Enhancing the Total Polyphenol Content and Antioxidant Activity of *Kaempferia parviflora* through Optimized Binary Solvent Extraction: Isolation and Characterization of Major PMF Metabolite

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Kaempferia parviflora (*K. parviflora*) is a herbaceous species with potent bioactivities contributed by its major compounds, polymethoxyflavones (PMFs). The abundance of PMFs varies by extraction technique and parameter. This study aims to optimize the influence of a binary solvent extraction system on the percentage yield, total phenolic content (TPC), total flavonoid content (TFC), and free radical scavenging activity, and subsequently isolate and characterize the PMFs. Optimization of the binary solvent extraction system (ethanol: water) was carried out at different solvent ratios (40 – 95 % ethanol), solid:solvent ratios (s/s) (1:10 – 1:50), and extraction durations (24 – 72 hours). PMFs were identified through thin-layer chromatography (TLC). The *K. parviflora* extract (0.05 g/ml) was further fractionated, followed by isolation of PMFs using column chromatography with an appropriate mobile phase. The isolated PMFs were elucidated through analytical and spectrochemical methods (^{1}H-NMR, ^{13}C-NMR, MS & FT-IR). The antioxidant activity of the *K. parviflora* extract and the isolated PMFs were confirmed by 2-diphenyl-1-picrylhydrazyl DPPH assay. The maximum yield of *K. parviflora* extract was 17.82 % (60 % ethanol, 1:20 s/s, 24 hours). The optimized extract achieved the highest TPC, TFC, and strongest inhibition (IC_{50}: 0.161 mg/ml). Correlation analysis indicated a moderate linear relationship between TPC, TFC, and IC_{50}. TLC analysis confirmed the presence of 3,5,7,4'-tetramethoxyflavone, 5,7-dimethoxyflavone, 3,5,7-trimethoxyflavone and 5,7,4'-trimethoxyflavone. Fractionation of *K. parviflora* extract yielded 22 subfractions of n-hexane and 13 of chloroform. The major PMF was characterized and identified as 5-hydroxy-3,7-dimethoxyflavone (5-H-3,7-DMF), which exhibited moderate free radical scavenging activity.

Keywords: *Kaempferia parviflora*; maceration; optimization; characterization; polymethoxyflavones

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Secondary metabolites in plants are associated with bioactive agents derived from primary metabolites and their intermediates in biosynthetic pathways that are not involved in plant development [1]. Diverse secondary metabolites have been discovered in traditional herbs, including flavonoids from the polyphenolic group with established medicinal therapeutics [2]. Flavones are a subclass of flavonoids with a double bond between C2 and C3 on heterocyclic pyran ring C, that possesses anti-inflammatory [3, 4], anti-cancer [5], antioxidant [6] and antidiabetes [7] properties.

Kaempferia parviflora (*K. parviflora*) is a herbal rhizome species widely used by traditional practitioners in Thailand to enhance energy, and treat ulcers and inflammation [8]. The therapeutic potential of the rhizome is attributed to its bioactive polymethoxyflavones (PMFs), which have been exclusively identified in *K. parviflora*, compared to other rhizome *Kaempferia* species [6]. The abundance of PMF scaffolds in the crude *K. parviflora* extract is influenced by various parameters in the extraction technique, as indicated by variations in the percentage yield, total flavonoid content (TFC), total phenolic content (TPC) and antioxidant activity. With the

rise of new extraction methods offering improved efficiency, the maceration technique continues to be favoured due to its low energy consumption, and is preferred for extracting thermolabile compounds [8]. The selection of a solvent for the maceration technique is crucial to extract the desired phytochemicals effectively. Water has been widely used as the extraction solvent given its strong polarity that promotes the release of polar soluble compounds and increases the crude extract yield [9]. Nevertheless, considering the hydrophobic and semi- to low polarity properties of PMFs, organic solvents such as ethanol are preferred to maximize the absorption of these phytochemicals [10]. In a study by Chaisuwan et al. (2021), a high concentration ethanol extraction of *K. parviflora* rhizome resulted in a greater 5,7-dimethoxyflavone content (3.49 g/100 ml extract) [11]. Meanwhile, optimization with ultrasound assisted extraction of *K. parviflora* demonstrated the highest percentage yield (14.72 %) through a 1:1 ratio of ethanol to water [12]. Additionally, the sample to solvent ratio and the extraction duration are known to influence the extraction yield and eventually, its free radical scavenging capacity. The anti-radical activity of the extract can be explored using a DPPH assay. DPPH is widely utilized in determining the radical scavenging capacity due to its stable free radical [13]. By employing a binary solvent maceration extraction system with an appropriate ratio of sample to solvent and extraction duration, the aqueous solvent may facilitate absorption while the ethanolic solvent maximizes the extraction of phenolic and flavonoid content from the *K. parviflora* extract.

As flavonoids are well known for their health-promoting benefits, various biological activities of *K. parviflora* including antioxidant activity [14] have been reported given its abundance; however the free radical scavenging activity of their bioactive compounds was limited. Multiple studies have successfully quantified and isolated PMFs from crude *K. parviflora* extracts which had 1 to 5 methoxy groups bonded to the flavone's skeleton structure, dominated by the methoxy group attached to C7 of aromatic ring A [6]. Several PMFs exhibited a hydroxyl group attached to the C5 position [6]. Therefore, this study focuses on determining the effects of different ethanol concentrations, solid to solvent ratios, and maceration durations on the yield percentage, total phenolic and flavonoid content, and free radical scavenging activity of a *K. parviflora* extract, and its correlation studies. The optimum parameters obtained were used to extract, isolate, and characterize the major PMF from the *K. parviflora* rhizome and determine its antioxidant activity.

EXPERIMENTAL

Chemicals

All analytical grade chemicals and reagents used in the study were purchased from reliable sources and institutes [Merck, ThermoFisher Scientific, and TARGETMOL]. TLC Silica gel Aluminium 60 F254 20 x 20 cm (Merck, Darmstadt, Germany) and silica gel for column chromatography (70-230 mesh; Thermo-Fisher Scientific, Massachusetts, USA) were also used.

