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ORIGINAL ARTICLE

# Bioactivity-Guided Isolation of Antioxidant Compounds from *Pouzolzia zeylanica* (L.) Benn

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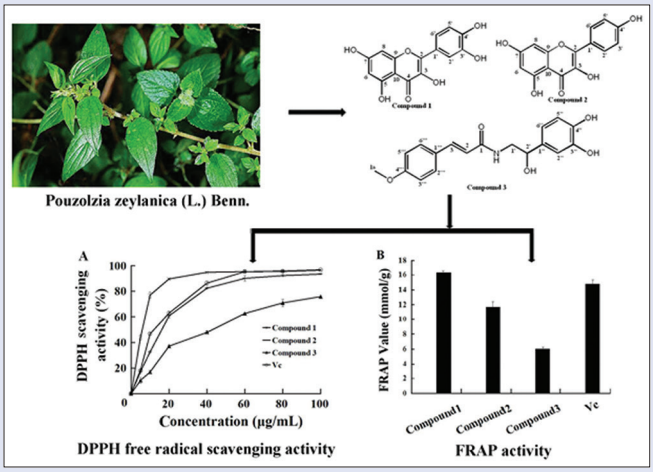
## ABSTRACT

**Background:** *Pouzolzia zeylanica* (L.) Benn. (Family: *Urticaceae*), which is widely distributed in China, is consumed as food and as a traditional herbal medicine for the treatment of numerous diseases. **Objective:** The aim of the present study was to explore the antioxidant compounds of *P. zeylanica*. **Materials and Methods:** A petroleum ether extract, an ethyl acetate extract (EAE), an *n*-butanol extract, and the remaining part were separated by liquid–liquid extraction from a 90% aqueous ethanol extract and a water extract of *P. zeylanica* and were evaluated using two complementary antioxidant assays, namely 2,2-diphenyl-1-picrylhydrazyl-free radical scavenging and ferric-reducing antioxidant power assays. **Results:** The EAE, which possessed the highest antioxidant activity, contained the highest total phenolic content (263.5 ± 4.8 mg/g) and total flavonoid content (388.3 ± 5.6 mg/g). In addition, three compounds with antioxidant activity, quercetin, kaempferol, and *N*-[2-(3-hydroxy-4-methoxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl) prop-2-enamide, were isolated from the EAE. **Conclusions:** These results demonstrate that the flavonoids and phenolic compounds from *P. zeylanica* could be used as natural antioxidants.

**Key words:** 2,2-diphenyl-1-picrylhydrazyl assay, antioxidant activity, ferric-reducing antioxidant power assay, flavonoids, *Pouzolzia zeylanica* (L.) Benn

## SUMMARY

- Five extracts of *Pouzolzia zeylanica* were evaluated by 2,2-diphenyl-1-picrylhydrazyl and ferric-reducing antioxidant power antioxidant assays
- The ethyl acetate extract possessed the highest antioxidant activity and contained the highest total phenolic content and total flavonoid content
- Three compounds with strong antioxidant activity were isolated from *P. zeylanica*
- The flavonoids and phenolic compounds from *P. zeylanica* could be used as natural antioxidants.




**Abbreviations used:** *P. zeylanica*: *Pouzolzia zeylanica* (L.) Benn., PE: Petroleum ether extract, EAE: Ethyl acetate extract, BE: n-butanol extract, RE: Remaining part, WE: Water extract, DPPH: 2,2-Diphenyl-1-picrylhydrazyl, FRAP: Ferric-reducing antioxidant power, TPC: Total phenolic content, TFC: Total flavonoid content, BHA: Butylated hydroxyanisole, BHT: Butylated hydroxytoluene,TPTZ: 2,4,6-Tris (2-pyridyl)-s-triazine, HPLC: High-performance liquid chromatography, CC: Column chromatography.

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## INTRODUCTION

Free radicals are produced during metabolism to maintain the redox balance in the body and to keep the internal environment steady. Most free radicals are related to a complex series of enzymatic and nonenzymatic reactions.[1,2] When homeostasis is disturbed, free radicals and lipid peroxides cannot be cleared rapidly, which will cause various diseases, such as cancer, diabetes, hypertension, and heart disease, as well as aging.[3] Moreover, free radicals and lipid peroxides are the main factors affecting food processing and storage and can cause deterioration in the texture, flavor, and nutritional value of food.[4] It is worth noting that



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antioxidants can protect against food deterioration caused by oxidative damage. In addition, antioxidants can play important roles in preventing or curing disease caused by oxidative damage in the digestive tract, as well as other tissues and organs.[5-7] However, a variety of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been shown to have potential carcinogenic effects, and they have been gradually rejected by the public.[8-10] Therefore, researching alternatives with high efficiency and low toxicity from plant sources inevitably became a trend in the development of novel antioxidants. Previous studies showed that flavonoids and phenolic compounds had important antioxidant properties, such as reducing oxidative damage, reducing lipid peroxidation, and quenching free radicals.[11] In addition, flavonoids and phenolic compounds also have a certain curative effects on atherosclerosis, cancer, and Parkinson's disease.[12,13]

