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Effects of extraction methods on antioxidants and methoxyflavones of *Kaempferia parviflora*

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Abstract

This research was aimed to determine the effects of extraction methods on antioxidant properties and methoxyflavone contents of *Kaempferia parviflora* (KP) rhizome extracts. The KP rhizomes were extracted by maceration using ethanol at the concentrations of 25, 50, 75 and 95% V/V for 7 days and 95% V/V ethanolic extraction with sonication-assisted extraction (SAE) for 15-45 mins. Antioxidant components (phenolics, flavonoids and anthocyanins) and antioxidant activities (DPPH and FRAP) were examined. Methoxyflavones of the KP extracts were identified by a GC-MS technique. It was found that extraction methods affected the antioxidant properties of the extracts. Increasing ethanol concentrations enhanced anthocyanins but not phenolics and flavonoids. Ethanol concentration at 75% V/V exhibited the greatest DPPH while 25% V/V ethanol showed the greatest FRAP values. In this study, 10 methoxyflavones from KP extracts were separated and identified by GC chromatograms. The content of 5,7-dimethoxyflavone increased from 1.1 g/100 mL extract to 48.10 g/100 mL extract as the ethanol concentrations increased from 25% to 95% V/V. SAE for 15-45 mins had little impact on antioxidant properties as well as methoxyflavone contents. In general, SAE enhanced the extraction of KP rhizomes by increasing 5,7-dimethoxyflavone contents.

1. Introduction

Kaempferia parviflora (KP) or black ginger, is a medicinal plant in the family Zingiberaceae. It is originally found in the North and Northeast of Thailand as well as in other tropical areas such as Malaysia, Sumatra and Borneo Island (Chen *et al.*, 2018). The rhizomes of KP are considered health-promoting herbs and are traditionally used as a folk medicine for managing a variety of diseases (Saokaew *et al*., 2017). Some pharmacological studies on KP have claimed valuable health benefits due to its active phytochemicals. Previous studies have identified methoxyflavones and their glycosides as predominant flavonoids from KP rhizome extracts (Azuma *et al*., 2008; Chaipech *et al*., 2012; Ninomiya *et al*., 2016; Asamenew *et al*., 2019). The presence of those compounds contributed to a wider range of medicinal effects such as cellular metabolism-regulating activity (Okabe *et al*., 2014), anticancer activity (Potikanond *et al*., 2017), vascular relaxation and cardioprotective activity (Wattanapitayakul *et al*., 2008), sexual enhancing activity (Lert-Amornpat *et al*., 2017), neuroprotective activity (Wattanathorn *et al*., 2013), antiallergic (Kobayashi *et al*., 2015), anti-inflammatory (Horigome *et al*., 2014), antioxidative activity (Horigome *et al*., 2014), anti-osteoarthritis activity (Kobayashi *et al*., 2018), anti-microorganism activity (Sornpet *et al*., 2017), and transdermal permeable activity (Tuntiyasawasdikul *et al*., 2015).

Increasing health consciousness has recently attracted the attention of food manufacturers towards the products from KP rhizome extracts, owing to their rich contents of mythoxyflavones. Several products are commercially available such as concentrated KP extracts in both powder and liquid forms, as well as KP, extract beverages (Yeasmin *et al*., 2021). Extraction processes represent an important step. The common methods of extractions include conventional methods such as maceration in solvents, soxhlet extraction, and ultrasound extraction or modern methods such as supercritical and subcritical extraction and pressurized liquid extraction (Routray and Orsat, 2012). Different types of solvents are used, out of which hot water bath extraction and soxhlet extraction method are the most common for extraction of plant bioactive compounds (Wang and Weller, 2006). Ethanolic extraction is commonly used for KP rhizome extraction (Saokaew *et*

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al., 2017). Another solvent extraction method such as sonication-assisted extraction is also used for the extraction of flavonoids (Wang and Weller, 2006).

In view of the importance of extraction methods as well as phytochemical properties of KP rhizome extracts, this study determined the effects of ethanol concentrations and sonication-assisted extraction on antioxidant properties and methoxyflavone contents of KP rhizome extracts. The findings could be useful for designing the process that could enhance the properties of KP rhizome extracts.

