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Phytochemical, Antioxidant Analysis *and In Vitro* Xanthine Oxidase Inhibitory Activity of *Kaempferia parviflora* and *Kaempferia galanga*

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ABSTRACT

Kaempferia galanga and *Kaempferia parviflora* are employed in the functional food and nutraceutical industries and have many health benefits. Both plants contain bioactive substances with anti-inflammatory and alternative therapeutic potential for gout-related inflammation. The purpose of this was to compare the phytochemical, antioxidant, and xanthine oxidase inhibiting activities of rhizome extracts from *K.galanga* and *K.parviflora* using *in vitro* assays. The test tube approach was employed to perform phytochemical screening analysis. The spectrophotometric method using *1,1-diphenyl-2-picryl-hydrazyl* was employed to measure the antioxidant activity. UV spectrophotometry was employed to determine the *in vitro* xanthine oxidase inhibitory activity with Allopurinol as a positive control. The results showed that tannins, alkaloids, flavonoids, and polyphenols were present as phytochemical constituents. Based on the Folin-Ciocalteu technique, the level of phenolic compounds in *K.parviflora* and *K.galanga* was 52.33 and 50.35 mgGAE/100g, respectively. *K.parviflora* contains 144.2 mg total flavonoid content and *K.galanga* has 20.98 mgQE/100 g. In *K.parviflora*, free radical scavenging activity was measured using the IC_{50} (DPPH method), with a value of 547.202 ± 3.88 µg/mL, and *K.galanga* with a value of 626.308 ± 5.06 µg/mL. *K.parviflora* also has the highest inhibitory effect with an IC_{50} value of 29.69 ± 3.27 µg/mL while *K.galanga* has 139.92 ± 0.51 µg/mL based on the xanthine oxidase inhibitory activity using *in vitro* assay. The findings helped to shed light on some of this species' phytochemical traits and indicate that both plants are worth future investigation for an anti-gout *in vivo* assay using an animal test.

***Keywords*:** Phytochemicals, Antioxidant, Xanthine oxidase, *Kaempferia parviflora*, *Kaempferia galanga*.

Introduction

Gout is a metabolic illness induced by monosodium urate crystal deposition in the joints.[1] Moreover, hyperuricemia is a condition in which uric acid levels are above normal due to excessive production or/and impaired excretion.[2] The purine catabolic pathway is responsible for the oxidation of hypoxanthine to xanthine and xanthine to uric acid at the last stage of biosynthesis.[1] Gout is a prevalent condition employed by excessive production and/or insufficient secretion of this molecule.[1] Gout patients frequently have an elevated level of oxidative stress.[3] Hyperuricemia and gout are pathological conditions defined by excessive or insufficient excretion of uric acid; a byproduct of purine metabolism naturally discharged in the urine. Chronic disorders such as hypertension, diabetes, and metabolic syndrome, as well as renal and cardiovascular diseases, are frequently connected with these conditions.[4] Oxidative damage to living tissues causes inflammation, arterial hardening, cancer, senescence, and gout arthritis. Xanthine oxidase (XO) is the principal biological generator of oxygen-derived free radicals. This demonstrates the significance of uricemia control and uric acid level monitoring. European Guidelines propose blood uric acid values of 6 mg/dL for patients with both chronic hyperuricemia and urate deposition.[5] On-pharmacological therapy, such as consuming a diet low in purine-rich foods, sugars, and alcohol, as well as those rich in vegetables and water is very important.[6] However, an advanced pharmacological therapy is needed when other methods are not effective in reducing uric acid levels.[1] Allopurinol lowers serum and urine urate levels by inhibiting Xanthine oxidase.[1] Previous studies also showed that reduced urate levels can minimize the incidence of flares and tophi.[7] Although allopurinol reduces uric acid, it is not the appropriate medicine for acute gout arthritis.[1] The drug can produce superoxide, as well as cause allergies.[8] It can also induce several serious responses, such as liver function problems, allopurinol hypersensitivity syndrome, and reaction toxicity, including gastrointestinal, renal, and gastrointestinal hemorrhage.[8] Several studies employed plant-based phytochemicals without side effects to cure hyperuricemia and gout, which serve as xanthine oxidase inhibitors.[9] Previous reports showed that flavonoids can be employed as active inhibitors of Xanthine oxidase. They also function as compounds that scavenge free radicals.[8]

