies showed that the activity on XO inhibition was correlated with their phenolic compounds particularly from flavonoid contents.¹⁹ Flavonoids such as chrysin, luteolin, kaempferol, quercetin, myricetin, and isorhamnetin can inhibit xanthine oxidase activity in a mixed-type mode.^{20,21} Therefore, further study was performed to quantify the total flavonoid and quercetin content itself after confirming its presence. It is found that *M. oleifera* seed ethanolic extract contain total flavonoids of 82.17 ± 0.684 mg QE/g dried extract and quercetin content of 0.5131 ± 0.0022 mg/g. This total flavonoid value was higher than the total flavonoid of *M. oleifera* seed previously reported from Kenya. Meanwhile, the quercetin concentration reported here was also higher than the quercetin content of *M. oleifera* seed from Carribean Island of Saint Lucia.^{18,22}

CONCLUSION

The antihyperuricemic activity *M. oleifera* seed ethanolic extract could be suggested by inhibiting the XO activities and mainly supported by the high concentration of total flavonoid and quercetin concentration. It is suggested that *M. oleifera* seed ethanolic extract can be developed as herbal medicine for treating hyperuricemia.

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CONFLICTS OF INTEREST

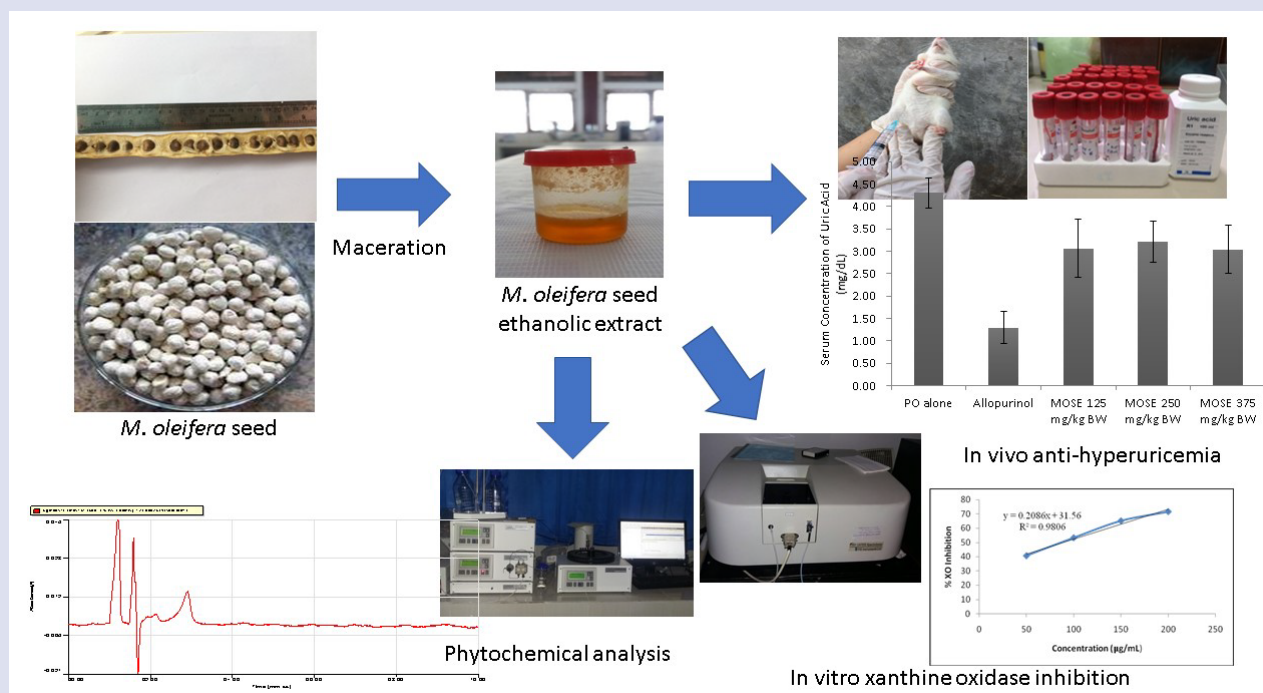
None.

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GRAPHICAL ABSTRACT



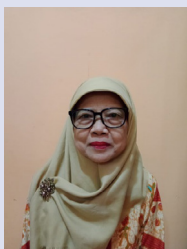
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