Plant Material

The *K. parviflora* rhizome originating from Thailand was purchased in dried form from Kelantan, Malaysia. Authentication of the rhizome was conducted at the Biodiversity Unit, Institute of Bioscience, Universiti Putra Malaysia, with voucher specimen KM0071/23.

Maceration Optimization

The extraction of *K. parviflora* powder (1 g) was executed based on a modified method adapted from Lee et al. (2022) at room temperature [15]. The experimental design followed a one-factor-at-a-time (OFAT) procedure, with the ethanol concentration, solid to solvent ratio, and maceration duration parameters shown in Table 1.

Table 1. Optimization parameters for the binary solvent maceration extraction system.

Series No.	Ethanol concentration (%)	Solid:solvent ratio (s/s)	Maceration duration (hour)
1	40	1:20	24
2	60	1:20	24
3	80	1:20	24
4	95	1:20	24
5	95	1:10	24
6	95	1:30	24
7	95	1:40	24
8	95	1:50	24
9	95	1:20	48
10	95	1:20	72

Extraction and Liquid-Liquid Extraction (LLE)

Based on the results obtained in the previous section, the optimal parameters were selected. Approximately 1 kg of *K. parviflora* powder was extracted at room temperature in the dark. The resulting extract was then filtered to remove plant materials, air-dried, concentrated in a rotary evaporator under reduced pressure, and subsequently freeze-dried overnight to yield dark purple crystals. The dried *K. parviflora* extract (109.5676 g, yield: 11.05 %) was carefully sealed from moisture and stored below 4 °C for further analysis. The dried ethanolic extract of *K. parviflora* was suspended in batches in 150 ml of a 1:1 ratio of water and ethanol. The suspended *K. parviflora* extract was partitioned successively with 150 ml of n-hexane, chloroform and water, and this process was repeated three times. Subsequently, all fractions were concentrated under reduced pressure and weighed using an analytical balance.

Total Phenolic Content (TPC)

The total phenolic content was measured by the Folin–Ciocalteu method with Gallic acid as the standard [16, 17]. A 200 µL aliquot of the extract dissolved in 95 % v/v methanol was added to 1 ml of Folin–Ciocalteu reagent (diluted with distilled water 10-fold). The mixture was vortexed and rested for 3 minutes, followed by the addition of 7.5 % w/v sodium carbonate (diluted with distilled water) to produce a 2 ml solution. The samples were then incubated in the dark at room temperature for 1 hour. The absorbance was recorded at 765 nm in triplicate. The results were expressed as gallic acid equivalent per gram of dried *K. parviflora* extract (mg GAE/g extract).

Total Flavonoid Content (TFC)

The total flavonoid content was quantified using the aluminium chloride colorimetric assay derived from Sittichai et al. (2022) with minor adjustments [17]. A total of 2 ml of the mixture was prepared by mixing the extract solution with 2 % w/v aluminium chloride solution in a 1:1 ratio. The mixture was kept in the dark at room temperature for 1 hour. The absorbance was recorded at 420 nm in triplicate. Quercetin was used as the standard, and the results were expressed as quercetin equivalent per gram of dried *K. parviflora* extract (mg QE/g extract).

Antioxidant Activity (DPPH) Assay

The antioxidant activities of both the extract and the PMF isolated from *K. parviflora* extract were evaluated by their scavenging activity on the stable free radical DPPH [18,19]. The isolated PMF was dissolved in 95 % MeOH (1000 µg/mL) and quercetin in MeOH (1000 µg/mL) was prepared as the standard. The PMF solution (50 µL) was mixed with 1000 µL of DPPH (0.1 mM in MeOH) and incubated in the dark for 30 min. The absorbance values of both the extract and isolated PMF were recorded at 517 nm, with ascorbic acid and quercetin used as positive controls.

The percentage of inhibition of the free radical DPPH by the *K. parviflora* extract and PMF was calculated using the formula:

% Inhibition = 100 × [(control absorbance – sample absorbance)/control absorbance].

Phytochemical Screening using Thin Layer Chromatography (TLC)

The *K. parviflora* extract was subjected to TLC using F254 plates (Merck, Darmstadt, Germany) to identify possible PMF secondary metabolites. Multiple solvents of different polarities were employed to establish the best solvent system with good separation and R_f value. A solvent system of n-hexane:ethyl acetate was chosen with a ratio ranging from 10:0 to 0:10. Following a modified method from Sharma & Janmeda (2014), both the *K. parviflora* extract and PMF standards (<0.01mg) were dissolved in an appropriate volume of chloroform [20]. TLC plates were prepared (7cm x 2cm), with a straight line drawn 0.7 cm from the bottom and 0.3 cm from the top of the plate. The crude extract was spotted adjacent to the PMF standards (5,7-dimethoxyflavone; DMF, 5,7,4'-trimethoxyflavone; 5,7,4'-TMF, 3,5,7-trimethoxyflavone 3,5,7-TMF, & 3,5,7,4'-tetramethoxyflavone; TEMF) using an elongated capillary tube along the drawn line. The plate was partially submerged in the hexane: ethyl acetate solvent system (7:3) below the bottom line in an airtight chamber. The PMF spots were visualized under UV light (λ: 254 & 365 nm). The R_f values of the crude extract and standard compounds were measured and compared.

Column Chromatography

Based on the TLC analysis, the combined hexane fractions (H1-H29) (8.7832 g) contained most of the PMF metabolites, so these were subjected to silica gel column chromatography (70-230 mesh; Merck, Darmstadt, Germany). The fraction was combined (H30) and eluted gradually from low to high polarity using the n-hexane/ethyl acetate solvent system [10:0] → [0:10], yielding eight sub-fractions (H31-H38). Sub-fractions H34 (0.1927 g) and H35 (0.1132 g) were confirmed through TLC analysis (hexane:ethyl acetate; 10:0 - 8:2) and then subjected to recrystallization to yield the purified sub-fraction H31 (305.9 mg). Sub-fraction H31 was then further analyzed by ^{1}H-NMR, ^{13}C-NMR, MS, and FT-IR.

