Studying the Extraction Conditions on Rosmarinic Acid Content and Antioxidant Activity of Basil (*Ocimum basilicum* L.)

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Abstract: *Basil (*Ocimum basilicum *L.), a popular herbal plant, is known for its ornamental and therapeutic importance. It has been described as having antioxidant, anti-inflammatory, hepatoprotective, immunomodulatory, antihyperglycemic, and antimicrobial properties. This study aimed to evaluate the effects of some factors on the rosmarinic acid concentration and antioxidant activity of basil extract, such as samples, solvents (water and 50% ethanol), solvent-to-sample ratios (from 1:20 to 1:70 g/mL), extraction temperature (70, 90, and 110 °C), and extraction time (30, 60, 90, and 120 min). Rosmarinic acid content was analyzed using the spectrophotometry method. The DPPH free radical scavenging experiment was also used to assess the extracts' antioxidant potential. The results showed that with the dried leaves, the ratio between the sample and 50% ethanol was 1:40 g/mL, extraction temperature of 90 °C, and extraction time of 60 min were the best conditions for obtaining rosmarinic acid from basil. The quantitative result also showed that basil extracts had a lot of polyphenols (dark green precipitate) and flavonoids (yellow precipitate). In addition, basil leaves had antioxidant properties with an IC_{50} value of 3103.18 µg/mL. These findings showed that basil extract may be an important source of antioxidant compounds, such as rosmarinic acid.*

Keywords: *antioxidant; basil; extract;* Ocimum basilicum *L.; rosmarinic acid*

■ INTRODUCTION

The body's imbalance in producing free radicals compared to the activity of antioxidants is the direct cause of numerous diseases, such as diabetes, multiple sclerosis, heart disease, Parkinson's disease, inflammation, Alzheimer's disease, atherosclerosis, stroke, and cancer. To reduce the risk of diseases caused by oxidative imbalance, the use of antioxidants is essential. However, there are worries over the potential harm synthetic antioxidants like butylated hydroxytoluene and butylated hydroxyanisole, which are often utilized, that may cause risk to human health. Because of that, scientists are now searching for antioxidants that come from plants. Among them, the high polyphenol content of herbs and spices frequently results in their decisive antioxidant action [1-2]. Antioxidants, dietary fiber, vitamins, and minerals give food its functionality; many of these elements are present in medicinal plants [3]. Natural antioxidants like polyphenols, present in aromatic and therapeutic herbs and may help reduce oxidative damage, are gaining popularity. Basil is one of the fragrant and medicinal herbs used in food manufacturing that has demonstrated encouraging health benefits [4].

About 150 species of the genus *Ocimum* L. have significant differences in plant morphology and biological characteristics, essential oil content, and chemical composition [5]. This genus derives from Asia, Central and South America, and Africa, but today, it is grown worldwide [6]. *Ocimum basilicum* L. is one of the most popular herbal plants belonging to the Lamiaceae family [7]. It is known as sweet basil or basil in English, and basilic, basilikum, and albahaca in French, German, and Spanish [8]. Basil is a spice commonly used in Italian and Southeast Asian cuisines [4]. In Vietnam, basil has been grown and popular since 1975 and is mainly used as a spice and a traditional medicinal plant [9]. Basil grows upright, branched, about 30–90 cm high, with smooth, light green leaves, simple, opposite, ovate,

pointed. Flowers are white to purple. Nutlets are ellipsoid, black, and pitted [10]. According to traditional Vietnamese medicine, basil treats many diseases, such as anti-inflammatory, deworming, dysentery, mental disorders, tooth decay, and bronchitis [11].

