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Research Article

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# Optimizing suitable solvent for phenylpropanoid extraction and antioxidant activities in *Agastache rugosa* hairy roots

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## ABSTRACT

*Agrobacterium*-mediated hairy roots (HRs) can induce genetic stability, rapid growth, and the synthesis of bioactive compounds in plant roots. *Agastache rugosa* is otherwise called Korea Mint and several studies have reported that this plant has been used for the treatment of various diseases due to the presence of a variety of bioactive compounds. *A. rugosa* HRs are rich in secondary metabolites than the seedling roots, and the HRs extract might be more useful in pharmacology, especially in cosmetology. This study aimed to select the suitable solvent for the extraction of phenylpropanoid compounds, total phenolic (TP), flavonoid (TF), and antioxidant activities (DPPH, ABTS scavenging activity, and reducing power assay). In this study, we extracted the *A. rugosa* HRs with three different extracts of solvent (water, MeOH, and EtOH) of *A. rugosa* HRs and analyzed the phenylpropanoid compounds, TP, TF, and antioxidant activities. The result showed that 70% MeOH extracts showed the highest activities in all assays, followed by the 70% EtOH, and water extracts. In addition, 70% of MeOH extracts showed the highest TP and TF (46.14±0.25 GAE mg/g DW and 65.46±1.41 QE mg/g DW, respectively) contents, which was 1.96- and 1.76- times higher than that of the water extracts. The phenylpropanoids in *A. rugosa* HR extracts were identified by using HPLC, and the results showed that 70% MeOH and EtOH showed the highest contents. Regarding these results, we can conclude that 70% MeOH is the optimal solvent to extract the *A. rugosa* HRs for the highest phenylpropanoid, TP, TF content, and antioxidant activities. This study might be useful for producing useful compounds at an industrial scale.

**KEYWORDS:** *Agastache rugosa*, Extraction of different solvents, Antioxidants, Phenolics, Flavonoids, Phenylpropanoids

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## INTRODUCTION

Oxidative processes are crucial for both physiological functions in living organisms and the preservation of food, with oxidation being vital for cellular life. However, the free radicals and reactive oxygen species (ROS) generated during these processes can pose detrimental effects on health by damaging cells and impacting cellular signaling pathways (Bae *et al.*, 1999; Antolovich *et al.*, 2002). Additionally, these radicals contribute to the chemical degradation of food, emphasizing the importance of understanding oxidative mechanisms (Colbert & Decker, 1991). Natural antioxidants, including phenolics and flavonoids found in a diverse array of plant-based foods such as fruits, vegetables, cereals, and spices, offer protective roles against oxidative stress (Shahidi & Zhong, 2015). These antioxidants can be extracted and utilized in pharmacological and nutraceutical applications due to their health benefits, contrasting with synthetic antioxidants that have been linked to adverse effects, leading to a shift towards natural alternatives (Choe & Yang, 1982; Antolovich *et al.*, 2002; Shahidi & Zhong, 2015).

The increased interest in natural antioxidants over synthetic ones underscores the exploration of plant-based sources for new antioxidant compounds, driven by their potential

health benefits and minimal adverse effects (Lim *et al.*, 2024). *Agastache rugosa*, commonly known as Korea Mint, is recognized for its medicinal properties and is part of the traditional pharmacopeia in East Asia (Yeo *et al.*, 2023). It has been associated with various health-promoting activities, including anti-inflammatory, antitumor, and antioxidant effects, attributed to its rich phenolic content, including rosmarinic acid found across its different tissues (Rampart *et al.*, 1986; Shin & Kang, 2003; Lee *et al.*, 2008; Desta *et al.*, 2016; Park *et al.*, 2019). Plant secondary metabolites such as phenolics and flavonoids play crucial roles in plant development and defense against environmental stresses (Douglas, 1996; Higdon *et al.*, 2007).

The advent of *Agrobacterium rhizogenes*-mediated transformation has facilitated the development of hairy roots (HRs) in plants, offering a novel approach to studying plant metabolism and the production of secondary metabolites. This technique involves the integration of the bacterium's root-inducing plasmid (Ri-plasmid) into the plant genome, leading to the creation of HRs that exhibit genetic stability, rapid growth, and the capability to synthesize bioactive compounds under minimal nutritional requirements (Shanks & Morgan, 1999; Biswas *et al.*, 2023). Such transgenic plants present an invaluable resource for biotechnological applications, including the enhanced production of valuable secondary metabolites (Makhzoum *et al.*, 2013; Halder *et al.*, 2019; Park *et al.*, 2021b).