Kaempferia galanga and *Kaempferia parviflora* are rich in bioactive phytochemicals, particularly polyphenols and flavonoids.[10,11] Several studies also revealed that they have great anti-inflammatory potential and were employed as an alternative with increased therapeutic activity and fewer side effects.[8,12] This indicates that they can be employed as an alternative treatment for gout-related inflammation.[13,14] *K.galanga* and *K.parviflora* were selected for this investigation based on their ethnomedicinal use for rheumatic illnesses, arthritis, joint fractures, gastritis, osteoarthritis, and inflammation.[15,16] Although they have extensive usage, no studies revealed the xanthine oxidase inhibitory activity. This indicates that it is important to analyze the inhibitory effect of bioactive substances in plants. Consequently, the purpose of this research is to use *in vitro* assays to determine whether or not *K.galanga* and *K.parviflora* rhizome extracts

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have any phytochemical, antioxidant, or xanthine oxidase inhibitor activity.

Materials and Methods

Material

The study material employed Hydrochloric acid (Merck), ammonia solution (Merck), Dragendorff reagent, Mayer's reagent, magnesium powder (Merck), diethyl ether (Merck), Lieberman-Burchard reagents, DPPH (Sigma Aldrich, p.a), sodium acetate trihydrate, quercetin, EDTA (Merck), folin ciocalteu reagent, ferric chloride, acetylsalicylic acid, methanol (E. Merck), chloroform (E. Merck), ethyl acetate (Smart lab), allopurinol, and xanthine oxidase (Sigma).

Preparation of K. parviflora and K. galanga Extracts

Kaempferia parviflora was obtained from Malaysia in January, 2021 and authenticated by a botanist from the Laboratorium Ekologi dan Konservasi Biodiversitas Hutan Tropis, Universitas Mulawarman Fakultas Kehutanan with specimen voucher number 11/UN17.4.08/LL/2021 while *Kaempferia galanga was* collected from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Kementerian Kesehatan Republik Indonesia in Februari, 2021 and further identified by botanist from the Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, Kementerian Kesehatan Republik Indonesia with specimen voucher number of YK.01.03/2/892/2021. The rhizomes were immediately washed under running water to remove the soil, then sliced and dried in an oven at 70 ℃ for 48 hours. The plant is then coarsely processed to produce plant powder. Then, extraction was performed using the maceration method with ethanol solvent at a powder-to-solvent ratio of 1:5 for three days, with daily stirring. The solution was then filtered, condensed with a rotary evaporator, and the resultant extract was refrigerated at 4 ℃ until further examination.[17]

Qualitative Screening phytochemical

The preliminary phytochemical analysis was carried out using the methods to identify the following compounds: saponin, tannin, steroid/terpenoid, alkaloid, flavonoid, glycosides, quinone, and polyphenol was performed according to the methods described by Arifudin and Bone[17] and Mardina.[18] The methods were employed to perform qualitative analysis with a test tube analysis described by Pooja[19] Table 1 summarizes the results of the phytochemical analysis .