2. Materials and Methods

2.1 Materials

Kaempferia parviflora Wall. ex Baker (Zingiberaceae), collected in October 2020 from Phetchabun province, Thailand, was supplied by Chaichada Co., Ltd. The rhizomes were dried at 60°C using a tray dryer and then ground to pass an 80-mesh screen (KP powder). Ethanol (analytical grade) and methanol (HPLC grade) were purchased from RCI Labscan Ltd. (Bangkok, Thailand).

2.2 Ethanolic extraction method

KP powder was extracted by the maceration method using ethanol at various concentrations (25, 50, 75 and 95% V/V). The KP powder was immersed in ethanol solution (solid: solvent ratio was 1:10 by weight), inside an airtight container for 7 days. The extracts were evaporated until the volume was reduced to 20% of the initial volume using a rotary evaporator (Roavapor® R-100, BÜCHI Labortechnik AG, Germany). The concentrated extracts were used for further analysis.

2.3 Sonication-assisted extraction method

Ethanol 95% V/V extraction with sonication-assisted extraction (SAE) was conducted in a sonication bath (GT Sonic G-Series, Korea). The sound wave was set at 40 kHz. The KP powder was immersed in 95% V/V ethanol solution (solid: solvent ratio was 1:10 by weight), in the sonication bath for 15, 30 and 45 mins. Extraction with hot water (45 mins) was used as the baseline comparison. The extracts were evaporated until the volume was reduced to 20% of the initial volume using a rotary evaporator (Roavapor® R-100, BÜCHI Labortechnik AG, Germany). The concentrated extracts were used for further analysis.

2.4 Antioxidant properties

The concentrated extracts (both ethanolic and SAE methods) were examined for their antioxidant properties.

Total phenolic contents of the extracts were measured according to the method described earlier (Luque-Rodríguez *et al*., 2007). The extract (0.4 mL) was added to 2 mL of 0.25 N Folin-Ciocalteu phenol reagents in water. After that, 1.6 mL of 7.5% (W/V) sodium carbonate was added to the mixture and heated in a water bath at 50°C for 5 mins. The absorbance was measured at 760 nm by a spectrophotometer (GENESYS™ 10S, Thermo Fisher Scientific Inc.) after cooling in darkness. The phenolic content was calculated from the standard curve of gallic acid, expressed as milligram of gallic acid equivalent (mg GAE)/mL concentrated extract.

Total flavonoids were assayed according to the method described elsewhere (Yang *et al*., 2009). The extract (0.25 mL) was mixed with 1.25 mL of distilled water and 75 µL of 5% sodium nitrite. After 6 mins, 150 µL of 10% aluminium chloride were added and left standing for 5 mins prior to mixing with 0.5 mL of 1 M sodium hydroxide and 775 µL of distilled water. The absorbance of the solution was determined at 510 nm. The calibration curve was established using catechin. Total flavonoid contents of the extracts were expressed as milligram catechin equivalent (mg CE)/mL concentrated extract.

Total anthocyanin contents were quantified using a modified pH differential method (Luque-Rodríguez *et al*., 2007). Samples were diluted in two buffer solutions: potassium chloride buffer 0.025 M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5) and then the absorbance was measured simultaneously at 525 nm and 700 nm, after 15 mins of incubation at room temperature using distilled water as blank. Total anthocyanins were expressed in mg of cyanidin-3-glucoside equivalents (CGE)/mL concentrated extract.

The ability of the extracts to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was determined according to the method described earlier (Nuengchamnong *et al*., 2009). A 50 µL of sample solution (in DMSO) was added to 200 µL of 100 mM DPPH solution in methanol. After an incubation period of 30 mins at room temperature in the darkness, the decrease in the absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) was used as a positive control for this assay. Inhibitory activities of various concentrations of the extracts were assessed to determine the half-maximal inhibitory concentration (IC_{50}) in µg/mL concentrated extract.

FRAP assay was performed as previously described elsewhere (Maier *et al*., 2009). The FRAP reagent was prepared from 2.5 mL of a TPTZ solution (10 mM) in hydrochloric acid (40 mM) and 2.5 mL of a ferric

chloride solution (20 mM) mixed with 25 mL of an acetate buffer (0.3 M, pH 3.6). For the determination of the antioxidant capacity, the FRAP reagent (1.5 mL) was mixed with 100 µL of distilled water and 100 mL of the appropriately diluted sample. The mixture was allowed to stand for 4 min at room temperature before the absorption was measured at 593 nm using Trolox as the standard. The results were expressed as mg Trolox equivalent (TE)/mL concentrated extract.