Given the diverse biological activities of *A. rugosa* and the potential of its HRs for producing bioactive compounds, identifying the most effective extraction solvent is critical for maximizing the yield of antioxidants, phenolics, flavonoids, and phenylpropanoids. This study aims to evaluate the antioxidant activities and quantify the total phenolic (TP) and total flavonoid (TF) contents, as well as phenylpropanoids, in HRs of *A. rugosa* using various extraction solvents. The goal is to establish optimal extraction conditions that facilitate the efficient recovery of these bioactive compounds, contributing to the development of natural antioxidant sources (Rababah *et al.*, 2010; Michiels *et al.*, 2012).

## MATERIALS AND METHODS

### Plant Materials

Seeds of *Agastache rugosa*, supplied by Aram Company located in Seoul, Korea, underwent a meticulous washing process under running tap water before being subjected to 70% (v/v) ethanol mixed with Tween 20 for 30 sec. Subsequently, the seeds were sterilized with 2% (v/v) sodium hypochlorite solution and then repeatedly washed thrice with sterile distilled water. Following sterilization, the seeds were carefully dried on sterile tissue paper. The dried seeds were then placed on half-strength Murashige and Skoog (MS) medium, which was augmented with 0.8% plant agar and 3% sucrose, and the pH was adjusted to 5.8. The prepared medium was sterilized through autoclaving. The seeds inoculated in the medium were cultivated in a controlled environment within a growth chamber, which was maintained at a constant temperature of 25 °C and 60% relative humidity. The growth conditions also included exposure to white fluorescent light with a flux rate of 30 μmol $s^{-1}$ $m^{-2}$ and a photoperiod of 16 h. After three weeks, leaves from mature *A. rugosa* plants were collected for hairy root induction. This process was carried out in accordance with the methodology described by Park *et al.* (2021a), employing the *A. rhizogenes* R1000 strain to induce hairy roots in the leaves. The leaves with induced hairy roots were then cultured in MS liquid medium under a constant temperature of 25 °C. The culture was agitated on a gyratory shaker set at 100 rpm, under a 16 hour light/8 hour dark cycle, and the medium was changed every 10 days. Upon reaching the appropriate growth stage, the hairy roots were harvested, subsequently freeze-dried, and then meticulously ground into a fine powder for further analysis and experimental use.

### Extraction Using Different Solvents

To extract bioactive compounds from *A. rugosa* HRs, three distinct solvents - water, 70% methanol (MeOH), and 70% ethanol (EtOH) were employed. The aqueous extraction involved mixing 1 g of *A. rugosa* HRs dried powder with 20 mL of distilled water in a 50 mL centrifuge tube, which was then kept in an 80 °C water bath for 12 h. For post-thermal extraction, the mixture was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was subsequently filtered using Whatman No. 2 filter paper.

For alcoholic extractions, a similar procedure was followed with slight modifications. One gram of HR-dried powder was treated with 20 mL of either 70% MeOH or 70% EtOH. The mixtures were then subjected to sonication for 1 hour using a JAC 4020 sonicator (KODO Technical Research Co., Ltd., Hwaseong, Korea). After centrifugation, the supernatants were collected and filtered through a 0.45 μm PTFE hydrophilic syringe filter.

### *In vitro* Antioxidant Activity Assessment

#### *Evaluation of DPPH radical scavenging activity*

The method for assessing DPPH radical scavenging activity was adapted from Lim *et al.* (2024). A 2 mM stock solution of DPPH in 99.9% MeOH was prepared, from this solution the working solution of 0.2 mM concentration was made. In a 96-well plate, 160 μL of the 0.2 mM DPPH solution was mixed with 40 μL of the sample across various concentrations (31.25 to 1000 μg/mL). The mixture was incubated in darkness for 20 min before measuring the absorbance at 517 nm with a SPECTROstar Nano plate reader (BMG LABTECH). The equation to calculate the percentage of scavenged DPPH radicals was calculated by using the formula as follows: Inhibition (%) = {[CA - (SA - BA)]/CA} x 100, where CA represents the control's absorbance, SA the sample's absorbance, and BA the blank's absorbance. The $IC_{50}$ value was estimated using a curve plotted based on the data, aligning with Lim *et al.* (2024).

