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Radical scavenging activities of *Kaempferia larsenii* Sirirugsa extract and prominent flavonoids in its rhizomes

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Abstract

Medicinal plants of the genus *Kaempferia* (Zingiberaceae) have long been valued in Thailand, where their fresh rhizomes, extracts and oils are utilized for traditional medicinal purposes. While the well-known *Kaempferia* plants, particularly *K. galanga* and *K. parviflora* have been scientifically proven to be effective medicinal herbs in antioxidative investigations and in a variety of pharmacological activities, *K. larsenii*, one of the members of this genus that is found in northeastern Thailand and has long been used by local practitioners for curative purposes, has never had the chemical constituents and antioxidant activity reported before. Thus, the purpose of this study was to determine the antioxidant capacity of the plant and the active chemical components accountable for its action. *K. larsenii* rhizome extract was subjected to a number of antioxidative tests, including 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl, superoxide anion and hydroxyl radical scavenging activities, as well as HPLC analyses. The HPLC method used a ZORBAX Eclipse Plus C18 column, a 1 mL/min flow rate of methanol and 0.1% (v/v) phosphoric acid (55:45) and a diode-array UV absorbance at 265 nm as the stationary phase, mobile phase and detection respectively. In the investigated assays, the plant extract exhibited remarkable antioxidant activity, with EC_{50} values ranging from 11.60 to 26.31 µg/mL. Its efficacy was significantly higher than that of the positive controls, which had EC_{50} values ranging from 13.08 to 29.91 µg/mL. According to the HPLC profile of the extract, the medicinal flavonoids kaempferol and quercetin were present in the sample at % of 12.7562 and 0.4976 (w/w) respectively. *K. larsenii* rhizome extract is a good source of antioxidants and has the potential to be incorporated into herbal medicinal preparations that would be beneficial for health.

Keywords

Antioxidant, flavonoid, *Kaempferia larsenii,* kaempferol, Pro rasi, radical scavenging activity

Introduction

Kaempferia larsenii Sirirugsa is an herbaceous plant in the genus *Kaempferia* that is a medium-sized rhizomatous herb in the Zingiberaceae family. *Kaempferia* is a well-known medicinal plant found in tropical Asia with nearly 40 species, 29 of which are found in Thailand (1, 2). The species of this plant genus, notably *K. galanga* and *K. parviflora*, have been utilized in ethnomedical treatments for antiflatulence, antipyretic, gastric ulcers, leucorrhoea, oedema and wound healing for decades (3, 4). On the other hand, *K. larsenii*, also known as Pro rasi in Thailand, has a long history of being used as a cura-

tive and remedy for insect bites among Thai traditional practitioners in the northeastern region (5, 6).

Previous pharmacological and phytochemical examinations of *Kaempferia* plants demonstrated their remarkable therapeutic properties and related phytoconstituents, confirming the importance of the plants for both traditional and medicinal purposes (7, 8). There has been no research on *K. larsenii*'s bioactivities and responding active compounds. Thus, the purpose of this research was to analyze and establish the bioactivities and phytochemicals of *K. larsenii* in order to provide relevant information for the future development of the plant's crude into herbal preparations.

K. larsenii is a tiny herbaceous plant that grows to a height of 5-15 cm. The rhizomes are little brown ovoids that are strung together in a short-beaded necklace. The leaves are single, alternating, upright and lanceolate in shape. The inflorescences appear on the leafy shoot terminal. The petals are purple in color (9). The morphological attributes presented could differentiate the plant from other *Kaempferia* plants. Furthermore, the plant's habitat in Thailand is unique. It is exclusively found in the region of Ubon Ratchathani (10). Prior studies on the phytochemical investigation of *Kaempferia* plants, e.g., *K. parviflora*, revealed the presence of 3,5,7,3′,4′-pentamethoxyflavone, which is a flavonoid compound that was found abundantly in ethanolic extracts of the rhizomes and the compound also showed good antioxidant potential, as evidenced by its capability to scavenge free radicals, as noted in the literature (8). In this study, the antioxidant power of *K. larsenii* rhizome extracts was first measured using 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide anion and hydroxyl radical scavenging assays. In addition, the flavonoid compounds in the active extracts of this plant were identified by HPLC and their amounts were also reported .