Compound Characterization

The one-dimensional (1D) ^{1}H-NMR and ^{13}C-NMR spectra of isolated sub-fraction H31 were obtained using a Jeol ECZS 400 MHz instrument (Kyoto,

Japan) at the Institute of Science, Universiti Teknologi Mara (UITM). Proton and carbon chemical shifts were reported in parts per million (ppm), with deuterated chloroform ($CDCl_3$) used as the solvent and internal standard. The molecular weight of the isolated PMF was determined by direct injection into the ion source of a Shimadzu 2010 GCMS (Kyoto, Japan) at the Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. The direct injection mass spectrometry (DIMS) analysis was performed under the following conditions: the system was started at an initial rate of 80 °C/min, towards a final temperature of 320 °C, with a holding time of 10 minutes. Mass spectra detection was carried out in positive ionization mode by scanning at 50 to 550 *m/z* with a speed of 1111 amu/s. The functional group of the isolated PMF was determined by FTIR within the wavenumber range of 400 to 4000 cm^{-1}, using a Bruker Invenio R (Ettlingen, Germany) at the Department of Chemistry, Faculty of Science, Universiti Putra Malaysia. The spectra were accumulated over 32 scans with a 4 cm^{-1} resolution.

Statistical Analysis

The yield percentage, TPC and TFC values obtained were expressed as mean ± S.D. The data were statistically analyzed using SPSS Version 27.0 (IBM, Armonk, N.Y., USA) with one-way ANOVA followed by Tukey's test. Mean values were considered statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

Percentage of Yield

The effects of different extraction parameters are summarized in **Table 2**. As depicted in **Figure 1**, the percentage yield of *K. parviflora* extract peaked with a solvent containing 60 % ethanol (17.82 ± 1.18 %). Ethanol is widely used as a solvent in plant extraction owing to its low toxicity and high polarity index, facilitating the extraction of various metabolites from natural products [21]. As many hydroxylated flavonoid and water-soluble compounds have been recorded in the *K. parviflora* rhizome, multiple studies have employed a binary solvent system of ethanol and water to enhance the extraction of PMF metabolites [22-24]. Generally, a higher water content in the extraction process leads to an increased product yield, which is attributed to the absorption of hydroxylated compounds, carbohydrates and proteins by the strong polarity of water molecules [25]. Nonetheless, the decrease in product yield observed as the ethanol content decreased from 60 % to 40 % (**Figure 1**), suggests the significant presence of hydrophobic and non-polar compounds in the *K. parviflora* rhizome, which are effectively dissolved in organic solvent [26]. This underscores the importance of utilizing a higher concentration of ethanol in the extraction process for this plant species.

In addition, a gradual increase in the *K. parviflora* extract yield was observed with higher solvent-to-sample ratios, with the highest yield recorded at 8.32 ± 1.43 % (1:50). The increasing trend in the crude *K. parviflora* extract yield aligns with previous studies examining the effects of solid-to-solvent ratios on the yields of crude plant species [27-29]. According to the principle of mass transfer, a high concentration gradient between the solid (*K. parviflora* extract) and solvent (ethanol) resulting from a higher solvent ratio increases the diffusion rate of solid primary and secondary metabolites towards the solvent, thereby further enhancing the product yield [30].

Table 2. Percentage yield, total phenolic content, total flavonoid content, and antioxidant activity of each *K. parviflora* extract. Data are expressed as mean ± S.D. (n = 3).

Series no.	Extract yield (%)	TPC (mg GAE/g extract)	TFC (mg QE/g extract)	DPPH• Scavenging Activity (%)	DPPH• Scavenging IC_{50} (µg/mL)
Ascorbic acid	-	-	-	95.00	4.94
1	11.51 ± 1.81	36.10 ± 1.67	21.89 ± 0.07	62.30	308.57
2	17.82 ± 1.18	43.20 ± 0.17	28.31 ± 0.08	70.38	258.78
3	12.23 ± 0.74	62.12 ± 2.77	44.28 ± 0.18	72.31	254.56
4	5.48 ± 1.23	82.63 ± 3.15	74.22 ± 0.43	73.89	161.28
5	6.04 ± 1.36	72.65 ± 1.85	65.65 ± 0.42	61.15	338.20
6	7.03 ± 0.25	72.30 ± 1.34	66.32 ± 0.23	63.46	316.29
7	8.13 ± 0.84	63.42 ± 5.96	62.17 ± 0.20	63.08	337.86
8	8.32 ± 1.43	53.92 ± 1.69	49.02 ± 0.17	58.85	384.03
9	6.65 ± 0.19	71.11 ± 3.43	65.26 ± 0.12	63.78	319.51
10	7.22 ± 0.28	68.82 ± 0.53	60.24 ± 0.14	41.73	541.72

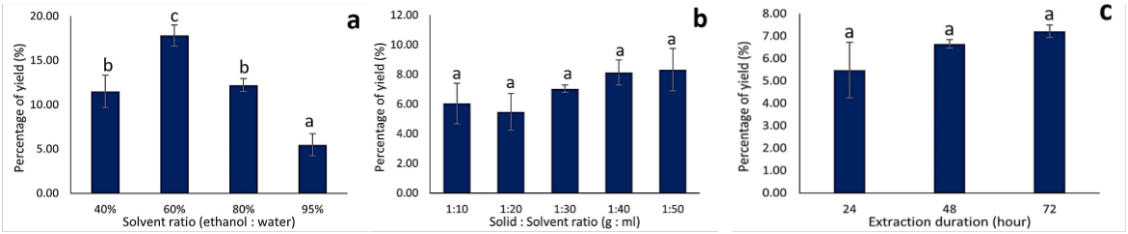

Figure 1. The effects of (**a**) solvent ratio, (**b**) solid: solvent ratio, and (**c**) extraction duration, on the percentage yield of *K. parviflora* extract. Data are expressed as mean ± S.D. (n = 3), with a significance of $P < 0.05$ indicated by different letters.