2.5 Methoxyflavone concentrations

Methoxyflavone concentrations were quantified by a GC-MS. The concentrated extracts were filtered through a 0.22 mm filter and then evaporated until dry. The extracts were re-dissolved with 1 mL of methanol prior to GC-MS instrumental analysis. The conditions of GC were as follows: column HP-5ms Ultra Inert, 30 m × 250 µm × 0.25 µm (Agilent 19091S-433UI, USA); oven temperature was programmed to raise from 200°C to 300°C at a rate of 15°C/min, and held at 300°C for 8.333 mins. The carrier gas was helium with a flow rate of 1.0 mL/min and a split ratio of 20:1. The Injection and transfer line temperatures were 280°C and 300°C, respectively. The sample (1 µL) was injected into the system. For electron ionization (EI), we used the ionization voltage of 70 eV. The temperatures used were 150°C for the MS Quad and 230°C for the MS Source. Full scan mass spectra were acquired at the mass range of 35-550 m/z with a scanning rate of 2 scans/s. The quality of methoxyflavones was identified by comparing them with the NIST MS library search program. For 5,7-dimethoxyflavone, it was identified couple with the retention time of the authentic compound (Sigma-Aldrich, St. Louis, MO, USA) and its quantity was performed using external standard ($R^2 = 0.9989$).

2.6 Statistical analysis

The experimental results were expressed as mean ± SD of duplicate observations. Data were analyzed statistically using SPSS® version 17 by ANOVA and Duncan's multiple range tests. A p-value of less than 0.05 was considered significant. The correlation coefficient methods among the contents of each antioxidant component and the value of each antioxidant capacity were determined by Pearson's correlation coefficient.

3. Results and discussion

3.1 Ethanolic extractions

3.1.1 Antioxidant properties

Antioxidant properties as determined by phenolic compounds, flavonoids, and anthocyanins are presented in Table 1. In addition, antioxidant activities as investigated by DPPH and FRAP are shown in Table 2.

From Table 1, it is obvious that ethanol concentrations affected the contents of phenolic compounds, flavonoids and anthocyanins of the extracts in different ways. The higher the ethanol concentrations, the lower the phenolic and flavonoid contents. In contrast, anthocyanins seemed to increase as the ethanol concentrations increased. In terms of antioxidant activity as evidenced by DPPH and FRAP (Table 2), ethanol concentration at 75% V/V exhibited the greatest DPPH while 25% V/V ethanol showed the greatest FRAP values. Pearson correlation between antioxidant components and antioxidant activities (data not shown) found that phenolics positively correlated to flavonoids and FRAP while they were negatively correlated to anthocyanins and DPPH. Flavonoids negatively correlated to anthocyanins and DPPH but they positively correlated to FRAP. Clearly that FRAP negatively correlated to DPPH. Those correlations were considered significant at a 0.01 level (2-tailed).

Table 1. Phenolic compounds, flavonoids and anthocyanins of the concentrated ethanolic extracts of KP powder.

Extraction conditions	Phenolic compounds (µg GAE/mL)	Flavonoids (µg catechin/mL)	Anthocyanins (µg CGE/mL)
25% ethanol	6.795±219^{a}	2.111±51.1^{a}	2.09±0.77^{d}
50% ethanol	5.277±100^{b}	1.496±35.8^{b}	4.34±0.44^{c}
75% ethanol	2.958±8.56^{c}	620.7±9.24^{d}	10.25±0.21^{b}
95% ethanol	2.864±23.2^{c}	686.2±3.20^{c}	24.90±0.77^{a}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

Table 2. Antioxidant activities of the concentrated ethanolic extracts of KP powder.