Quantitative Screening Phytochemical

Determination of Total Phenolic Content (TPC)

TPC was measured using UV-vis spectrophotometry at 765 nm. A concentration range of 3–50 µg/mL of gallic acid was utilized as a standard. In addition, 100 mg of material was weighed before being dissolved in 1 mL of 96% methanol (ethanol extract) and distilled water (water extract). 10 mL of methanol with a concentration of 96% were added to a volumetric flask. The materials were centrifuged after 10 minutes of vortexing to extract the supernatant. The procedure continued with the addition of 500 µL of sample and 5 mg/mL of gallic acid solution, followed by the addition of 0.5 mL of Folin–Ciocalteu reagent. Eight minutes were spent shaking and incubating the mixture. The mixture was supplemented with 10 mL of distilled water and 3 mL of a 10% Na_2CO_3 solution. The sample was then incubated in the dark for two hours before being examined with a 765 nm wavelength UV-vis spectrophotometer. Each sample was examined three times, and the results were expressed as milligrams of gallic acid per one hundred grams of extract.[21]

Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric method was employed to figure out how much TFC was in the sample. In order to measure quercetin, a standard calibration curve was made. Then, 5 mg of quercetin stock solution was put into 1.0 mL of methanol (5–180 µg/mL) and dissolved one by one. A 2% $AlCl_3$ solution was made by weighing and dissolving 1 g of $AlCl_3$ in 50 mL of distilled water in a beaker glass under a fume hood. Next, 0.6 mL of diluted standard quercetin extracts or solutions were mixed with 0.6 mL of 2% aluminum chloride. After being mixed, the mixture sat at room temperature for 60 minutes. A Varian UV-Vis spectrophotometer was employed to measure the absorbance of the reaction mixtures against a blank at a wavelength of 420 nm. The calibration curve ($Y = 0.0162x + 0.0044$, $R2 = 0.998$) was employed to figure out how much TFC was in the test samples. The result was given as mg of quercetin equivalent (QE) per gram of dried plant material. There were three copies of every measurement.[21]

DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging assay

In this research, the DPPH radical scavenging abilities of *K.parviflora* and *K.galanga* extracts were tested using a previously described methodwith a minor modification.[22] Additionally, 5 mL of a 5–200 µg/mL extract solution in methanol was added to 3 mL of DPPH solution. After homogenizing the mixture, it was put in a dark area for 30 minutes. The mixture's absorbance was measured at a wavelength of 517 nm. Methanol is utilized as the blank. As a positive control, vitamin C was employed as a comparison. Every measurement was conducted in triplicate .

Screening of Xanthine Oxidase Inhibitory Activity

In this study, allopurinol (100 µg/mL) served as the inhibitory test standard. 300 µL of 50 mm sodium phosphate buffer (pH 7.5), 100 µL of sample solution dissolved in deionized water, 100 µL of freshly manufactured enzyme solution, and 100 µL of deionized water were combined to create the reaction mixture. The mixture was then incubated for 15 minutes at 37°C. After adding 200 µL of substrate solution, the mixture was incubated at 37°C for 30 minutes. The reaction was finally halted by adding 200 µL of 0.5 M hydrochloric acid (HCL). Using a UV/VIS spectrophotometer against a blank, the absorbance was measured and read at 295 nm. Enzyme solution was replaced with the phosphate buffer. As a control, 100 µL of dimethyl sulfoxide (DMSO) was added to the reaction mixture in place of the test chemicals to obtain the greatest uric acid production. All determinations were performed in triplicate.[23]

% xanthine oxidase (XO) inhibition = $(1 - \beta/\alpha) \times 100$

A means the activity of xanthine oxidase without the test extract, and β means the activity of xanthine with the test ectract.

Statistical analysis

The data from both readings were put together and given as mean ± standard deviation (SD). The significance level was set at $p < 0.05$, and an Independent sample T-Test was employed to compare and evaluate the mean values. In addition, nonlinear regression analysis was employed to determine the IC_{50}.[2] IBS SPSS Statistics version 23 for Windows was employed for all statistical analyses.