*Pouzolzia zeylanica* (L.) Benn., which is distributed throughout China, is one of the most commonly used traditional herbal medicines and can be cooked into soups consumed for the treatment of some diseases. It has been used to treat acute mastitis, edema, dysentery, indigestion, abdominal pain, infantile malnutrition, urine negative embolism, bruises, hematemesis, and traumatic hemorrhage.[14,15] It contains phenolic compounds proven to have antioxidant activity, such as L-epicatechin, celereoin, scutellarein-7-O-α-L-rhamnoside, quercetin, and quercetin-3-O-malonyl-β-D-glucoside.[16]

Therefore, the main purpose of the present research was to obtain bioactive extracts and compounds from *P. zeylanica*. First, the antioxidant activities of the petroleum ether extract (PE), ethyl acetate extract (EAE), *n*-butanol extract (BE), remaining part (RE), and water extract (WE) of *P. zeylanica* were evaluated. Then, the relationships between the antioxidant activities and the total phenolic and the total flavonoid contents (TFCs) of these extracts were investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH)-free radical scavenging and ferric-reducing antioxidant power (FRAP) assays. In addition, the EAE, which had the highest antioxidant activity, was further separated, and the antioxidant activities of the isolated compounds were also evaluated.

## MATERIALS AND METHODS

### Chemicals and instruments

DPPH was purchased from Sigma-Aldrich (St. Louis, USA). 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) was obtained from Shanghai Baoman Biotechnology Co. Ltd. (Shanghai, China). Sodium phosphate, ascorbic acid (Vc), hydrogen peroxide (30%, v/v), and standard rutin were purchased from Chengdu Kelong Chemical Works (Chengdu, China). All other chemicals used were of analytical grade. Absorbance measurements were recorded using an ultraviolet (UV)-visible spectrophotometer from Shimadzu, PharmaSpec-2450 (Tokyo, Japan). High-performance liquid chromatography (HPLC) analysis was carried out on a Reverse-Phase Agilent 1260 Series Instrument (Palo Alto, USA). A digital thermostatic water bath was purchased from Shanghai Yuejin Medical Instrument Works (Shanghai, China). The vacuum drying oven was from Shanghai Yiheng Technology Co. Ltd. (Shanghai, China).

### Preparation of *Pouzolzia zeylanica* extracts

The plant material was purchased from An Guo Chang'an Chinese Medicinal Herbs Co. Ltd., (Hebei, China) in October 2011. The specimen was identified by the research associate Songyun Qin, Chongqing Academy of Chinese Material of Medica, and a voucher specimen has been deposited at Natural Products Chemistry Laboratory, Department of Pharmacy, Chongqing University, China. The air-dried material (10 kg) was extracted with 90% EtOH three times for 1 h and each at 80°C. The debris was removed by filtration through paper, and the filtrate was then concentrated on a rotary evaporator and then dried in a vacuum drying oven. The ethanol extract was finally fractionated using different solvents as shown in Figure 1.

### Determination of total phenolic content

The total phenolic content (TPC) of different extracts was determined using the Folin–Ciocalteu method with gallic acid as the standard.[17] In brief, the extract or gallic acid (1 mL) was added to a 10 mL volumetric flask along with 200 μL of Folin–Ciocalteu reagent; after 30 s, 2 mL of 15% $Na_2CO_3$ solution was added, and the solution was diluted with distilled water. The blank was prepared using the same procedure without the addition of the sample. The mixtures could react at ambient temperature for 60 min, and the absorbance of the solutions were recorded at 750 nm. The calibration curve was prepared based on gallic acid concentrations, and the TPC was expressed as milligram gallic acid equivalents per gram dry mass of *P. zeylanica* (mg GAE/g DPZ).

### Determination of total flavonoid content

The TFC of each extract was determined by a slightly modified version of a previously reported method.[18] Briefly, 1 mL of the extract or standard solution of rutin was added to a 10 mL volumetric flask along with 300 μL of 5% sodium nitrite. After 6 min, 300 μL of 10% aluminum nitrate was added. After a further 6 min, 4 mL of 4% aqueous sodium hydroxide was added to the mixture, and the solution was diluted with distilled water. Again, the blank was prepared by the same method but without the addition of the sample. The absorbance of each sample was recorded after 13 min at 504 nm. The calibration curve was prepared based on the rutin concentrations, and the TFC was expressed as milligram rutin equivalents per gram dry mass of *P. zeylanica* (mg RUE/g DPZ).