#### *Determination of ABTS radical scavenging activity*

Following modified methodologies from Lim *et al.* (2024), the ABTS radical scavenging activity of *A. rugosa* HR extracts was assessed.

An ABTS+ solution was prepared by reacting 7 mM ABTS powder with 2.5 mM potassium persulfate and incubating in darkness for 16 h. The solution's absorbance was adjusted to 0.7±0.02 at 734 nm. A mix of 20 µL of the sample with 180 µL of ABTS+ solution was prepared, and absorbance was measured at 734 nm.

### *Assessing reducing power*

The reducing power assay, indicative of the conversion from $Fe_3^+$ to $Fe_2^+$, was conducted based on a modified protocol from Lim *et al.* (2024). This involved mixing six different concentrations of the extract with phosphate buffer and potassium hexacyanoferrate, followed by incubation, addition of trichloroacetic acid, centrifugation, and mixing with ferric chloride. The absorbance was measured at 700 nm, with higher values indicating stronger reducing power.

### *Total phenolic and flavonoid content estimation*

The TP content was quantified following the Folin-Ciocalteau reagent reduction method described by Lim *et al.* (2024). The sample extracts were diluted to 5 mg/mL with their respective solvents and reacted with Folin-Ciocalteau reagent and sodium carbonate. Absorbance was measured at 760 nm, and TP content was calculated using a gallic acid calibration curve.

The TF content was determined by Lim *et al.* (2024). The process included mixing the diluted extracts with sodium nitrite and aluminum chloride, followed by incubation and absorbance measurement at 415 nm. TF content was estimated using a quercetin calibration curve.

### *Phenylpropanoid compounds identification*

Phenylpropanoid compounds in A. *rugosa* HR extracts were identified and quantified using high-performance liquid chromatography (HPLC) as outlined by Lim *et al.* (2024). The HPLC analysis employed a $C_{18}$ column with a mobile phase gradient of acetic acid and MeOH. The flow rate was set at 1.0 mL/min, and compounds were detected at 280 nm. Identification was based on retention time comparisons and spike tests, with quantification against calibration curves (Table 1) for each compound carried out according to the protocol described by Lim *et al.* (2024).

## Statistical Analysis

The analysis of variance (ANOVA) method in SPSS 20 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. At the $p<0.05$ level, significant differences were established using Duncan's multiple-range test. The results were expressed as mean values with a standard deviation (SD) from each result in triplicate.

# RESULTS

## *In vitro* Study for Antioxidant Activities

*In vitro*, antioxidant activities measurement is a widely acknowledged method for assessing the antioxidant capacity to neutralize free radicals. This method was employed to evaluate the scavenging abilities of A. *rugosa* HR extracts against radicals generated by DPPH and ABTS assays. The results shown in Figure 1a illustrate that across all tested concentrations, the extract prepared with 70% MeOH exhibited the highest efficiency in DPPH radical scavenging activity, surpassing both the 70% EtOH and aqueous extracts. Specifically, at the concentration of 1000 µg/mL, the 70% MeOH extract demonstrated a radical scavenging activity of 83.62±0.14%, nearing 90% effectiveness. This was comparatively higher than the 70% EtOH extract, which showed an activity of 81.34±0.15%, and significantly superior to the water extract's activity of 77.27±1.18%, which did not exceed more than 80% effectiveness. The half-maximal inhibitory concentration ($IC_{50}$) values for DPPH radical scavenging showed that the MeOH extract requiring the lowest concentration to achieve 50% inhibition (0.38±0 mg/mL), followed by the EtOH extract (0.44±0 mg/mL), and the water extract (0.59±0 mg/mL) (Table 2).

The evaluation of ABTS radical scavenging activity yielded analogous findings to those of the DPPH assay. Until reaching a concentration of 250 µg/mL, the scavenging capabilities of the alcohol-based extracts (MeOH and EtOH) did not significantly differ (Figure 1b). However, at 500 µg/mL, the MeOH extract exhibited a markedly higher scavenging activity of 75.06±0.87%, compared to the EtOH extract (60.36±1.83%), which showed a lower activity. These results were further supported by the $IC_{50}$ values obtained for the ABTS assay, which paralleled the observations from the DPPH assay, indicating noticeable differences between the extracts (Table 2). The consistent pattern observed across both DPPH and ABTS assays underscores the superior antioxidant potential of the 70% MeOH extract of A. *rugosa* HRs, highlighting its effectiveness as a source of natural antioxidants.