Basil has many bioactive compounds, such as monoterpene hydrocarbons, oxygenated monoterpenes, oxygenated sesquiterpene hydrocarbons, sesquiterpenes, triterpenes, polyphenols, flavonoids, aromatic compounds [12]. The primary polyphenols are phenolic acids such as rosmarinic acid (RA), chicoric acid, ferulic acid, and caffeic acid [3,13]. RA is an important secondary metabolite found in various plant species, particularly the Lamiaceae and Boraginaceae families [14]. RA, an ester of caffeic acid with *R*-(+)-3-3,4-dihydroxylphenyl lactic acid, was first isolated from rosemary (*Rosmarinus officinalis*) in 1958 [15]. The chemical formula of RA is $C_{18}H_{16}O_8$, with a molecular weight of 360.318 g/mol and a melting point of 172–174 °C [16-17]. RA is a slightly yellow powder in crystalline form, soluble in most polar organic solvents but somewhat soluble in water [18]. Many studies have reported that RA is basil's most biologically active compound due to its potent antioxidant, anti-aging, anticancer, antiviral, and antimicrobial properties [5,13,19]. RA is also used to preserve food because of its antioxidant and antibacterial properties [20]. For example, RA-rich *Perilla frutescens* (L.) extract was used to preserve fresh seafood [21], or RA-rich extract from *Rosmarinus officinalis* was used as an antioxidant in candy [22].

Nowadays, research on the extraction of bioactive compounds from fruits and vegetables to be added back into food or pharmaceuticals is a trend to increase nutritional value, prolong product shelf life, and increase the ability to treat some diseases [23]. Therefore, research on methods to extract RA compounds from basil for use in food or pharmaceuticals is to meet this trend. Polyphenols are susceptible to oxidation. Therefore, the extraction process to determine the contents of polyphenols requires very caution. Despite the development of new extraction techniques today, the conventional method dominates many laboratories mainly due to its simplicity and low economic outlay. The extraction yield can be widely regulated by the choice of suitable solvents, optimal temperature, and time [24]. This study was conducted to find appropriate extraction conditions and antioxidant activity of extract from the basil plant in Binh Duong province, Vietnam, creating a premise for research on applying antioxidants from the basil plant in testing the production of functional foods.

■ EXPERIMENTAL SECTION

Materials

Basil plants were grown in Tan Uyen district, Binh Duong province, Vietnam. These plants were transported to the experimental center of Thu Dau Mot University and washed to remove dust, yellow, and pest-infested leaves (Fig. 1). The aerial plant parts, including leaves and stems, were dried at 50 °C in an oven and ground into powder (sieve size 1 mm) to ensure sample uniformity [13] (Fig. 2). Dried powders from the basil

Fig 1. Basil (*Ocimum basilicum* L.) in Vietnam

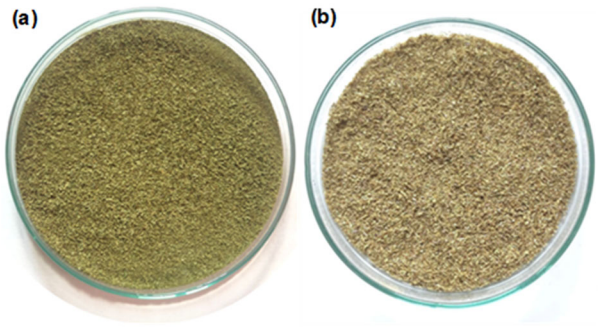

Fig 2. Dried powders from basil (a) leaves and (b) stems

leaves and stems had a moisture content of 11.33 and 11.20%, respectively. They were stored in sealed glass jars to avoid dehumidification at room temperature and used to extract and analyze qualitative polyphenols and flavonoids, RA content, and antioxidant activity. The solvents used for extraction are ethanol (99.5% purity, CEMACO - Vietnam) and distilled water. Lead(II) acetate trihydrate ($Pb(CH_3COO)_2{\cdot}3H_2O$) and ferric trichloride anhydrous used ($FeCl_3$) for qualitative analysis were produced by Himedia, India. RA (98% purity), used as a standard in spectrophotometry analysis, was obtained from Sigma-Aldrich. The study was carried out from September 2023 to April 2024 in the experimental center of Thu Dau Mot University, Vietnam.

Instrumentation

A magnetic stirrer with heating (Heidolph MR Hei-Tec) was used to extract in this research. JASCO UV-vis-NIR V770 spectrophotometer was used to analyze RA content and antioxidant activity.

Procedure

Qualitative analysis of the polyphenols and flavonoids in basil extract

Medicinal powders from the dried leaves and stems (1.0 g) were added to 50 mL of ethanol 50% in a 100 mL Erlenmeyer flask, then boiled on a magnetic stirrer heater at 70 °C for 60 min. The mixtures were filtered, and the clear liquid was collected. The tests were presented as shown in Table 1 [25].