### 2,2-Diphenyl-1-picrylhydrazyl-free radical scavenging assay

The DPPH radical scavenging activities of the crude extract and isolated pure compounds were assessed according to a previously reported method with minor modifications.[19-21] Briefly, 1 mL of sample solution (solubilized in ethanol) was added to 3 mL of 0.1 mM ethanolic DPPH. After 30 min at ambient temperature in darkness, the absorbance of mixture was measured at 517 nm. The percentage of DPPH radical scavenging activity (%) of the sample was calculated by the following equation:

$$[1- (A_s - A_0)/A_b] \times 100\%$$

Where $A_s$ is the absorbance of samples containing antioxidant, $A_0$ is the absorbance of the antioxidant in solvent, and $A_b$ corresponds to the absorbance of the DPPH solution.[22]

### Ferric-reducing antioxidant power assay

The ferric-reducing antioxidant power (FRAP) assay of the crude extract and isolated pure compounds was estimated according to a previously reported method with minor modifications.[23] Sample solution (80 μL) was added to a cuvette along with 2.4 mL of FRAP reagent (10 parts 300 mM sodium acetate buffer at pH 3.6, 1 part 10 mM TPTZ solution, and 1 part 20 mM $FeCl_3 \cdot 6H_2O$ solution), and the mixture was incubated at 37°C for 30 min. The absorbance was recorded at 593 nm. The result was expressed as mmol Fe (II) equivalent per gram sample in dry mass.

### Isolation and purification of bioactive compounds from ethyl acetate extract

Of all the extracts of *P. zeylanica*, the EAE fraction possessed the highest TFC and TPC and the highest antioxidant activity. The EAE