Results and Discussion

K.galanga and *K.parviflora* are plants that grow in tropical and subtropical regions, specifically in tropical Asia, including China, Indonesia, Malaysia, Thailand, Taiwan, and India.[10,13] Rhizome of *K.parviflora* is black and has a slightly bitter taste while *K.galanga* is white and has a lighter taste. Furthermore, plants from the Zingiberaceae family have several biological activities, such as antioxidant, antibacterial, anti-inflammatory, pain reliever, gastritis, dyspepsia, and anticancer.[24,25] In this study, the samples were extracted using the maceration method using 96% ethanol. The extraction yield was 21.14% for *K. parviflora* and 12.21% for *K.galanga*. Based on organoleptic results, dried *K. parviflora* was odorless and black, while dried *K.galanga* was odorless and dark brown. Another study using the maceration method obtained a yield of 13.12% with the n-hexane solvent, while 5.52% was recorded from the ethyl acetate solvent.[11] In *K.parviflora*, soxhlet method, ultrasonic maceration, and infusion were carried out with 95% ethanol, methanol, and water as solvents, but the yield value was unknown.[24] The yield analysis in this study is important because it can be employed to determine the success rate of the extraction process, where the higher the yield value, the higher the compound components produced.

Phytochemical analysis showed positive results for *K. parviflora* and *K.galanga* for the presence of tannins, alkaloids, flavonoids, and polyphenols (Table 1). Wang *et al* stated that *K.galanga* has various types of secondary metabolite compounds, such as terpenoids,