Longer extraction times significantly increased the crude extract yield of *K. parviflora* rhizome, reaching 7.22 ± 0.28 % after 72 hours **(Figure 1(c))**. Although no specific study has been conducted on different maceration extraction times for *K. parviflora* rhizome, Krongrawa et al. (2022) noted an increase in *K. parviflora* extract yield with a longer extraction duration using the ultrasound assisted extraction technique [12]. Longer extraction durations are favoured as they allow for greater mass transfer of metabolites into the solvent [31]. However, it is noteworthy that prolonged extraction, as indicated in a study by Muflihah et al. (2021), may increase yields but could negatively impact on free radical scavenging capacity, suggesting the possible decomposition of bioactive compounds [32], as depicted in **Figure 4(c)**. The yield of crude extracts can vary depending on the plant species and extraction techniques employed. Thus, shorter and optimized extraction times are preferredtime is preferred to save time and cost, especially in industrial-scale extraction processes, and to prevent the decomposition of bioactive compounds within the plant extract [33].

Total Phenolic Content

The TPC values of crude *K. parviflora* extracts under different parameters are shown in **Figure 2**. Notably, the extract with the lowest yield of 5.48 ± 1.23 %, obtained using the highest concentration of ethanol (95 %), a lower solid: solvent ratio (1:20), and a 24-hour extraction time yielded the highest phenolic content (82.63 ± 3.15 mg GAE/g extract). Interestingly, a higher water ratio significantly reduced the TPC to the lowest value recorded (36.10 ± 1.67 mg GAE/g extract (**Figure 2(a)**), despite demonstrating the highest yield (**Figure 1(a)**).

These results are in agreement with a recent study that found water to be unsuitable solvent for extracting high phenolic content from *K. parviflora* rhizome, although a different extraction technique was employed [34]. In addition, increasing the solvent volume per gram of *K. parviflora* rhizome initially led to a higher TPC recorded at a ratio of 1:20 (g: ml), before significantly decreasing to 53.92 ± 1.69 mg GAE/g extract (**Figure 2(b)**). This suggests that a large volume of solvent was ineffective in extracting phenolic content from *K. parviflora*, despite reports of increased phenolic content obtained with higher solvent volumes in other plant species [35]. A study by Krongrawa et al. (2022) reported a slight increase in TPC with prolonged extraction time; however, the longest duration was only 30 minutes using ultrasound assisted extraction [12]. Phenolic compounds in plant species are crucial as they possess redox properties responsible for antioxidant capacity. Various studies have shown a correlation between high TPC values and the enhanced antioxidant capacity of plant extracts, which contribute to various biological activities [32].

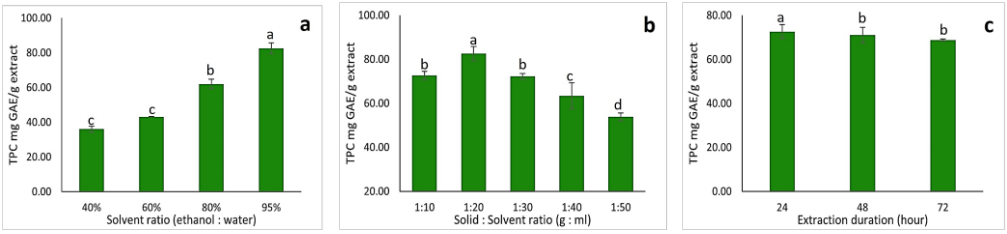

Figure 2. Effects of (**a**) solvent ratio, (**b**) solid:solvent ratio and (**c**) extraction duration on the total phenolic content values of *K. parviflora* extracts. Data are expressed as mean ± SD (n = 3), with a significance of $P < 0.05$ represented by different letters.

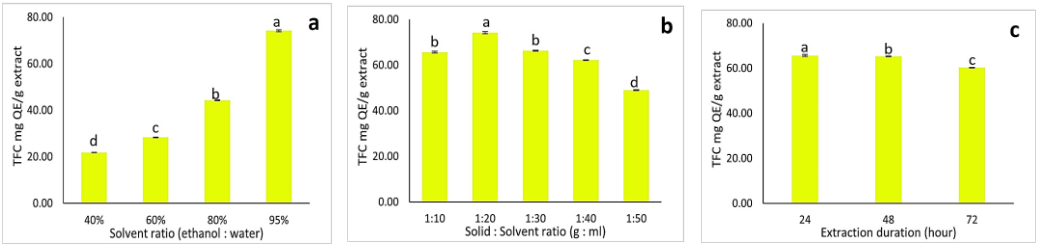

Figure 3. Effect of (**a)** solvent ratio, (**b)** solid:solvent ratio, and (**c)** extraction duration, on the total flavonoid content values of *K. parviflora* extracts. Data are expressed as mean ± S.D. (n = 3), with a significance of $P < 0.05$ represented by different letters.

Total Flavonoid Content

The total flavonoid content (TFC) of *K. parviflora* extracts under different parameters is shown in **Figure 3**. The quercetin standard curve was successfully plotted with the equation: $y = 0.0079x + 0.0094$ ($R^2 = 0.998$). As seen in **Figure 3(a)-(c)**, the TFC followed a pattern similar to the TPC, with 95 % ethanol, a 1:20 solid:solvent ratio, and a 24-hour extraction duration achieving the highest TFC value (74.22 ± 0.43 mg QE/g extract).

The TFC of all samples were lower than the TPC values (**Table 2**), consistent with previous studies conducted by Nisoa et al. (2023) and Zuraida et al. (2018), despite the use of different extraction techniques [34, 36]. The higher TPC values may be attributed to the presence of phenolic acids and some flavonoids, particularly flavanols, which are commonly found in ~~the~~ plant extracts [37]. Multiple studies have reported that *K. parviflora* rhizome ~~is~~ predominantly contains semi- to low-polarity flavones with multiple methoxy-substitutions which are poorly soluble in water and represent a major group of compounds [38]. Thus, ethanol is a preferable solvent over water for extracting these bioactive compounds, as it enhances semi-polar interactions and absorption of compounds in the *K. parviflora* rhizome [32].