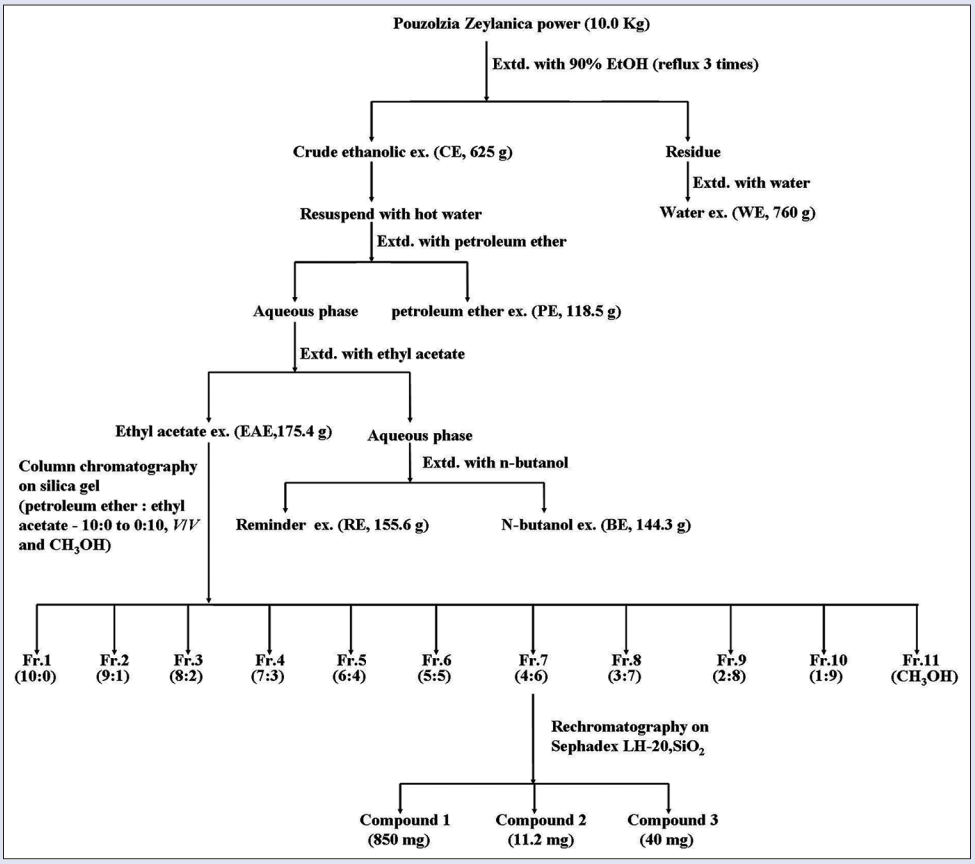


**Figure 1:** Schematic diagram of extractions and antioxidant compounds from *Pouzolzia zeylanica*

was therefore chosen for additional separation and purification. As presented in Figure 1, the active EAE (175.4 g) was separated by column chromatography (CC) on normal-phase silica gel, eluted with a solvent mixture of petroleum ether/ethyl acetate (100:0–0:100, v/v), and then eluted with methanol to generate 11 major fractions. Compounds were visualized under UV light (254 and 365 nm) and by spraying the thin-layer plates with anisaldehyde-$H_2SO_4$ reagent. Fraction 7 was purified by CC on silica gel (petroleum ether/ethyl acetate, 3:2), followed by purification on a Sephadex LH-20 column, eluting with MeOH to give quercetin (compound 1, 850 mg) and kaempferol (compound 2, 11.2 mg). Fraction 7 was further purified by CC on silica gel (petroleum ether/ethyl acetate, 1:1) and preparative thin layer chromatography ($CH_2Cl_2$/ethyl acetate, 1:1) to afford *N*-[2-(3-hydroxy-4-methoxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphe nyl) prop-2-enamide (compound 3, 40 mg). As shown in Appendix 1 the structures of the isolated compounds were identified by comparison of their $^1$H NMR and $^{13}$C NMR data with the reported values.

## High-performance liquid chromatographic analysis

HPLC analyses were carried out on a Reverse-Phase Agilent 1260 Series Instrument. Separations were achieved using an Agilent ZORBAX SB-C18 (4.6 mm × 150 mm; particle diameter, 5 μm) column at 30°C. The mobile phase consisted of 0.4% phosphoric acid in water in pump C and 100% methanol in pump D at a flow rate of 0.8 mL/min. The gradient elution program was as follows: 20% D (0–5 min), 20%–30% D (5–7 min), 30% D (7–12 min), 30%–45% D (12–17 min), 45% D (17–25 min), and 45%–60% D (25–30 min), maintained at 60% D to 40 min, and returned to 20% D in 5 min. The injection volume was 1 μL. UV detection was at 360 and 320 nm. UV absorption spectra were scanned in the range of 200–400 nm. The identities of the analytes were confirmed by comparing their chromatographic retention times and UV spectra to pure compounds. The contents of the individual antioxidant compounds in the EAE were determined from calibration curves prepared from the corresponding purified compounds.

## Statistical analysis

The results are expressed as the mean ± standard deviation of three independent determinations. In addition, the data were analyzed using SPSS V.16 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was used to determine the differences among the means. $P$ <0.05 was considered statistically significant.

# RESULTS AND DISCUSSION

## Total flavonoid and total phenolic contents

It has been shown that polyphenols are important antioxidant compounds in the fight against deleterious oxidative damage, and finding promising new antioxidants among flavonoids and phenolic compounds has great potential.[24,25] Hence, in this study, contents of the total flavonoids and the total phenols in the crude extract were determined.

The extraction yield, TPC, and TFC of each extract of *P. zeylanica* are shown in Table 1. The overall extractable compounds (extracted with 90% ethanol in water, v/v) were 6.25% ±0.45% of the total dry weight. The EAE showed the highest extraction yield (28.07% ±1.62%) among all the extracts, followed by RE, BE, and WE, while the PE had the lowest extraction yield (7.6% ±0.36%). In addition, the extraction yield among all the extracts was significant ($P$ < 0.05).

The TPC of each *P. zeylanica* extract was determined using the regression equation of the calibration curve ($y = 0.0081x + 0.0722$; $R^2 = 0.9995$) and expressed as gallic acid equivalents. The results showed that the EAE had the highest TPC (263.5 ± 4.8 mg GAE/g DPZ), followed by BE, WE, and PE, while the RE had the lowest TPC (26.0 ± 2.4 mg GAE/g DPZ). In addition, there were significant differences among the TPC of all extracts ($P < 0.05$). This suggests that the phenolic compounds responsible for the higher antioxidant activity could be isolated from the EAE fraction.

The TFC of each *P. zeylanica* extract was determined using the regression equation of the calibration curve ($y = 0.0012x + 0.0035$, $R^2 = 0.9985$) and then expressed as rutin equivalents. The results showed that the TFC was affected by the extraction solvents in the following order: EAE > BE > WE > PE > RE ($P < 0.05$). The EAE had the highest TFC (388.3 ± 5.6 mg RUE/g DPZ), and the RE had the lowest TFC (37.5 ± 1.2 mg RUE/g DPZ), making the TFC of EAE approximately ten times higher than that of the RE. The TFC values of the *P. zeylanica* extracts were in good agreement with the TPC of the *P. zeylanica* extracts ($r = 0.987$).