Effect of the different plant parts and solvent types on RA content in basil extract

The dried samples from basil leaves and stems (1.0 g) were extracted with 50 mL water or ethanol 50% at 70 °C for 60 min on a magnetic stirrer heater. The extracts were filtered and used directly to determine RA content.

Effect of the solvent-to-sample ratio on RA content in basil extract

In the previous experiment, 1.0 g dried samples were extracted with a suitable solvent at 70 °C for 60 min on a magnetic stirrer heater. The ratio variations were performed from 1:20 to 1:70 g/mL. Finally, the extracts were passed through filter paper and used directly to analyze RA concentration.

Effect of extract temperature on RA content in basil extract

As in the prior experiment, the dried samples were extracted with a solvent at an appropriate ratio for 60 min on a magnetic stirrer heater. The extract temperatures were 70, 90, and 110 °C. After 1 h the mixtures were filtered to analyze RA content.

Effect of extract time on RA content in basil extract

The samples were extracted with a solvent at an appropriate ratio on a magnetic stirrer heater at a proper extract temperature (determined in the previous experiment). The extract times were 30, 60, 90, and 120 min. After that, the mixtures were filtered to determine RA concentration.

Spectrophotometry analysis of RA content

RA content was determined based on a bright yellow reaction when RA and zirconium(IV) oxide chloride formed a complex, resulting in the highest absorption at 362 nm [26].

Preparation of standard solutions and reagents. RA standard solution was prepared with a concentration of 0.001 M (0.0901 g dissolved in 96% ethanol and made up to 250 mL. Zirconium(IV) oxide chloride reagent solution had a concentration of 0.5 M was prepared by dissolving 1.6117 g in distilled water and made up to 10 mL [26].

RA calibration curve construction. RA standard solution samples with volumes of 25, 50, 75, 100, and 125 µL were pipetted into different test tubes, and 4.6 mL of 96% ethanol was added. Finally, 200 µL of zirconium(IV) oxide chloride solution (0.5 M) was added to each test tube to reach a total volume of 5 mL, mixed well, and left to stand. After 5 min, the absorbance

Table 1. Preliminary phytochemical test

Compound	Reagent	Reaction to identify chemical compounds
Flavonoids	$Pb(CH_3COO)_2$ 10%	Yellow precipitate
Polyphenols	$FeCl_3$ 5%	Dark green precipitate

was measured on a spectrophotometer at 362 nm. The blank sample had the same composition as the analyzed sample but did not contain RA. The regression equation was constructed to represent the linear dependence between RA concentration (5–25 μM) and absorbance (required $R^2 \geq 0.99$) [26].

Quantification of RA in the sample. First, 4.6 mL of ethanol and 200 μL of a 0.5 M zirconium(IV) oxide chloride solution were combined with 200 μL extract solutions. The absorbance of the reaction mixture at 362 nm was measured using a spectrophotometer after 5 min. The concentration of RA was determined based on an equation $y = 0.0231x - 0.0444$ ($R^2 = 0.9995$) with RA concentration from 5 to 25 μM.

Analysis of antioxidant activity

The antioxidant activity of the extract was evaluated through the ability to scavenge DPPH free radicals [27].

Preparation of DPPH reagent solution. First, 0.6 mM DPPH solution was prepared by dissolving 5.915 mg DPPH with an amount of absolute ethanol sufficient to dissolve DPPH completely. Then, the mixture was put in a volumetric flask, and enough ethanol was added to make 25 mL.

Preparation of test sample. The basil extract was evaporated at 50 °C to dryness. The extract was dissolved in absolute alcohol in a series of concentrations: 3000, 1000, 500, 250, and 125 μg/mL. Vitamin C as a positive control was diluted in absolute ethanol into a concentration range of 50, 25, 12.5, 6.25, and 3.125 μg/mL. Absolute ethanol is used as the negative control. The DPPH free radical scavenging assay test was carried out, as shown in Table 2.