Extraction conditions	DPPH (IC_{50}, mg/mL)	FRAP (mg trolox/mL)
25% ethanol	5.37±0.01^{d}	5.051±33.7^{a}
50% ethanol	8.59±0.13^{c}	3.718±40.5^{b}
75% ethanol	20.0±0.08^{a}	2.144±67.4^{d}
95% ethanol	12.5±0.86^{b}	2.457±175^{c}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

Generally, the extraction efficiencies are governed by several factors such as the type of solvent, solvent concentration, time, temperature, pH, number of steps, liquid-to-solid ratio and particle size of the plant material. Due to the difference in extraction conditions, it is difficult to compare the phytochemical contents obtained from the extractions as they were reported in the literature. The contents of antioxidant components in this study were relatively high when compared to those previously reported (Tonsomboon *et al*., 2021). The

black colour of KP rhizomes could be the rich source of anthocyanins which are natural water-soluble pigments widely existing in plants, and with strong antioxidant activity.

KP rhizome extracts with the greatest content of phenolic and flavonoid compounds were obtained using distilled water extraction at 90°C for 2 hrs, and a solid/ solvent ratio of 1:25 g/mL (Ab Rahman *et al*., 2018). Another study reported no significant difference in antioxidant properties when KP powders were extracted using ethanol at various concentrations, 20-80% V/V (Patanasethanont, Nagai, Matsuura *et al*., 2007). The ethanolic extract of KP was reported to exhibit better pharmacological qualities than those of the aqueous extract. Some biological active compounds in the rhizome of KP could relatively be hydrophobic, which were more easily partitioned into ethanol solvent than into water solvent (Patanasethanont, Nagai, Yumoto *et al*., 2007). Antioxidant activities as evidenced by DPPH and FRAP reported in this study were also high. It has been reported that KP extracts exhibited dose-dependent radical scavenging activity. Almost all of the radicals were destroyed by 200 µg/mL of KP extract treatment (Lee *et al*., 2018). This study found that the great antioxidant properties of KP extracts could be obtained by ethanolic extraction, 50-75% V/V concentration. Maceration for a long period (7 days) in this study could also enhance the extraction capacity.

3.1.2 Methoxyflavone contents

GC chromatograms of the concentrated ethanolic extracts of KP powder are shown in Figure 1. In the current study, a total of 10 methoxyflavone aglycones were separated and identified from KP extracts. Based on earlier structural identification studies (Sutthanut *et al*., 2007; Asamenew *et al*., 2019), the main structure of methoxyflavones extracted from KP rhizomes includes benzene A ring with 2 substituent groups at positions 5 and 7, an aromatic B ring with 2 substituent groups at positions 3′ and 4′, and C ring with a substituent group linking on position 3. The substituent groups might be -H, -OH, or -OCH_3. The structure of methoxyflavones obtained from the concentrated extract in this study is illustrated in Figure 2.

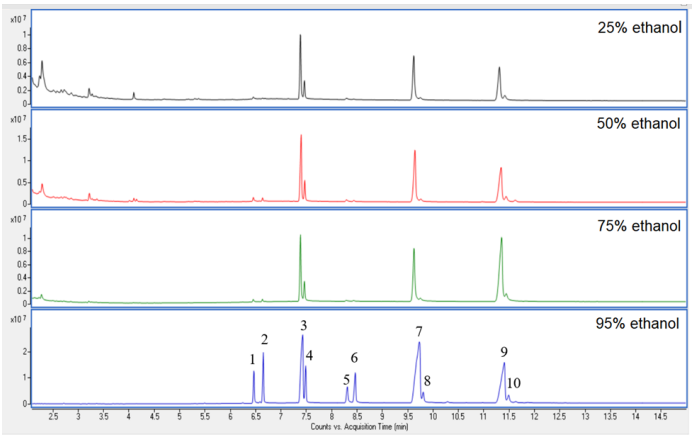
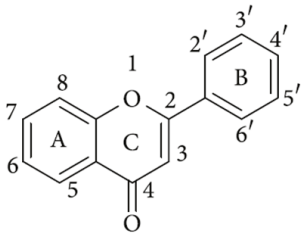


Figure 1. GC-Chromatograms of 10 methoxyflavones from the concentrated ethanolic extracts of KP powder using different concentrations of ethanol (25-95% V/V). Peak identifications are as follows: (1) 5-hydroxy-7-methoxyflavone; (2) 3,4'-dimethoxy-7-hydroxyflavone; (3) 5,7-dimethoxyflavone; (4) 3,5,7 trimethoxyflavone; (5) 5-hydroxy-4',7-dimethoxyflavone; (6) 7,4'-dimethoxy-3-hydroxyflavone; (7) 5,7,4'-trimethoxyflavone; (8) 5-hydroxy-3,7,3',4'-tetramethoxyflavone; (9) 3,3',4',5,7-pentamethoxyflavone; (10) 3',4',5,7-tetramethoxyflavone.