## 2,2-Di-phenyl-1-picrilhydrazyl-free radical scavenging activity

The ability of extracts of *P. zeylanica* to scavenge free radicals was assessed using the DPPH radical scavenging activity assay. The DPPH assay was published in 1958 and is widely used for the quantitative determination of the antioxidant capacity of biological samples and food. DPPH is a deep-violet stable-free radical that has one electron and has strong absorption at 517 nm.[26]

All the extracts of *P. zeylanica* were capable of scavenging DPPH radicals in a concentration-dependent manner [Figure 2]. The scavenging effects of EAE, BE, WE, PE, and RE on DPPH radicals increased when their concentrations were increased from 0.05 to 0.30 mg/mL. As shown in Figure 2, the $EC_{50}$ values of radical scavenging activity toward DPPH were 47.12 ± 5.07, 61.88 ± 4.38, 76.51 ± 5.62, 177.47 ± 7.73, 334.20 ± 20.71, and 13.00 ± 0.46 μg/mL for EAE, BE, WE, PE, RE, and Vc, respectively. The scavenging activity of different solvent extracts from *P. zeylanica* toward the DPPH radical decreased in the following order: Vc > EAE > BE > WE > PE > RE. The DPPH scavenging activity values were significantly correlated with the TPC ($r = 0.891$) and TFC ($r = 0.916$) in all the extracts. The correlation study between DPPH radical scavenging activity and the TPC and TFC values in all extracts were significant ($P < 0.05$). These results indicated that the phenolic compounds, especially flavonoids that are present in all extracts of *P. zeylanica*, were the major constituents responsible for scavenging the DPPH radical. These results were consistent with other investigations.[26]

**Table 1:** The results of extraction yield and total phenolic and total flavonoid contents of the five *Pouzolzia zeylanica* extractions

| Extraction | Extraction yield (%) | TPC (mg GAE/g DPZ) | TFC (mg RUE/g DPZ) |
|---|---|---|---|
| EAE | 28.07±1.62$^a$ | 263.5±4.8$^a$ | 388.3±6.3$^a$ |
| BE | 23.09±1.76$^c$ | 189.5±2.6$^b$ | 308.3±3.6$^b$ |
| WE | 7.60±0.36$^e$ | 145.8±3.2$^c$ | 251.7±1.8$^c$ |
| PE | 18.96±0.89$^d$ | 87.0±1.6$^d$ | 105.0±1.2$^d$ |
| RE | 24.90±1.26$^b$ | 26.0±0.8$^e$ | 37.5±0.2$^e$ |

Values are the mean±SE, ($n$=3). Different letters (a-e) next to the means indicate the quantity decreasing from high to low. EAE: Ethyl acetate extract; BE: *n*-Butanol extract; WE: Water extract; PE: Petroleum ether extract; RE: Remaining extract; TPC: Total phenolic content; TFC: Total flavonoid content: SE: Standard error

## Ferric-reducing antioxidant power activity

The reducing abilities of all the extracts from *P. zeylanica* were determined using the FRAP assay. The principle is that antioxidants cause the reduction in the ferric–TPTZ (Fe[III]-TPTZ) complex to the blue ferrous–TPTZ (Fe[II]-TPTZ) complex, and the absorbance of the ferrous–TPTZ complex can be determined at 593 nm. Greater FRA*P* values indicate stronger ferric-reducing ability.

In this study, the EAE from *P. zeylanica* exhibited the strongest ferric-reducing ability (4.35 ± 0.18 mmol/g), while the RE showed the lowest (0.29 ± 0. 02 mmol/g) [Figure 2]. The FRA*P* values of all the extracts (EAE, BE, WE, PE, and RE) and Vc were found to be 4.35 ± 0.18, 3.03 ± 0.14, 1.21 ± 0.08, 0.45 ± 0.04, 0.29 ± 0.02, and 14.81 ± 0.54 mmol/g, respectively. The reducing power of all extracts from *P. zeylanica* decreased in the following order: Vc > EAE > BE > WE > PE > RE. The reducing power of *P. zeylanica* extracts showed strong correlations with the TPC ($r = 0.953$) and TFC ($r = 0.932$) values, and these correlations were significant ($P < 0.05$).

## Antioxidant activities of the isolated compounds from ethyl acetate extract

Flavonoids are a major class of compounds found in food and plant extracts that have been the focus of increasing interest. The relative significance of the positions and extents of hydroxylation on the flavonoid rings significantly influence the antioxidant properties of each compound.[27,28] In this study, three compounds (two flavones and one amide) were isolated from the EAE, and the antioxidant activities of these compounds were examined. As shown in Figure 3, compound 1, compound 2, and compound 3 demonstrated significant antioxidant activities with $EC_{50}$ values of DPPH radical scavenging activity of 5.47 ± 0.40, 15.80 ± 0.89, and 35.18 ± 3.03 μg/mL, respectively; these results can also be expressed as (1.81 ± 0.13) × $10^{-2}$, (5.52 ± 0.31) ×