**Table 1: Calibration curve and coefficient factor values of individual phenylpropanoids**

| | Calibration curve | Coefficient factor |
|---|---|---|
| Gallic acid | y=32.89591693x - 26.17370908 | $R^2$ = 0.99996 |
| Caffeic acid | y=39.98286487x - 65.70752695 | $R^2$ = 0.99989 |
| (-)-Epicatechin | y=8.59893686x - 8.335554043 | $R^2$ = 0.99999 |
| Epicatechin gallate | y=13.8273647x - 67.80924713 | $R^2$ = 0.99927 |
| Sinapic acid | y=16.97022716x+7.311514962 | $R^2$ = 0.99987 |
| Benzoic acid | y=7.525240872x - 37.38700057 | $R^2$ = 0.99970 |
| Rutin | y=8.09714215x - 105.546569 | $R^2$ = 0.99954 |
| Quercetin | y=14.00604622x - 148.3452191 | $R^2$ = 0.99973 |
| Kaempferol | y=17.40369286x - 66.08927083 | $R^2$ = 0.99983 |
| Apigenin | y=28.98298976x+29.49766667 | $R^2$ = 0.99998 |

**Table 2: The $IC_{50}$ values of DPPH and ABTS from three different extracts of *A. rugosa* HRs**

| | $IC_{50}$ of DPPH (mg/mL) | $IC_{50}$ of ABTS (mg/mL) |
|---|---|---|
| Water | $0.59\pm0^{c}$ | $0.51\pm0.01^{c}$ |
| M70 | $0.38\pm0^{a}$ | $0.35\pm0.01^{b}$ |
| E70 | $0.44\pm0^{b}$ | $0.39\pm0.01^{a}$ |

Using Duncan's multiple tests (ANOVA, $p<0.05$), different letters in the values indicated represent statistically significant variations among the means. The results represent the mean±SD. Water water extracts, M70 70% MeOH, E70 70% EtOH.