Materials and Methods

Chemicals and reagents

Sigma-Aldrich, Singapore, provided the kaempferol (HPLC purity: 95%) and quercetin (HPLC purity: 95%) used in the study. Sigma-Aldrich, Singapore and S.N.P. Scientific Co., Ltd., Thailand respectively, supplied the chemical reagents ABTS and DPPH, as well as the essential reagents and disposable accessories for all of the antioxidant studies. Merck KGaA, Germany and S.N.P. Scientific Co., Ltd., Thailand provided us with all of the chemical reagents and disposable HPLC equipment accessories.

Plant material

Fresh *K. larsenii* rhizomes were gathered at one year of age in July 2019 in Muang Sam Sip district, Ubon Ratchathani province, Thailand's northeastern region, where the plant is endemic (6). The plant sample was identified by Asst. Prof. Dr. Thaya Jenjittikul of the Department of Plant Science, Faculty of Science, Mahidol University, Thailand. The Suanluang Rama IX Royal Botanic Garden in Bangkok, Thailand, has been designated as the repository for the plant samples, and a voucher specimen was coded as SL 9427.

Preparation of plant extract

Fresh *K. larsenii* rhizomes (1 kg) were rinsed with tap water, dried and cut into small pieces before being pulverized in a blender. The ground plant sample (300 g) was extracted for 3 hours with 95% ethanol using the Soxhlet device. At 40 °C, a rotating evaporator was used to take out the crude extract from the plant. The crude extract was weighed, resulting in an extraction yield of 2.84% w/w. Before being subjected to biological activity experiments, the ethanolic extract of *K. larsenii* rhizomes was diluted in methanol to reach concentrations of 1-300 µg/mL.

ABTS radical scavenging assay

The experiment was carried out on a 96-well plate according to the standard method (11) and absorbance at 734 nm was measured using a microplate reader (BioTek Synergy HT, USA). Each well contained 200 µL of the ethanolic extract and 200 µL of the newly prepared ABTS+ solution (the ABTS+ solution was made by reacting 7 mM ABTS (5 mL) with 2.45 mM potassium persulfate (5 mL) at room temperature for 16 hrs. The mixture was then diluted with distilled water to achieve an absorbance of 1.00 at 734 nm. For 6 mins, the reaction mixture was kept at room temperature. As a positive control, Trolox® was used. The following equation was used to estimate the % of ABTS radical scavenging.

Percent scavenging = $[(A_0\text{-}A_1)/A_0] \times 100\%$

The absorbance of the control (without the sample) is A_0, while the absorbance of the sample is A_1. The assessments were carried out in triplicate and the EC_{50} values (the sample concentration that results in 50% scavenging of maximal activity) were calculated using a program (Quest Graph™, AAT Bioquest, Inc., USA).

DPPH radical scavenging assay

The experiment was performed in a 96-well plate using the standard protocol (12) and absorbance at 517 nm was observed with a microplate reader (BioTek Synergy HT, USA). Each well contained a 200 µL extract of the doses examined as well as 0.2 mM DPPH in methanol (200 µL). The reaction mixture was vigorously agitated before being left at room temperature for 30 mins. As a positive control, L-ascorbic acid was employed. The DPPH radical scavenging % was calculated using the same equation as in the ABTS radical scavenging activity assay.

Superoxide anion radical scavenging assay

In order to assess the activity of superoxide anion radical scavenging in the extract, methods were followed using the published report (13). Methanolic extracts in various concentrations (200 µL) were combined with 50 mM nitroblue tetrazolium, 78 mM NADH, 16 mM Tris-HCl buffer pH 8.0, and 10 mM of phenazine methosulfate in 200 µL of the mixture. For 5 mins, the reaction mixture was allowed to stand at room temperature. A microplate reader (BioTek Synergy HT, USA) was used to measure the absorbance of the reaction mixture at 560 nm. An L-ascorbic acid positive

control was employed in the experiment. The percentage of superoxide anion radical scavenging was accomplished by following:

Percent scavenging = $[1-(A_1-A_2)/A_0] \times 100\%$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the sample, and A_2 is the absorbance without phenazine methosulfate. The EC_{50} values for the tested extract and the positive control were also calculated and presented in the result.