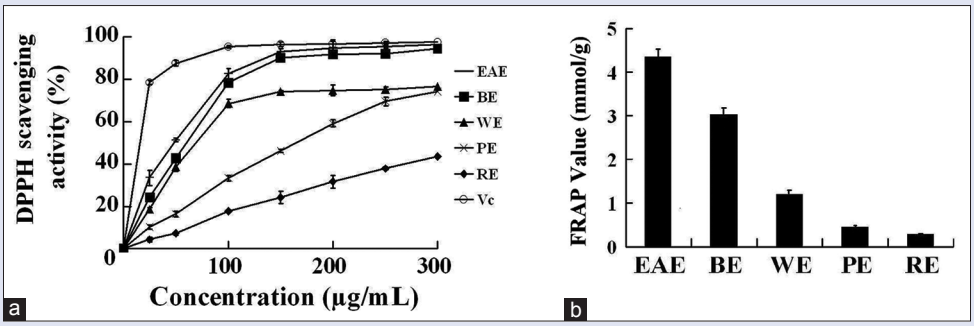


**Figure 2:** 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (a) and ferric ion-reducing activity (b) of the five extracts from *Pouzolzia zeylanica*. EAE: Ethyl acetate extract; BE: *n*-Butanol extract; WE: Water extract; PE: Petroleum ether extract; RE: Remainder extract

$10^{-2}$, and (10.69 ± 0.92) × $10^{-2}$ μM. The results of the DPPH radical scavenging assay of the isolated compounds from the EAE of *P. zeylanica* were determined in comparison to Vc as the standard antioxidant. The activity of quercetin was twice as high as that of the reference antioxidant. The activity of kaempferol was similar to that of the reference antioxidant. Compound 3 also had good DPPH radical scavenging activity, but the activity was inferior to the other compounds. The FRA*P* values of the three compounds and ascorbic acid were 16.28 ± 0.32, 11.71 ± 0.68, 6.02 ± 0.26, and 14.81 ± 0.54 mmol/g, respectively. This is the first report of the antioxidant activity of compound 3 based on its DPPH and FRAP activities. The antioxidant activities of the isolated compounds decreased as follows: 1 > 2 > 3. It was reported that compound 3 from the EAE of *P. zeylanica* has potential antineoplastic activity and DPPH radical scavenging activity. The results of the present work also suggest that the activity of *P. zeylanica* against acute mastitis, dysentery, indigestion, abdominal pain, infantile malnutrition, edema, urine negative embolism, bruises, hemoptysis, hematemesis, and traumatic hemorrhage could be explained by the presence of the antioxidant flavones and amides. Therefore, further studies on the mechanistic properties of flavonoid antioxidant activities are necessary for understanding and preventing diseases linked to reactive oxygen species.

## High-performance liquid chromatography analysis of active compounds from the ethyl acetate extract

The contents of antioxidant active compounds from EAE were obtained; 1 (16.95 ± 0.620 mg/g), 2 (1.14 ± 0.056 mg/g), and 3 (1.21 ± 0.028 mg/g) (Precision and repeatability results could be found in Appendix 1).

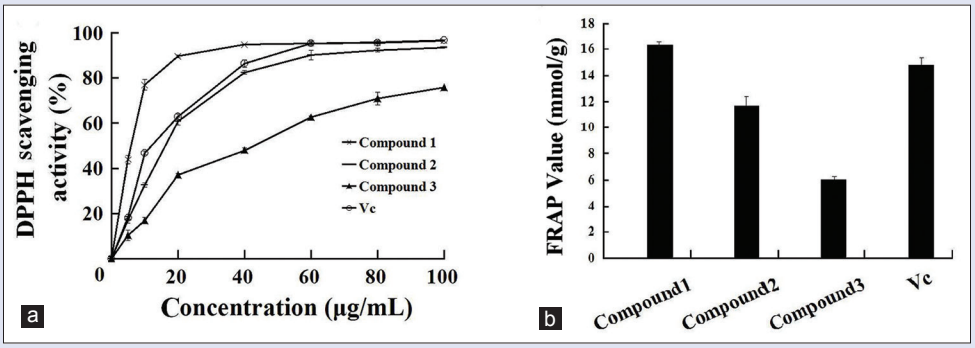


**Figure 3:** 2,2-Diphenyl-1-picrylhydrazyl radical scavenging activity (a) and ferric ion-reducing activity (b) of the isolated antioxidant compounds from the ethyl acetate extract of *Pouzolzia zeylanica*