Antioxidant Activity

The high TPC and TFC values in the extract, as discussed in the previous section, resulted in the highest DPPH scavenging activity of 73.89 % (**Figure 4(a)**) and the strongest IC_{50} value of 161.28 µg/ml (**Table 2**). Interestingly, ethanol concentration had minimal impact on free radical scavenging capacity at higher extract concentrations (500 µg/ml) (**Figure 4(a)**) compared to the effects of higher solvent:solid ratios and longer extraction durations (**Figure 4(b)** & **4(c)**).

K. parviflora extracts obtained using ethanol concentrations ranging from 40 % to 80 % unexpectedly demonstrated high antioxidant activities despite having lower TPC and TFC values. This suggests the presence of specific bioactive compounds with potent antioxidant activity along with minor bioactive groups such as terpenoids and terpenes, capable of scavenging free radicals [39]. However, a longer extraction duration (72 hours) led to a notable decrease in the free radical scavenging activity as evidenced by a weaker IC_{50} value of 541.72 µg/ml (**Figure 4(c)**). This reduction in antioxidant activity may be due to the decomposition of bioactive compounds caused by prolonged solvent exposure [33].

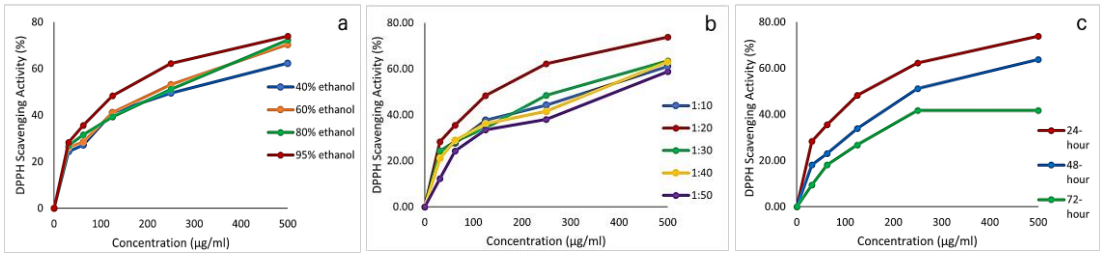

Figure 4. Effects of (**a)** solvent ratio, (**b)** solid:solvent ratio, and (**c)** extraction duration on the DPPH scavenging activity of *K. parviflora* extracts.

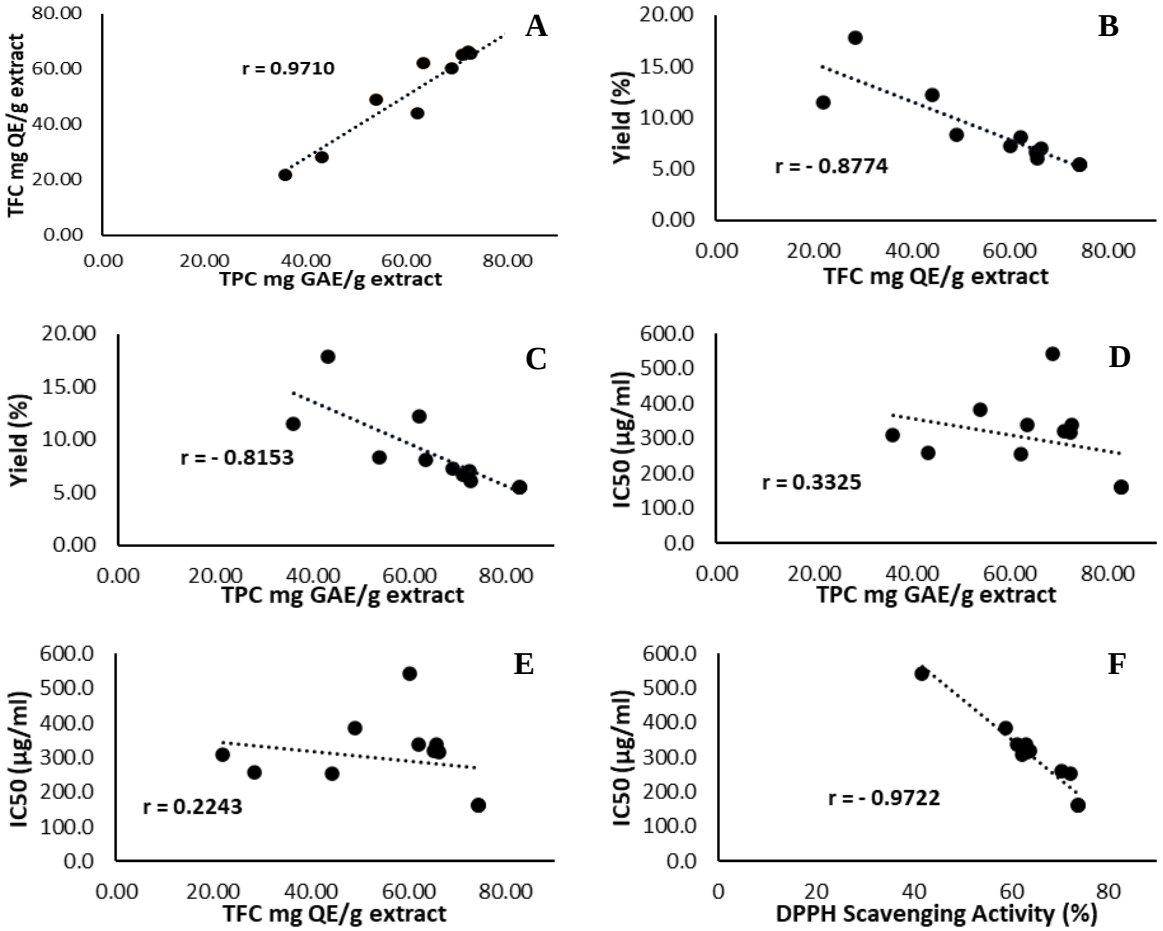

Figure 5. Scatter plots of Pearson's correlation analysis between (**A**)* TFC & TPC, (**B**)* Yield & TFC, (**C**)* Yield & TPC, (**D**) IC_{50} & TPC, (**E**) IC_{50} & TFC, (**F**)* IC_{50} & DPPH Scavenging Activity. * indicates significant correlations at $p < 0.01$, with correlation coefficient (*r*) shown for each plot.