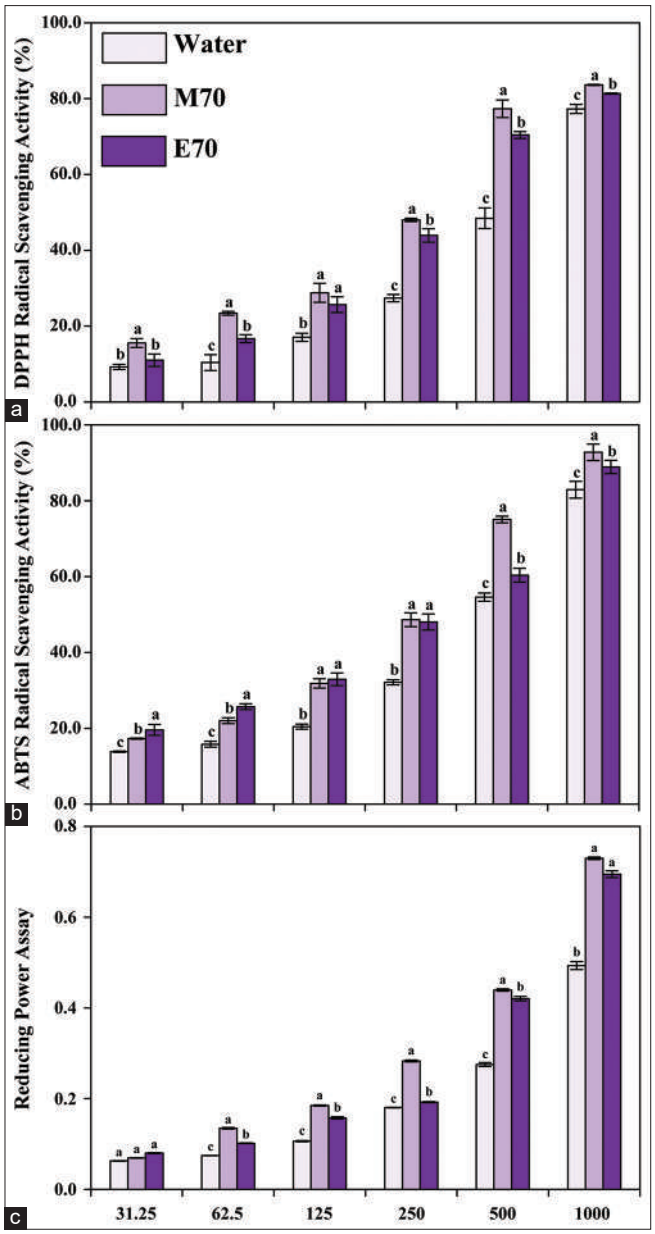


**Figure 1:** *In vitro* antioxidant activities of three different solvent extracts of *A. rugosa*. (a) DPPH radical scavenging assay, (b) ABTS radical scavenging assay, and (c) reducing power assay. The result bar was calculated as the mean of triplicate data with SD. Different letters (a-c) mean significantly different values among the mean values ($p < 0.05$, ANOVA). Water - water extracts, M70 - 70% MeOH, E70 - 70% EtOH

The reducing power of A. *rugosa* HR extracts was confirmed to be increasing with an increase in the concentration of samples. Although there was no significant difference between the MeOH and EtOH alcoholic extracts (0.73±0.01 and 0.69±0, respectively) at a concentration of 1000 µg/mL, overall, it was found that the MeOH extract showed high reducing power. On the other hand, it was found that the water extract (0.49±0.01) showed a reducing power value about 1.5 times lower than the MeOH extract, which showed the highest activity (Figure 1c). Based on the above results, we can assume that MeOH extraction results in the highest extraction of bioactive compounds involved in antioxidant activity.

### Quantification of TP and TF Contents

To quantify the TP content in A. *rugosa* HR extracts, the study employed the Folin-Ciocalteau assay, expressing results in mg of gallic acid equivalent (GAE) per gram of dry weight (DW) (Table 3). The MeOH extract emerged with the highest phenolic content 46.14±0.25 GAE mg/g DW, closely followed by the EtOH extract with 45.03±0.17 GAE mg/g DW. However, the water extract showed significantly lower TP content at 23.54±0.29 GAE mg/g DW. For TF content quantification, in this study we utilized a colorimetric method sensitive to the reaction between flavonoids and specific reagents, yielding results in mg of quercetin equivalent (QE) per gram of DW. Consistent with TP content findings, the MeOH extract demonstrated the highest TF content at 65.46±1.41 QE mg/g DW, followed by the EtOH extract at 62.43±1.1 QE mg/g DW, and water extract at 37.28±1.23 QE mg/g DW. These results align with the antioxidant activity assessments, indicating that MeOH extraction surpasses other methods in extracting phenolic and flavonoid compounds from A. *rugosa* HRs. The data thus far concludes that while both MeOH and EtOH extractions are effective, water extraction proves less efficient in harnessing the bioactive compounds from A. *rugosa* HRs.

### Identification and Quantification of Phenylpropanoids

Figure 2 presents the identification and quantification of ten distinct phenylpropanoid compounds extracted from A. *rugosa* HRs using three different solvent methods. While the water extracts exhibited superior quantities of certain compounds in comparison to the alcoholic extracts (70% MeOH and 70% EtOH). The compounds that were significantly higher in the alcoholic extracts were (-)-epicatechin, sinapic acid, kaempferol, and apigenin.

A closer examination revealed that (-)-epicatechin, kaempferol, and apigenin were most abundantly found in the MeOH extracts, with concentrations of 0.07±0 mg/g dry DW for (-)-epicatechin, 0.11±0 mg/g DW for kaempferol, and 0.02±0.01 mg/g DW for apigenin. On the other hand, sinapic acid showed the highest concentration in the EtOH extracts, recording a value of 0.03±0 mg/g DW. These four compounds were not detected in the water extracts. This discrepancy underscores the differential extraction efficiencies of water versus alcoholic solvents in isolating specific bioactive compounds from A. *rugosa* HRs.

## DISCUSSION

The critical role of solvent selection in the extraction process has been emphasized by Lee *et al.* (2022), who pointed out that variations in solvent polarity, such as MeOH and EtOH significantly influence the types of compounds extracted. This highlights the importance of understanding and accurately applying extraction strategies to optimize research procedures and improve the yield and quality of the final product (Rababah *et al.*, 2010; Michiels *et al.*, 2012; Abubakar & Haque, 2020).