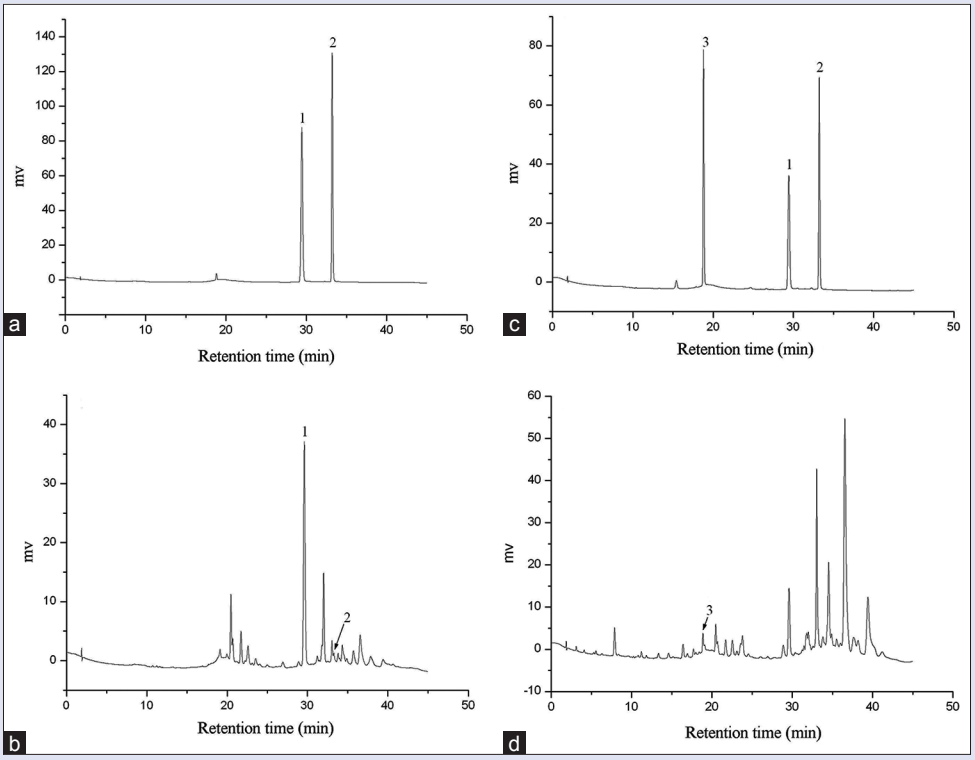


**Figure 4:** High-performance liquid chromatography chromatograms of a standard mixture (a and c) and an ethyl acetate extract of *Pouzolzia zeylanica* (b and d); a and b at 360 nm; c and d at 320 nm. 1 (quercetin), 2 (kaempferol), and 3 (N-[2-(3-hydroxy-4-methoxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl) prop-2-enamide)

Compound 1 and compound 2 were identified by comparison to known standards at 360 nm in HPLC analysis, and compound 3 was compared at 320 nm [Figure 4]. The content of these three compounds from the EAE decreased in the following order: 1 > 3 > 2. As shown in Figure 4, compound 1 was the main flavonoid in the EAE from *P. zeylanica*. Lower contents of compound 2 and compound 3 were found in the EAE. This is the first report of the contents of compound 1, compound 2, and compound 3 in *P. zeylanica*.

## Correlation between antioxidant activity and flavonoids

According to the Pearson's correlation analysis in each assay mentioned above, the whole experiment showed high correlations between the antioxidant activities and the TPC and TFC values of all extracts. The Pearson's correlations between the TPC and the DPPH radical scavenging activity and ferric ion-reducing activity in all extracts were 0.891 and 0.953, respectively. Significant correlations have been found between the TFC and the DPPH radical scavenging activity (0.916) and ferric ion-reducing activity (0.932) in all extracts. Therefore, it can be presumed that flavonoids and phenolic compounds are the major antioxidant compounds in *P. zeylanica*. It was demonstrated that the EAE contained the highest total phenolic and flavonoid contents, and we therefore preferentially chose to further purify the ethyl acetate fraction to isolate its active antioxidant compounds. Three antioxidant compounds (1, 2, and 3) were isolated from the EAE. It can be demonstrated that compound 1 was the major constituent in the EAE based on HPLC analysis, which further verified the conclusion that flavonoids and phenolic compounds were the major antioxidant compounds.

# CONCLUSIONS

Through two *in vitro* antioxidant models, we can prove that the EAE from *P. zeylanica* possesses the highest antioxidant activity and TPC and TFC among the five parts. In addition, we chose the EAE for further separation. Two flavones and one amide were isolated from the EAE fraction, namely quercetin, kaempferol, and N-[2-(3-hydroxy-4-methoxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl) prop-2-enamide. The antioxidant capacities of these three compounds were also investigated by DPPH-free radical scavenging and FRAP assays, demonstrating their antioxidant activities. Furthermore, the contents of antioxidant active compounds in the EAE were studied. Our findings suggest that the extracts and phytocompounds from *P. zeylanica* can act as potential antioxidants; however, further investigation is required to study the *in vivo* antioxidant efficacy, and the separation of other fractions of *P. zeylanica* should be explored.

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## Conflicts of interest

There are no conflicts of interest.

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# APPENDIX

## APPENDIX 1

## STRUCTURAL DETERMINATION OF ACTIVE COMPOUNDS FROM THE ETHYL ACETATE EXTRACT

The chemical structures of the isolated compounds from the ethyl acetate extract were confirmed by comparing their $^{1}$H NMR and $^{13}$C NMR spectra to published data.