Correlation Analysis

Pearson's correlation coefficient was adopted to examine linear relationships between parameters. The analysis depicted a significant positive correlation was observed between TFC and TPC ($r = 0.9710$; $p < 0.01$) while strong negative correlations were noted between TFC and extract yield ($r = 0.8774$; $p < 0.01$) and TPC and extract yield ($r = 0.8153$; $p < 0.01$) (**Figure 5(A)-(C)**). The free radical scavenging activity (IC_{50}) of *K. parviflora* extracts was mainly attributed to their phenolic content that exhibited a moderate correlation ($r = 0.3325$) (**Figure 5(D)**). These findings align with multiple studies reported on other plant species, highlighting the role of phenolic groups as major contributors to antioxidant activity [16, 32, 40, 41]. Nonetheless, the moderate correlation suggests the potential involvement of other secondary metabolites such as flavonoids, terpenoids, and tannins, in the antioxidant activity of *K. parviflora* extracts (**Figure 5(E)**) [39].

Extraction & Isolation

Solvent partitioning of crude *K. parviflora* extracts yielded multiple fractions from n-hexane, chloroform, and water (**Figure 6**). Despite yielding a small quantity, the combined n-hexane fraction contained a significant amount of PMF compounds in the form of a clear yellowish solution. In contrast, the chloroform extract, with higher polarity, dissolved most compounds [15], resulting in a sticky dark purplish-brown fraction likely contributing to its greater weight. The water fraction appeared colourless and contained the lowest PMF content, as multiple fractionations completely partitioned most metabolites into the n-hexane and chloroform fractions. These results align with those reported by Lee et al. (2022) on the *K. parviflora* rhizome, in which the n-hexane fraction contained the highest amount of PMFs and was subjected to silica gel chromatography for isolation of the PMF compounds [15]. Sub-fractions H1-29 and H30 were combined and subjected to column chromatography, yielding multiple fractions. However, only sub-fractions H34 and H35 were considered pure, forming yellowish needle-shaped crystals. The purity of the isolated PMFs were subsequently confirmed through TLC analysis using an 8:2 hexane:ethyl acetate solvent system.

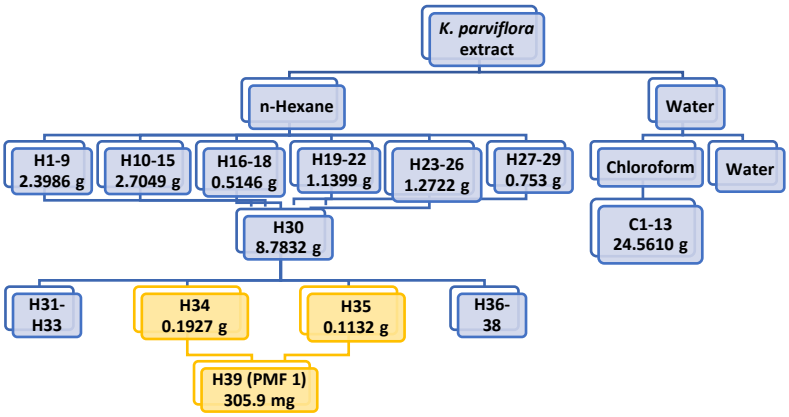

Figure 6. Schematic diagram for the isolation of PMFs from *K. parviflora* extract.

Identification of PMFs by TLC Analysis

PMFs in the crude extract were identified using TLC, to isolate bioactive compounds for subsequent spectroscopic analysis [42]. Utilising a hexane: ethyl acetate solvent system (6:4), PMF compounds were detected upon exposure to 365 nm ultraviolet light **(Figure 7)**. Confirmation of PMFs in the crude *K. parviflora* extract was achieved by comparing the R_f values with standard PMFs. The R_f values and corresponding colours of PMFs are presented in **Table 3.**

Table 3. R_f values and corresponding colours of identified PMFs visualized under UV light (365 nm).

PMF	R_f value (Crude extract & standard PMF)	Colour under UV light (365 nm)
5,7-dimethoxyflavone	0.47	Blue
5,7,4'-trimethoxyflavone	0.41	Blue
3,5,7-trimethoxyflavone	0.58	Green
3,5,7,4'-tetramethoxyflavone	0.52	Blue

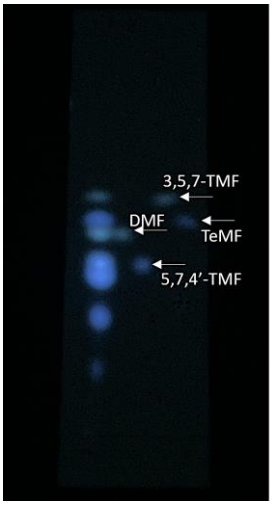

Figure 7. TLC plate of the n-hexane fraction and standard PMFs visualized under UV light (365 nm).