Hydroxyl radical scavenging assay

The experiment was conducted according to a published protocol in a 96-well plate (12), and absorbance at 562 nm was determined using a microplate reader (BioTek Synergy HT, USA). Each well contained 200 µL of the extract and 1.5 mM $FeSO_4$, 140 µL of 6 mM hydrogen peroxide, and 20 µL of 20 mM sodium salicylate. For 1 hr, the reaction mixture was incubated at 37 °C. As a positive control, L-ascorbic acid was employed. The percentage of ·OH radical scavenging was calculated using the same equation as for superoxide anion radical scavenging. A_2 in the equation, on the other hand, was the absorbance in the absence of sodium salicylate. The EC_{50} values for both the extracted and the positive control were further demonstrated.

Preparation of sample solutions and chromatographic apparatus

Dried extracts of *K. larsenii* rhizomes were precisely weighed and diluted with small portions of methanol to yield a sample solution with a concentration of 20 mg/mL. The phytochemicals kaempferol and quercetin were chosen as the analytical markers in this investigation according to the presence of these compounds in *Kaempferia* plants that were noted in previous reports (14-16). The reference material for the compounds was precisely weighed and diluted in methanol before being prepared for standard solutions at concentrations ranging from 10-400 µg/mL. Prior to analysis, the sample and serial standard solutions were filtered through a 0.45 µm nylon membrane. Each filtered sample (20 µL) was injected into a photodiode array detector (DAD)-equipped HPLC instrument (1260 Infinity Series, Agilent Technologies, USA). The OpenLab ChemStation software was used to record and evaluate the data collected from the HPLC apparatus.

Quantification of kaempferol and quercetin in extracts

The chromatographic signals of kaempferol and quercetin in the extracts were separated from the other compounds on a ZORBAX Eclipse Plus C18 column (150 mm × 4.6 mm i.d., 5 µm) using the isocratic mode of the mobile phase system, which consisted of methanol and 0.1% (v/v) phosphoric acid in a 55:45 ratio. This procedure was partially modified from the analytical method of flavonoid components in the extract of sea buckthorn using HPLC (17). The mobile phase flow rate was set to 1.0 mL/min and the HPLC column chamber was kept at room temperature. A signal from the chemicals was obtained at a wavelength of 265 nm. In each sample, the total analysis duration was set at 10 mins. The kaempferol and quercetin levels in each sample were calculated using a calibration curve of standard solutions and expressed as a % (w/w) of the ethanolic extract in each sample. The quantification of these components in the extract was carried out in triplicate.

Statistical analysis

Each experiment was conducted in groups of 3. The experimental data was presented in the form of a mean ± standard deviation (SD). For statistical comparisons, the *t*-test was utilized with a confidence interval % of 95% (SPSS Statistics version 26, USA) .

Results and Discussion

In vitro antioxidant capacity

K. larsenii rhizome ethanolic extracts showed effective activity in *in vitro* antioxidative tests including ABTS, DPPH, superoxide anion and hydroxyl radical scavenging assays with EC_{50} values of 11.60 ± 0.87, 18.56 ± 0.98, 26.31 ± 0.63 and 26.07 ± 0.73 µg/mL respectively, which are summarized in Table 1 and Fig. 1.

Table 1. The EC_{50} value of the rhizome ethanolic extract and positive control

Assay	EC_{50} (µg/mL)	
	Rhizome extract	Positive control
ABTS radical scavenging activity	11.60 ± 0.87[a]	13.08 ± 0.35[b]
DPPH radical scavenging activity	18.56 ± 0.98[a]	19.12 ± 0.36[a]
Superoxide anion radical scavenging activity	26.31 ± 0.63[a]	29.91 ± 0.89[b]
OH radical scavenging activity	26.07 ± 0.73[a]	26.82 ± 0.48[a]

All values are expressed as mean ± SD (n = 3). Trolox® was used as a positive control in ABTS radical scavenging assay. L-ascorbic acid was used as a positive control in DPPH, superoxide anion and ·OH radical scavenging activity assays. At a 95% confidence interval, a significant difference in mean values was noticed between the tested extract and the control group in each row, superscripted with different lowercase letters.