In previous studies, 25 methoxyflavones and their glycosides were identified and quantified as predominant flavonoids from rhizome extracts of KP (Azuma *et al*., 2008; Chaipech *et al*., 2012; Ninomiya *et al*., 2016) and



Methoxyflavones	Substitutions				
	3	5	7	3’	4’
(1) 5-hydroxy-7-methoxyflavone		-OH	-OCH_3		
(2) 3,4'-dimethoxy-7-hydroxyflavone	-OCH_3		-OH		-OCH_3
(3) 5,7-dimethoxyflavone		-OCH_3	-OCH_3		
(4) 3,5,7 trimethoxyflavone	-OCH_3	-OCH_3	-OCH_3		
(5) 5-hydroxy-4',7-dimethoxyflavone		-OH	-OCH_3		-OCH_3
(6) 3'-hydroxy-3,4',7-trimethoxyflavone	OCH_3		-OCH_3	OH	-OCH_3
(7) 5,7,4'-trimethoxyflavone		-OCH_3	-OCH_3		-OCH_3
(8) 5-hydroxy-3,7,3',4'-tetramethoxyflavone	-OCH_3	-OH	-OCH_3	-OCH_3	-OCH_3
(9) 3,3',4',5,7-pentamethoxyflavone	-OCH_3	-OCH_3	-OCH_3	-OCH_3	-OCH_3
(10) 3',4',5,7-tetramethoxyflavone		-OCH_3	-OCH_3	-OCH_3	-OCH_3

Figure 2. Structure of mythoxyflavones from the concentrated ethanolic extracts of KP powder.

they are available as constructed LC-MS libraries (Asamenew *et al*., 2019). The presence of these compounds contributed to a wider range of medicinal effects as mentioned earlier. Ethanolic extraction conditions used in this study provided the KP extracts with confirmed 10 bioactive methoxyflavones (Figure 2).

As 5,7-dimethoxyflavone is the major phytoconstituent of KP extracts, contributes to the biological effects especially vasorelaxation properties (Chen *et al*., 2018). This study quantified the contents of 5,7-dimethoxyflavone of the concentrated KP extracts by comparing the chromatogram areas with the standard and the results are shown in Table 3.

Table 3. Contents of 5,7-dimethoxyflavone from the concentrated ethanolic KP extracts.

Extraction conditions	5,7-dimethoxyflavone (g/100 mL extract)
25% ethanol	1.11±0.02^{b}
50% ethanol	2.14±0.43^{b}
75% ethanol	3.49±0.70^{b}
95% ethanol	48.1±9.62^{a}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

In this study, the content of 5,7-dimethoxyflavone increased as the ethanol concentrations increased. The extraction using 95% V/V ethanol increased the content of 5,7-dimethoxyflavone to about 48 folds. Ethanol extraction is recommended for phytochemical extractions of KP. Ethanol has been reported to provide extracts with better pharmacological qualities than aqueous extract (Patanasethanont, Nagai, Yumoto *et al*., 2007). Other extraction conditions such as time, temperature, solid ratio and material particle size could also affect the contents of bioactive compounds. The 5,7-dimethoxyflavone content of KP rhizomes determined by TLC-densitometry and TLC image analysis were found to be 2.15±0.64 and 1.96±0.51 g/100 g dry solid (Pitakpawasutthi *et al*., 2018).

3.2 Sonication-assisted extraction method

Sonication-assisted extraction (SAE) method was used to assist the ethanolic extractions of KP powder in comparison with hot water extraction. Antioxidant components as indicated by phenolic compounds, flavonoids and anthocyanins are illustrated in Table 4. In addition, DPPH and FRAP which represented antioxidant activities are shown in Table 5.