Quercetin (1): obtained as a yellowish powder, $C_{15}H_{10}O_7$. $^{1}$H NMR (500 MHz, DMSO, δ, ppm, J/Hz): 6.18 (1H, d, J = 1.5 Hz, H-6), 6.40 (1H, d, J = 2.0 Hz, H-8), 7.67 (1H, d, J = 2.0 Hz, H-2'), 6.88 (1H, d, J = 8.5 Hz, H-5'), 7.54 (1H, dd, J = 8.5, 2.0 Hz, H-6'). $^{13}$C NMR (125 MHz, CDCl3, δ, ppm): 146.8 (C-2), 135.7 (C-3), 175.8 (C-4), 103.0 (C-4a), 160.7 (C-5), 98.2 (C-6), 163.9 (C-7), 93.4 (C-8), 156.2 (C-8a), 122.0 (C-1'), 115.1 (C-2'), 145.1 (C-3'), 147.7 (C-4'), 115.6 (C-5'), 120.0 (C-6'). The data were in agreement with the reported literature values.[1]

Kaempferol (2): obtained as a yellowish powder, $C_{15}H_{10}O_6$. $^{1}$H NMR (500 MHz, DMSO, δ, ppm, J/Hz): 6.18 (1H, br. s, H-6), 6.43 (1H, br. s, H-8), 8.05 (2H, br. d, J = 9.0 Hz, H-2' 6'), 6.92 (2H, br. d, J = 9.0 Hz H-3' 5'). $^{13}$C NMR (125 MHz, $CDCl_3$, δ, ppm): 146.8 (C-2), 135.6 (C-3), 175.9 (C-4), 103.0 (C-4a), 160.7 (C-5), 98.2 (C-6), 163.9 (C-7), 93.5 (C-8), 156.2 (C-8a), 121.6 (C-1'), 129.5 (C-2' 6'), 115.4 (C-3' 5'), 159.2 (C-4'). The data were in agreement with the reported literature values.[2]

N-[2-(3-Hydroxy-4-methoxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl) prop-2-enamide (3): obtained as white crystals, $C_{18}H_{19}NO_5$. $^{1}$H NMR (500 MHz, $CD_3OD$, δ, ppm, J/Hz): 6.47 (1H, d, J = 15.5 Hz), 7.45 (1H, d, J = 16.0 Hz), 3.45 (1H, dd, J = 14.0, 8.0 Hz), 3.54 (1H, dd, J = 14.0, 5.0 Hz), 4.73 (1H, dd, J = 8.0, 5.0 Hz), 7.11 (1H, d, J = 1.0 Hz), 6.79 (1H, d, J = 7.5 Hz), 7.01 (1H, d, J = 8.0 Hz), 7.22 (1H, d, J = 8.5 Hz), 6.78 (1H, d, J = 8.5 Hz), 3.86 (3H, s, OMe). $^{13}$C NMR (125 MHz, $CD_3OD$, δ, ppm): 169.5 (C-1), 118.6 (C-2), 142.3 (C-3), 48.3 (C-1'), 73.4 (C-2'), 134.7 (C-1"), 111.5 (C-2"), 149.8 (C-3"), 149.2 (C-4"), 116.4 (C-5"), 123.3 (C-6"), 128.2 (C-1'"), 128.5 (C-2'", 6'"), 116.1 (C-3'", 5'"), 158.0 (C-4'"), 56.4 (C-1α). The data were in agreement with the reported literature values.[3]

The structures of the compounds isolated from *Pouzolzia zeylanica* are shown in Supplementary Figure 1.

## RELATIONSHIP BETWEEN EXTRACTION SOLVENTS AND THE CONTENTS OF TOTAL PHENOLIC CONTENT AND TOTAL FLAVONOID CONTENT

The relationship between different percentages of extraction solvents and the contents of total phenolic content (TPC) and total flavonoid content (TFC) in the extracts was evaluated. As shown in Supplementary Table 1, the contents of TPC and TFC were the highest in 90% ethanol extract compared to other extracts and decreased with decreasing ethanol concentration in the extraction solvent. These results showed that alcohol concentrations 90% was the best to prepare phenolic and flavonoid compounds from *P. zeylanica*. We can speculate that flavonoid aglycones with low polarity were the primary phenolic and flavonoid compounds of *P. zeylanica*.

## PRECISION AND REPEATABILITY OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY EXPERIMENT

The standard solution was analyzed for five replicates. The value of peak area was detected as the condition mentioned above, and the relative standard deviation (RSD) of compounds 1, 2, and 3 was 1.56%, 1.13%, and 0.63%, respectively.

The repeatability of real-sample analysis was examined for six times as the method mentioned above, and the RSD of compounds 1, 2, and 3 was 1.95%, 1.38%, and 0.96%, respectively.

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