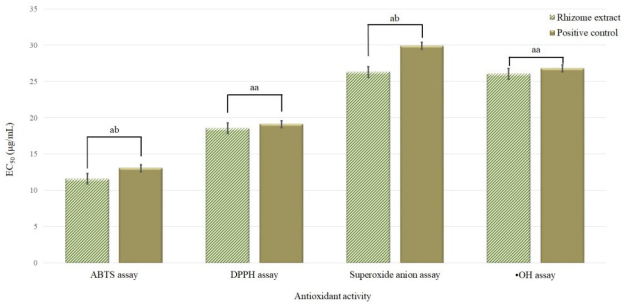

Fig. 1. Antioxidant activity of the rhizome ethanolic extract as determined by ABTS, DPPH, superoxide anion and ·OH radical scavenging activity assays; the first bar graph in each group represented the EC_{50} values of the examined extracts, while the second bar graph represented the positive controls' values; the distinct lowercase letters (ab) showed a significant difference (p < 0.05), whereas the identical letters (aa) proved the similarity of the means.

As a result of the antioxidant activity study, the ethanolic extract of *K. larsenii* rhizome was found to be effective in all of the radical scavenging activity experiments. The EC_{50} values of the plant rhizome extracts that were tested were consistently lower than those of the controls. This means that the rhizome extract of the plant was much

better at getting rid of free radicals than the positive control. Furthermore, in ABTS and superoxide anion radical scavenging assays, the statistical difference between the extracted sample and a positive control was significant ($p < 0.05$). Besides that, the examined extract was found to be just as powerful as a positive control, L-ascorbic acid, at $p < 0.05$ in DPPH and hydroxyl radical scavenging activities. Our study found that the antioxidant activity of the plant rhizome extract was interesting and appeared to have the potential to be subjected to further in-depth pharmacological testing in order to provide information for future development.

These findings were found to correlate with antioxidant properties in the other *Kaempferia* plants, such as *K. angustifolia* and *K. galanga*, with their rhizome extracts exhibiting strong antioxidant properties. In the ABTS radical scavenging assay, the ethanolic extract of *K. larsenii* displayed the strongest antioxidant activities, similar to the previous investigation of the alcoholic extract of *K. angustifolia* (18). Furthermore, the antioxidant effects of *K. larsenii* extract discovered in this study were similar to those of *K. galanga* extract, which demonstrated potent antioxidant capabilities in ABTS and DPPH assays (19, 20). The antioxidant power of *K. larsenii* extract was found in the same way as in previous studies, which revealed that extracts from *Kaempferia* species had a greater effect than a positive control (18, 19, 21).

As an outcome, our observations could be used to support the use of *Kaempferia* plants as a natural source of antioxidant materials. But for the first time, this report included scientific information about the antioxidant capacity of the extract of *K. larsenii* (Pro rasi) rhizomes. This could be useful information for plants in the *Kaempferia* species.

Kaempferol and quercetin content in extracts

The HPLC chromatogram of the ethanolic extract of *K. larsenii* rhizome demonstrates peaks of the chosen flavonoid compounds quercetin and kaempferol at retention times of 4.9 and 7.9 mins respectively and the chromatogram of the extract that was overlaid with the chromatograms of selected markers was also shown in Fig. 2. The standard-spiked technique with appropriate recovery and relative standard deviation % of average values of 103.26 and 1.43% for quercetin and 104.20 and 1.72% for kaempferol respectively, revealed that the analytical method employed in this study was accurate and precise. Table 2 summarizes the average % of quercetin and kaempferol per dried extract (% w/w) of 0.4976 ± 0.0511 and 12.7562 ± 0.0525 respectively. The quantities of quercetin and kaempferol detected in the extracts varied greatly. Kaempferol was found in the samples much more often than quercetin, which suggests that kaempferol is a considerable chemical component of the *K. larsenii* rhizome and could be used as a chemical marker to study the quality of rhizome extracts from the plant.

Kaempferol was discovered to be an important constituent in plant members of the Zingiberaceae family. It was discovered as a phytochemical in several *Kaempferia*

Table 2. Flavonoid content in the ethanolic extract of *K. larsenii* rhizome

Flavonoids	Content (% w/w)
Kaempferol	12.7562 ± 0.0525
Quercetin	0.4976 ± 0.0511

All values are expressed as mean ± SD ($n = 3$).