In this study, SAE with different times (15-45 mins) had little impact on antioxidant components as well as antioxidant activities. Phenolics and flavonoids slightly decreased as SAE time increased. Anthocyanin contents were not significantly different. The SAE conditions used in this study (15-45 mins) provided the KP extracts with fewer antioxidant components than those of hot water extraction (Table 4). For antioxidant activities, the extracts from SAE showed better DPPH but, unfortunately, they exhibited lower FRAP values than those from water extraction (Table 5). Moreover, 5,7-dimethoxyflavone of the concentrated KP extracts from SAE of KP powder was also quantified by comparing the chromatogram areas with the standard. The results are shown in Table 6.

Table 4. Phenolic compounds, flavonoids and anthocyanins of the concentrated extracts from SAE of KP powder.

Extraction conditions	Phenolic compounds (µg GAE/mL)	Flavonoids (µg catechin/mL)	Anthocyanins (µg CGE/mL)
Hot water 45 min	4.082±42.6^{a}	692.8±41.5^{b}	368.3±73.3^{a}
SAE 15 min	1.585±97.9^{b}	792.0±24.4^{a}	95.17±13.2^{b}
SAE 30 min	1.088±144^{c}	712.9±55.1^{b}	80.10± 8.90^{b}
SAE 45 min	1.160±63.6^{c}	754.8±0.80^{ab}	153.4±27.7^{b}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

Table 5. Antioxidant activities of the concentrated extracts from SAE of KP powder.

Extraction conditions	DPPH (IC_{50}, mg/mL)	FRAP (mg trolox/mL)
Hot water 45 min	15.48±2.47^{c}	2.060±14.4^{a}
SAE 15 min	39.82±8.62^{b}	1.625±6.19^{b}
SAE 30 min	58.11±2.30^{a}	1.362±103^{c}
SAE 45 min	42.09±1.19^{b}	1.305±59.2^{c}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

Table 6. Contents of 5,7-dimethoxyflavone of the concentrated extracts from SAE of KP powder.

Extraction conditions	5,7-dimethoxyflavone (g/100 mL extract)
Hot water 45 min	0.03±0.01^{c}
SAE 15 min	0.11±0.02^{b}
SAE 30 min	0.14±0.02^{b}
SAE 45 min	0.29±0.02^{a}

Values are presented as mean±SD. Values with different superscript within the same column are significantly different ($p \leq 0.05$).

Sonication uses ultrasonic waves to disrupt cell walls, exposing the surface area of cellular fragments to the solvent and thus increasing the rate of mass transfer of compounds to the extraction medium (Toma *et al*., 2001). Sonication has been used as a tool to extract phenolics from various plant materials (Onofre and Hettiarachchy, 2007). The conditions of SAE used in this study (15-45 mins) showed little impact on antioxidant

components and antioxidant activities, suggesting that the studied conditions might be insufficient to extract those compounds. However, SAE at 45 mins provided the highest content of 5,7-dimethoxyflavone. Although, only little impact on the increase of 5,7-dimethoxyflavone was observed as it increased from 0.11 to only 0.29 g/100 mL concentrated extract. SAE was used to assist the ethanolic extraction of KP and the best bioactive activity was found from the macerated extract, better than the extracts obtained by sonication and reflux (Wongsrikaew *et al*., 2012). In the current study, although maceration using ethanol provided the extracts with good qualities (Tables 1-3) the use of SAE could help in terms of reduced extraction time. The maceration in this study took 7 days and the contents of 5,7-dimethoxyflavone ranged from 1.11 to 48.10 g/100 mL concentrated extracts, while SAE took only 15 to 45 min to obtain contents of 5,7-dimethoxyflavone ranging from 0.03 to 0.29 g/100 mL extract.

4. Conclusion

KP rhizome extracts have claimed valuable benefits for a variety of diseases, owing to their rich source of bioactive mythoxyflavones. Extraction methods play a major role in controlling the phytochemical properties of KP rhizome extracts. This study found that ethanol concentrations affected antioxidant properties by altering antioxidant components and their activities, depending on the studied components. The total of 10 methoxyflavones obtained from the KP extracts was identified by GC chromatograms. Contents of the selected methoxyflavone, 5,7-dimethoxyflavone, were quantified. The maximum content of 48.10 g/100 mL concentrated extract was obtained using 95% V/V ethanolic maceration extraction. Sonication which uses ultrasonic waves enhanced the ethanolic extraction efficiency.

Conflict of interest

The authors declare no conflict of interest.

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