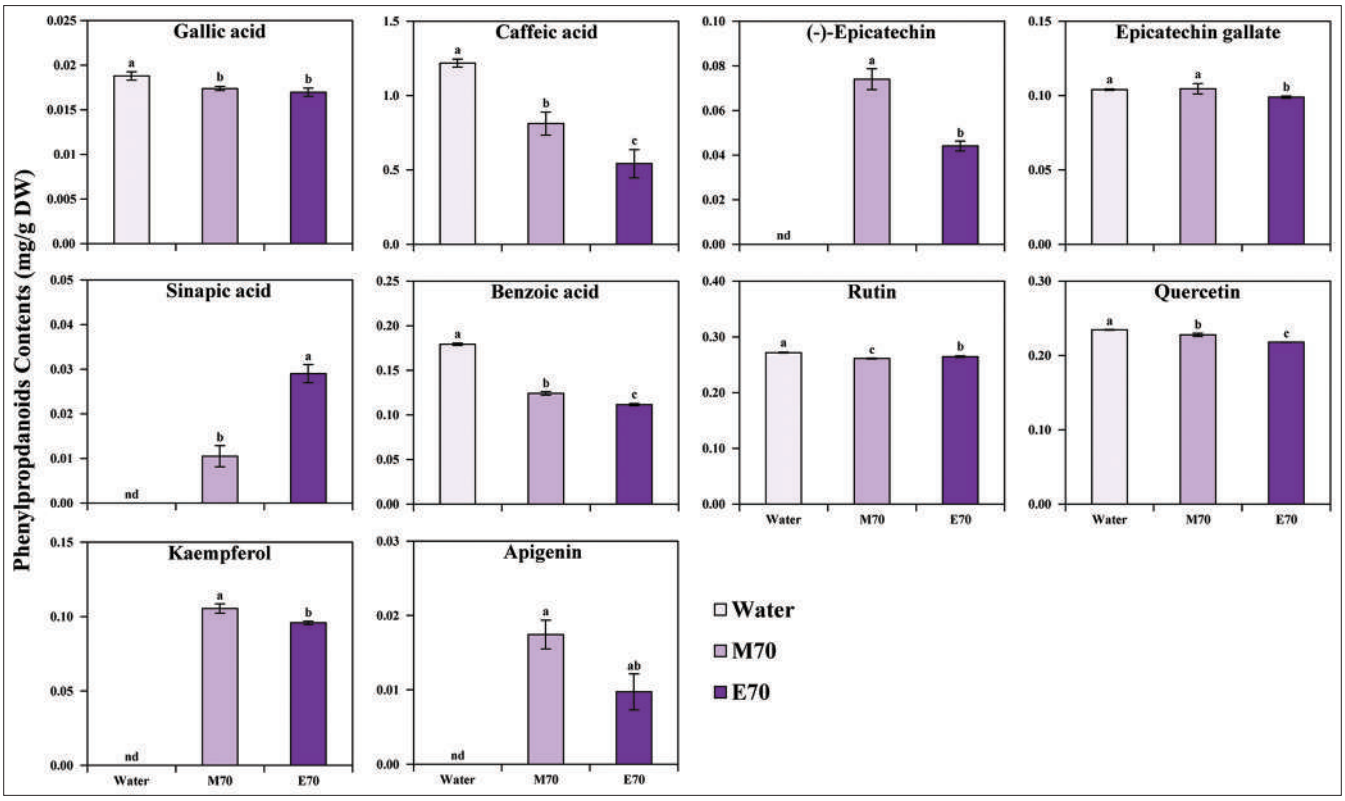


**Figure 2:** Phenylpropanoid compounds were identified from three different extracts of *A. rugosa* HRs. Using Duncan's multiple tests (ANOVA, $p<0.05$), different letters indicate statistically significant differences among the means. The result represents the mean±SD. Nd - non-detected. Water - water extracts, M70 - 70% MeOH, E70 - 70% EtOH

Table 3: The total phenolics and flavonoid contents from three different extracts of *A. rugosa* HRs

| | Total Phenolic Content (GAE mg g DW) | Total Flavonoid Content (QE mg/g DW) |
|---|---|---|
| Water | $23.54\pm0.29^{c}$ | $37.28\pm1.23^{c}$ |
| M70 | $46.14\pm0.25^{a}$ | $65.46\pm1.41^{a}$ |
| E70 | $45.03\pm0.17^{b}$ | $62.43\pm1.1^{b}$ |

Using Duncan's multiple tests (ANOVA, $p<0.05$), different letters in the values indicated represent statistically significant variations among the means. The results represent the mean±SD.