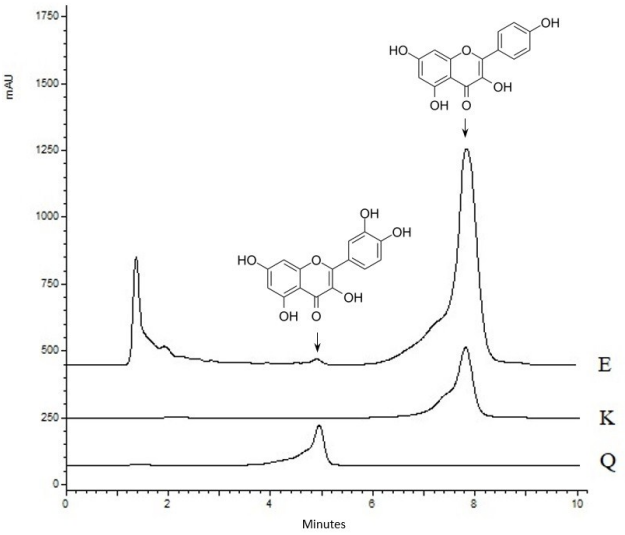

Fig. 2. Overlay chromatograms showing the peak of the flavonoids quercetin (Q), kaempferol (K) and *K. larsenii* extract (E)

species, including *K. augustiflora*, *K. galanga*, *K. parviflora* and *K. rotunda*, as previously reported (18-22). Thus, the existence of kaempferol, a flavonoid, in *K. larsenii* in this study was linked to the chemotaxonomy of the plants that contained this compound in their extracts and essential oils (23, 24). Moreover, one of the medicinal flavonoids, quercetin, was discovered for the first time in this investigation in *K. larsenii*, a plant species of the *Kaempferia*. In addition, kaempferol was recognized to have a wide range of biological functions, including the ability to reduce oxidative stress in both *in vitro* and *in vivo* tests, using both direct and indirect pathways (23). Kaempferol was discovered to scavenge several types of radicals, restrict reactive oxygen species (ROS)-generating enzymes and boost the production of antioxidant enzymes (25). It also shows intriguing anticancer properties by inhibiting the migration and proliferation of many cancer cell lines rather than gallic acid, a natural phenolic molecule that has been proven to have potent antioxidant functionality (26). Besides that, the minor quercetin component observed in *K. larsenii* has been found to have a broad spectrum of pharmacologically relevant flavonoid actions comparable to kaempferol (27). For the mechanism of action of flavonoids, the quantity of phenolic hydroxyl groups in the structure of flavonoids was shown to be proportional to their antioxidant activity. According to the findings, the antioxidant properties of flavonoid compounds were found to rise with higher concentrations, to have a dose-dependent relationship, and to exhibit a synergistic effect (28, 29). As a result, discovering these active chemicals in this plant could lead to the conclusion that they were responsible for the apparent antioxidant benefits. The exploration of these key medicinal compounds in this plant suggests that *K. larsenii*, like other well-known useful *Kaempferia* species, is one of the

promising medicinal plants for the development of natural medicines due to its potent antioxidant properties.

Conclusion

The *Kaempferia* species *K. larsenii* Sirirugsa was discovered to be a medicinally useful plant in this study based on the potent antioxidant properties of its rhizome extract observed in the investigated radical scavenging experiments. Furthermore, the HPLC profile of the extract indicated a strong peak of the potential therapeutic flavonoid kaempferol, a chemotaxonomic component found in the *Kaempferia* plants, suggesting that it corresponds to the other curative plants in the genus. As a consequence, this analytical method might be one of the most effective protocols to evaluate the authenticity of *Kaempferia* plants. This research could imply that *K. larsenii* rhizome extract has the potential to be employed as a natural antioxidant in herbal preparations and other related products. In addition to studying the different therapeutic phytochemical components, more research on the plant should include a number of pharmacological tests and an assessment of its toxicity. Exploring the antioxidant capacities and active components of this plant could provide significant information for advancement and identify it as one of the intriguing plants and potent herbs in the genus *Kaempferia*.

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Authors contributions

OT and PS created and developed this investigation's plan. OT conducted the plant authentication, pharmacognostic tests and biological activity with an emphasis on radical scavenging experiments. PS contributed to the chemical analysis of the investigated plant. Together, they exhaustively summarized and discussed all of the results and prepared the manuscript. The final manuscript was read and approved by all writers. PS is accountable for this research paper as the guarantor.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None.

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