Characterization of Isolated PMF

The ^{1}H-NMR spectra of the isolated compound confirmed the position of the proton and its flavone skeleton structure (**Table 4**). The presence of two methoxylated groups was evidenced by strong signals at δ_H 3.86 and δ_H 3.87 ppm. In addition, a strong doublet peak, corresponding to two meta-coupled aromatic protons δ_H 6.36 ppm (1H, *d*, J = 2.2 Hz, H6) and δ_H 6.45 ppm (1H, *d*, J = 2.2 Hz, H8), indicated 5,7-disubstitution on the flavone's ring A system. As predicted, the absence of a strong singlet proton peak within the spectral region confirmed the existence of a mono-substituted group C3 on heterocyclic pyran ring C. Meanwhile, multiple overlapping signals between δ_H 7.51 ppm (3H, m, H3', H4', H5') and δ_H 8.05 ppm (2H, dd, H2', H6') completed the proton assignments on aromatic ring C. A hydroxy group signal was detected at δ_H 12.58 ppm, strongly deshielded from the typical region range between δ_H 8.00 and δ_H 10.00 ppm for hydroxy groups on the flavone aromatic ring [43], which is known as a marker for 5-hydroxylated flavones [44]. The resonance effect of the planar benzene ring and extensive conjugation of the C2-C3 double bond and the C4-carbonyl group on heterocyclic ring C [45, 46] leads to a stronger intramolecular hydrogen bond interactions with the C5-OH group, leading to a significant deshielding effect [47]. This exceptional effect has been consistently reported in 5-hydroxylated flavones isolated from various plant species (**Table 5**).

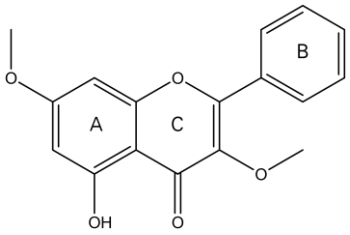

Figure 8. Chemical structure of 5-hydroxy-3,7-dimethoxyflavone showing flavone skeleton and positions of hydroxyl and methoxy groups.

Table 4. ^{1}H-NMR and ^{13}C-NMR chemical shift data (δH and δC) of PMF isolated from *K. parviflora* correspond to proton and carbon positions in the flavone structure.

C no.	δ_H (ppm)	δ_C (ppm)
2	-	156.02
3	-	139.76
4	-	179.05
5	-	162.10
6	6.36 (d, J = 2.2)	98.06
7	-	165.66
8	6.45 (d, J = 2.2)	92.26
9	-	157.00
10	-	106.27
1'	-	131.05
2'	8.05 (dd)	128.48
3'	7.51 (m)	128.71
4'	7.51 (dd)	131.05
5'	7.51 (m)	128.71
6'	8..05 (dd)	128.48
5-OH	12.58 (s)	-
3-OCH_3	3.86 (s)	60.49
7-OCH_3	3.87 (s)	55.93

Table 5. ^{1}H-NMR chemical shift data (δH) of PMFs isolated from different plant species.

Isolated flavone	Plant species	δH (ppm)				Reference
		C5-OH	C2'-OH	C6'-OH	C4'-OH	
5-hydroxy-2′,6′-dimethoxyflavone	*Hottonia palustris*	12.69				[56]
5,2'-dihydroxy-6-methoxyflavone		12.66				
5-hydroxy-2′,3′,6′-trimethoxyflavone		12.66	10.13			
5,6′-dihydroxy-2′,3′-dimethoxyflavone		12.65		9.74		
5-hydroxy-7-methoxyflavone	*Kaempferia parviflora*	12.77				[57]
5-hydroxy-7,4'-dimethoxyflavone		12.82				
Chrysin	*Passiflora incarnata L*	12.78				[58]
Apigenin	*E. splendens*	12.97			10.51	[59]

The 15 carbons of the di-substituted methoxyflavone skeleton structure were confirmed by their ^{13}C-NMR signals (**Table 4**). The disubstituted methoxylated groups were detected at C3 and C7 (δC 139.75 & 165.66 ppm), respectively. This pattern closely resembles those of penduletin and viscosine, compounds previously isolated from *Tillandsia bergeri*, which also exhibit similar methoxy group substitutions [42]. Additionally, oxygenated carbon signals detected between δC 155 to 180 ppm correspond to the parental structure of flavones **(Table 4),** as reported previously [43, 48]. The carbonyl group at C4 was the most deshielded, appearing at δC 179.05 ppm, while the hydroxy group, confirmed by its proton signal, showed a strong peak at δC 162.10 ppm (C5). Meanwhile, oxygenated carbon (C2 & C9) signals were observed at 156.02 and 157.00 ppm, respectively, consistent with similar flavones isolated from various plant species [42, 49]. Based on the NMR analysis, the isolated PMF was characterized as 5-hydroxy-3,7-dimethoxyflavone (**Figure 8**).

In the positive ionization mode of DIMS, the base ion peak of the isolated PMF was observed at *m/z* value $[M+H]^{+}$ of 297.10, confirming its molecular formula as $C_{17}H_{14}O_5$ (**Figure 9**). The loss of *m/z* 17 indicated hydroxy group degradation, confirming the formation of hydroxylated PMF (**Figure 10**). In addition, other prominent fragment ions were observed at *m/z* 135, 105, and 77. The peak at *m/z* 135 was a common product of a ring C cleavage between the oxygen of the pyran ring and the C-2 conjugated double bond [50]. The fragment peak at *m/z* 105 indicated ring fission and suggested the presence of a mono-substituted methoxy group on aromatic ring A, as demonstrated by previous literature on the fragmentation of isolated flavones [51]. In addition, the peak at *m/z* 77 corresponded to a monosubstituted aromatic ring B, confirming that the methoxy and hydroxy groups were exclusively bonded to aromatic ring A and the heterocyclic ring C (**Figure 10**).