Numerous studies have already demonstrated that different solvents yield extracts with varying antioxidant properties and biological activities, underscoring the necessity of choosing an appropriate extraction solvent based on the specific goals of the research (Huang *et al.*, 2006; Santos & Gonçalves, 2016; Lim *et al.*, 2024). This study was initiated to identify an extraction solvent that maximizes the content of phenolic and flavonoid compounds as well as antioxidant activity in *A. rugosa* HRs. Our findings confirm that 70% MeOH serves as the most efficient solvent for extracting bioactive compounds from *A. rugosa* HRs.

While the antioxidant potential of *A. rugosa* tissues, including flowers, leaves, and stems, is well documented (Desta *et al.*, 2016; Shtereva *et al.*, 2016; Park *et al.*, 2019), there has been less focus on the roots, particularly HRs, and on identifying optimal extraction solvents and techniques. We assessed the antioxidant activities of *A. rugosa* HR extracts by measuring DPPH and ABTS radical scavenging activities, along with reducing power. The results indicated that the 70% MeOH extract exhibited the highest antioxidant activities, suggesting that 70% MeOH is the optimal solvent for these purposes. Similarly, for the quantification of TP and TF, the 70% MeOH extract showed the highest contents. These outcomes are instrumental in selecting extraction solvents for *A. rugosa* HRs aimed at maximizing antioxidant activity and the extraction of phenols and flavonoids. Fu *et al.* (2011) demonstrated a correlation between TP contents and antioxidant activity across 62 plant species, affirming that an increase in TP content is associated with enhanced antioxidant activity, which aligns with our observations.

Plants produce a vast array of secondary metabolites that serve various functions, such as aiding in germination, defense against environmental stressors, and contributing to antioxidant activities (Wink, 2008; Halim *et al.*, 2022). Specifically, Park *et al.* (2019) reported that culturing *A. rugosa* HRs effectively accumulates rosmarinic acid, known for its potent antioxidant properties (Rampart *et al.*, 1986; Ly *et al.*, 2006). Additionally, our study identified phenylpropanoids in *A. rugosa* HR extracts, which are crucial for plant development, growth, and response to external and internal stimuli (Solecka, 1997; Korkina, 2007; Ortiz & Sansinenea, 2023). Pei *et al.* (2022) noted that phenylpropanoids act as precursors to flavonoids in *Scutellaria baicalensis*. Notably, compounds such as (-)-epicatechin, sinapic acid, kaempferol, and apigenin, which possess a range of biological activities, were absent in the water extract of *A. rugosa*

HRs. Epicatechin, for example, provides antioxidant protection to bovine spermatozoa under oxidative stress (Tvrda *et al.*, 2019) and has been explored as a therapeutic agent for diabetes in humans by reducing blood sugar levels (Abdulkhaleq *et al.*, 2017). Sinapic acid, with its antioxidant, anti-inflammatory, anticancer, neuroprotective, and antibacterial properties (Chen, 2016; Bi *et al.*, 2017), along with kaempferol and apigenin, known for their antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (Hofer *et al.*, 2020; Kashyap *et al.*, 2022; Periferakis *et al.*, 2022), was not detected in the water extract, underscoring its lower antioxidant efficacy in this study.

## CONCLUSION

From this study, we have established the appropriate extraction solvent in A. *rugosa* HRs to have the highest antioxidant activities and the highest contents of phenolics, flavonoids, and phenylpropanoids. Among the tested solvents, 70% MeOH showed the highest efficiency in the present assays. Thus, we can conclude that 70% MeOH is the best choice for antioxidants and extraction of TP, TF, and phenylpropanoid compounds, whereas different solvents may need to be chosen depending on the needs of the other purposes. However, we suggest extraction solvents with good antioxidant activity that can be further exploited in pharmacological and therapeutic applications.

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