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phenolics, flavonoids, polysaccharides, and essential oils.[26] Another study revealed that the extracts of petroleum ether, chloroform, and methanol extracts contain sterols, triterpenoids, flavonoids, and resins. Meanwhile, the aqueous extracts showed the presence of saponins.[12] Other studies on *K.parviflora* water extract stated that it contains polyphenolic and flavonoid compounds,[27] while the ethanol extract has a high volatile oil.[28] Analysis using GC-MS showed the presence of various methoxyflavones compounds, including 5-hydroxy- 3,7,3,4-tetramethoxyflavone, which has potential anti-inflammatory activity.[24] The phenol compound content of the ethanol extracts of *K. parviflora* and *K. galanga* was determined using the Folin-Ciocalteu test. The result shows that there was no significant difference between the total phenol content of *K. parviflora* and *K.galanga* with a value of 52.33 mg and 50.35 mg Gallic Acid Equivalent (GAE)/100g (Table 2). Polyphenols are phytochemical compounds that are naturally found in plants. Various studies revealed that polyphenols have several health benefits.[29] The value of flavonoid content in the extract was employed to assess the type of phenolic compound.[30] The test results for TFC were determined based on the standard curve of quarcetin.[20] Table 2 shows that TFC in *K.parviflora* caemployed a significant ($p < 0.05$) decrease in the total flavonoid content with a value of 144.2 mg and *K.galanga* 20.98 mg Quercetin Equivalent (QE)/100g. Chaisuwan, *et al*.[30] reported that *K.parviflora* extract had 10 methoxyflavones that were isolated and identified using GC chromatograms, whereas Kumar,[31] reported that the rhizome of *K.galanga* contained two highly bioactive flavonoids, kaempferol and kaempferide. These compounds can easily dissolve in polar solvents, and the solvent employed in this study was ethanol, which made them to be well drawn in the extraction process.[30] Previous studies,[32] revealed that their damage can be caemployed by temperature during the process. Flavonoids are very sensitive to heat, hence, they can be degraded at excessive temperatures. To avoid the degradation of flavonoid and phenolic compounds, the extraction must be carried out below 80˚C to produce greater content.[9] Phenolic compounds are widely recognized for their therapeutic benefits, particularly in the prevention of certain diseases like diabetes, hypertension, and cancer, as well as their potency as antioxidants and anticholinesterase agents.[30] The antioxidant activity of the ethanolic extracts of *K.parviflora* and *K.galanga* was evaluated using the DPPH technique at a standard concentration ranging from 5 µg/mL to 200 µg/mL.30 The results of DPPH scavenging activities by the two species extracts are presented in Figure 1. For the IC_{50} determination, a standard curve of DPPH was developed, where lower values suggest stronger scavenging activity. The IC_{50} were determined with linear regression against the DPPH scavenging percentage. The linear regression equation obtained in the antioxidant activity test was employed to determine the value. Radical scavenging activity using DPPH assay with $IC_{50} < 50$ µg/mL was declared as a very strong, while 50 - 100 µg/mL was categorized as strong. Value of 100 - 150 µg/mL was categorized as moderate, while values of > 150 µg/mL is weak.[21] The lower the value obtained, the stronger the antioxidant activity produced. Vitamin C as a standard showed an IC_{50} of 3.77 µg/mL, which was in the very strong category. This is because Vitamin C is an important antioxidant that can significantly reduce the side effects of reactive oxygen species, thereby preventing damage to macromolecules in the oxidative reactions.[21] The present study showed that the IC_{50} for DPPH assay are 547.202 ± 3.88 µg/mL and 626.308 ± 5.06 µg/mL for *K.parviflora* and *K.galanga*, respectively. This result is related to the total value of flavonoids produced in both plants, where the value of the flavonoids produced *in K.parviflora* is higher than *K.galanga*. Previous studies suggested that total phenolics and flavonoids contributed to the antioxidant capacity observed with DPPH radical scavenging.[33] Antioxidants' influence the scavenging due to their ability to donate hydrogen for the conversion of the radicals into stable diamagnetic compounds, such as diphenyl picrylhydrazine.[21] Furthermore, they are phenolic and flavonoid compounds because of their ability to neutralize free radicals, destroy singlet and triplet oxygen, dissolve peroxides, and chelate metal ions. The redox reactions of phenolics is due to the presence of hydroxyl groups in aromatic rings. This test correlates strongly with TPC, where the antioxidant activity increases along with the concentrations of these compounds.[30] The inclusion of glycosides and free hydroxyls in the structure of flavonoids contributes to their radical scavenging activity, with a chelating mechanism of action. The ability of these molecules in antioxidants to bind metal ions is closely related to the free radicals that are bound to a compound.[23] The process of hydrogen donation and tocopherol regeneration by flavonoids can help to break the long radical reaction. Biochemical and pharmacological activity is a broad spectrum of flavonoids and correlates with antioxidant and antiradical activity in a plant.[32] The independent t-test conducted on both plants at a confidence level of 95% yielded insignificant findings ($p>0.05$). Consequently, it was determined that there was no significant difference between the two test groups. (*K.parviflora* and *K.galanga*). From the result obtained in both plants indicate that the antioxidant activity cannot be extracted using the DPPH method. Therefore, it is important to carry out tests with other methods and compare them with this study. Meanwhile, the results for XO Inhibitory activity by both plant extracts showed in Figure 2. On the XO inhibition test, allopurinol (standard) showed IC_{50} value of 1.07 ± 2.50 µg/mL, while *K.parviflora* and *K.galanga* showed good *in vitro* XO inhibitory activity with IC_{50} values of 29.69 ± 3.27 µg/mL and 139.92 ± 0.51 µg/mL.

Table 1: Phytochemical Composition of *K. parviflora* and *K. galanga*

Name of samples	K.parviflora	K.galanga
Saponin	-	-
Tannin	+	+
Steroid/Terpenoid	-	-
Alkaloid	+	+
Flavonoid	+	+
Glycosides	-	-
Quinone	-	-
Polyphenol	+	+

(-): non identified; (+): identified

Table 2: Total phenol and flavonoid contents of *K. parviflora* and *K.galanga*

Sample	Total Phenol (mgGAE/100g)	Total Flavonoid (mgQE/100g)
K.parviflora	52.33 ± 0.03a	144.2 ± 0.20a
K.galanga	50.35 ± 0.05a	20.98 ± 0.09b

Values are expressed as mean ± standard deviation of replicate experiments (n = 3). Values with the same superscript alphabet along the same column are not statistically different at $p < 0.05$. GAE: Gallic acid equivalent; QE: Quercetin equivalent.