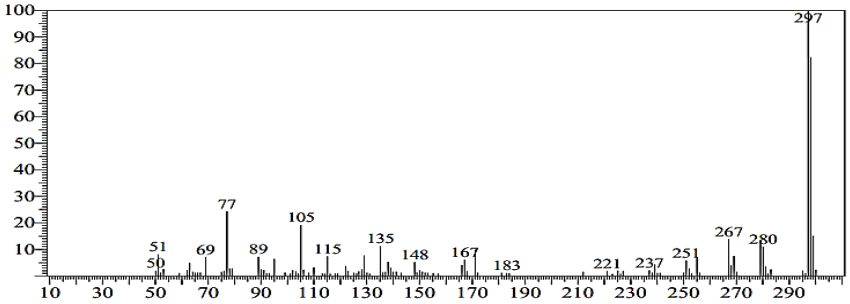

Figure 9. Mass spectrum (MS) of PMF isolated from *K. parviflora.*

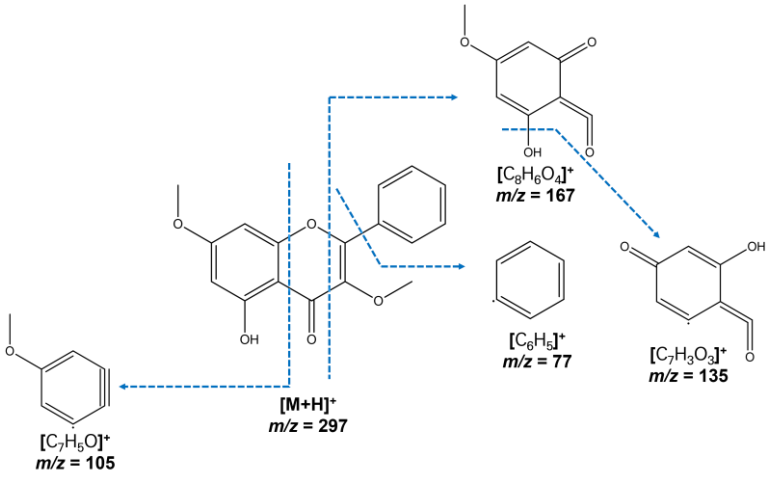

Figure 10. Proposed schematic fragmentation of 5-hydroxy-3,7-dimethoxyflavone.

The FT-IR spectrum of the isolated PMF was created by signals produced by bond stretching, bending, and vibrations characteristic of each functional group present [52]. The spectrum is depicted in **Figure 11** and summarized in **Table 6**. Peaks at 3233.68 cm^{-1} and 1376.74 cm^{-1} were attributed to hydroxy group stretching and bending, respectively, consistent with previous reports on quercetin [53]. The C-H stretching bands of aromatic and methoxy groups were observed between 2704.45 to 3080.73 cm^{-1}, within the range reported for rutin (2842.96 cm^{-1}), tangeritin (2848 cm^{-1}), and nobiletin (2829 cm^{-1}) [54, 55].

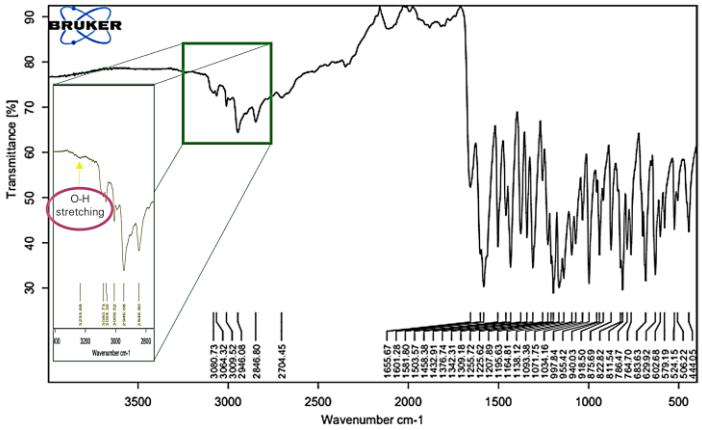

Figure 11. FT-IR spectrum of 5-hydroxy-3,7-dimethoxyflavone.

Table 6. FT-IR spectral bands of 5-hydroxy-3,7-dimethoxyflavone.

Wavenumber (cm^{-1})	Group	Compound class
3233.68	O-H stretching	Hydroxy group
2704.45 – 3080.73	C-H stretching	Aromatic
2846.80	C-H stretching	Methoxy group
1655.67	C=O	Carbonyl
1650-2000	C-H bending	Aromatic (overtone)
1581.80 & 1601.28	C=C stretching	Cyclic alkene
1376.74	O-H bending	Hydroxy group
683.63	C-H bending	Monosubstituted aromatic

Table 7. Percentage inhibition of free radicals by quercetin (standard) and 5-hydroxy-3,7-dimethoxyflavone.

DPPH Free Radical Scavenging Activity	(%)
5-Hydroxy-3,7-dimethoxyflavone	10.02
Quercetin	97.42

Antioxidant Activity of 5-Hydroxy-3,7-dimethoxyflavone

The radical scavenging activity of the isolated compound was estimated by its inhibition activity on the stable free radical DPPH, with quercetin serving as standard. As depicted in **Table 7**, the isolated compound demonstrated moderate anti-free radical activity, with a 10.02 % inhibition at 1000 µg/ml in comparison to quercetin (IC_{50}: 67.17 µg/ml). The IC_{50} value of PMF was undetectable due to its low solubility at higher concentrations, as previously reported by Leardkamolkarn et al. (2009) for PMFs isolated from the same plant species [60]. Nonetheless, the inhibitory activity of the isolated compound was comparable to tangeritin (11.20 %) [61] and superior to nobiletin (< 5 %) [62], which may be attributed to hydroxylation at C5 of the PMF [61]. The structure-activity relationship of flavones demonstrates that the position and substitution of methoxy and hydroxy groups on the flavone's B-ring significantly influences radical scavenging activity [63].

CONCLUSION

The *K. parviflora* rhizome demonstrated the highest total phenolic and flavonoid content and strongest antioxidant capacity, when extracted using 95 % ethanol, a 1:20 solid:solvent ratio, and a 24 hour extraction duration. Strong correlations were found between TFC, TPC and extract yield, while moderate correlation was observed between TPC, TFC and free radical scavenging activity (IC_{50}). The PMF isolated from the optimized extraction process was successfully identified as 5-hydroxy-3,7-dimethoxyflavone. This compound demonstrated a free radical scavenging ability comparable to other prominent PMFs in the DPPH assay. Further studies on this isolated PMF could provide more insight into its biological activities and potential applications.

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