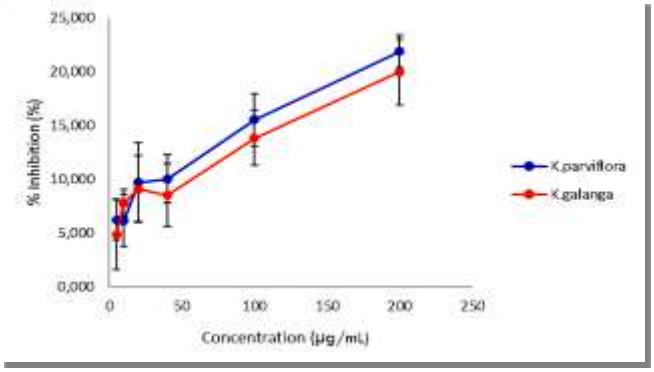

Figure 1: DPPH radical scavenging ability of ethanol extract of *K.parviflora* and *K.galanga*

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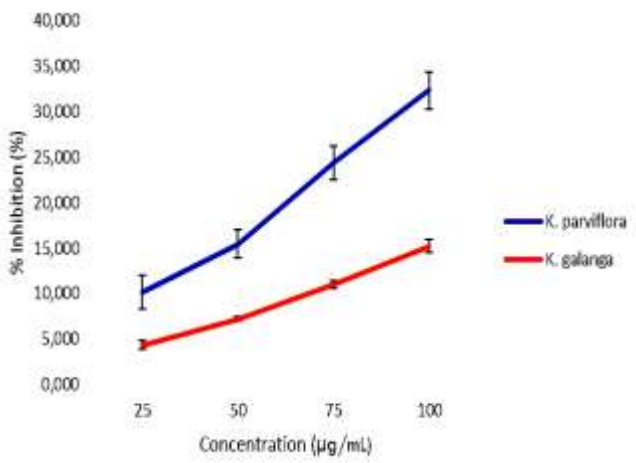

Figure 2: *In vitro* xanthine oxidase inhibitory activity of *Kaempferia parviflora* and *Kaempferia galanga*

The result of screening phytochemicals, total phenolics, and flavonoids shows that both plants are rich in flavonoids and polyphenols. Previous reports showed that phenolic and flavonoid compounds can inhibit XO activity.[32] The XO inhibitory activity of some plants is proportional to their TPC.[23] Other studies revealed that the structure-activity relationship of flavonoids determines their XO inhibitory and radical scavenging properties.[32] Through interaction with its molecular target, this relationship regulates the inhibition of XO. A previous study stated that the substitution of hydroxyl with glycoside or methyl group decreases the inhibitory activity of flavonoids.[8] The glycosylation at specific places can also interfere with the enzyme-binding process, thereby leading to a reduction in the inhibitory potency.[31] This is related with TPC and TFC of *K.parviflora*, which is higher than *K.galanga*. These results indicated that the flavonoids contained in both samples play a role in XO inhibitory activity. These findings were in line with previous studies that these molecules are one of the phenolic groups with the ability to inhibit XO activity.[23] This is due to the presence of bioactive compounds, namely flavonol.[34] These results from this study clearly show the XO inhibitory potential of the extracts of *K.parviflora* and *K.galanga*. The plants in this study have the potential to be employed as novel alternatives with improved medicinal outcomes and less adverse effects. Consequently, further testings should be carried out to determine the phytochemicals with XO inhibitory properties in the samples. I*n vivo* antihyperuricemia testing with test animals as well as XO inhibitory activity testing in serum and liver can also be performed in the future.

Conclusion

The present study showed that the ethanol extract of *K. parviflora* and *K. galanga* displayed positive for the presence of several secondary metabolites. Both plants possess *in vitro* xanthine oxidase inhibitory activity with IC_{50} values of 29.69 ± 3.27 µg/ml and 139.92 ± 0.51 µg/mL for *K. parviflora* and *K. galanga*, respectively. Further research should concentrate on *In vivo* antihyperuricemia animal testing and XO inhibitory activity testing in blood and liver.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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