The mixture was incubated in the dark at 37 °C for 30 min, and the absorbance was measured at 517 nm. The percentage of inhibition of DPPH free radicals was calculated using the Eq. (1);

Table 2. Test of DPPH free radical scavenging assay

Sample	Sample solution (mL)	Absolute alcohol (mL)	DPPH solution (mL)
Blank	0	4.0	0
Control	0	3.5	0.5
Sample	0.5	3.0	0.5

$$\%\text{inhibition} = \left[1 - \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right] \times 100\% \quad (1)$$

where $\text{Abs}_{\text{sample}}$ was the sample absorbance and $\text{Abs}_{\text{control}}$ was the control absorbance. The IC_{50} value (μg/mL of extract) is the concentration that inhibits 50% of DPPH free radicals.

Statistical analysis

Experimental data were analyzed by Statgraphics Centurion XV software with a significant difference of a 5% level and showed the mean ± SD with three replications.

■ RESULTS AND DISCUSSION

Qualitative Analysis of Flavonoids and Polyphenols in Basil Extract

Phenolic compounds are plant secondary metabolites, such as flavonoids and phenolic acid. They play essential biochemical roles and benefit human health due to their antioxidant properties [28]. In particular, RA is a phenolic compound with strong antioxidant activity, anti-HIV, and inhibition of cell division in some cancer cell lines. The important medicinal properties of RA have been and are being studied [29]. Therefore, the qualitative determination of flavonoids and polyphenols in basil extract was carried out to preliminarily investigate the presence of these compounds so that the RA extraction process can achieve higher efficiency.

When adding the reagent to basil extract, a yellow precipitate (test tube no. 2) and a dark blue precipitate (test tube no. 3) appeared (Fig. 3). In particular, the amount of precipitate collected in these tubes was large.

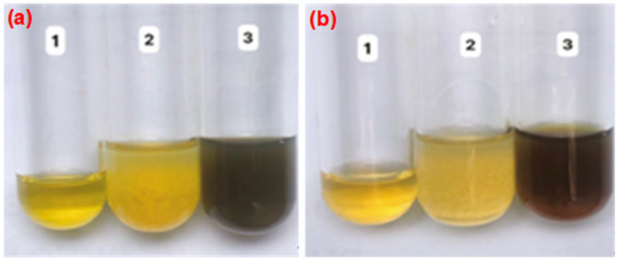

Fig 3. Qualitative analysis in basil of (a) dried leaves and (b) dried stems. (1) Sample before reaction, (2) qualitative of flavonoids, and (3) qualitative of polyphenols

Table 3. The presence of flavonoids and polyphenols in basil extract

Test	Dried sample	
	Leaves	Stems
Flavonoids	+	+
Polyphenols	+	+

(+) presence

It showed that the amount of flavonoids and polyphenols in basil extract was high (Table 3).

Many studies recorded that basil contained many polyphenols and flavonoids [4-5,30]. According to Güez et al. [5], polyphenols and flavonoids were present in basil with 23780 and 15982 mg/mL of extract, respectively. The main compounds in basil include RA, gallic acid, caffeic acid, chlorogenic acid, rutin, and quercetin. These compounds have antioxidant and anti-inflammatory activities and can be used as medicinal herbs. Song et al. [30] also reported that RA had the highest percentage of polyphenols, followed by chicoric and caffeic acids.

Effect of Different Plant Parts and Solvent Types on RA Content in Basil Extract

The efficiency of extracting secondary compounds from plants often depends significantly on the parts of the plant and the type of extraction solvent used. To obtain extracts that can be used in food and pharmaceutical products later, distilled water and 50% ethanol were chosen to conduct the extraction with two types of raw materials from basil (leaves and stems). The results of the different effects between raw material parts and solvent types on RA content were shown in Fig. 4. In Fig. 4, RA content in the leaves was higher than in the stems. Many previous studies reported that RA was the main phenolic compound found in both leaves and stems, but in stems, RA was found in minor quantities [31-32]. Leaves comprise most of the whole plant and are often used in food processing and medicine. Therefore, leaves were used as samples for the next test.

The efficiency of RA extraction from plant materials depends on the type of solvent used, especially the polarity of the solvent. RA is more soluble in most organic solvents but less soluble in water [18]. Different organic solvent/water mixtures produced different polar solvents for extracting polyphenols. An ethanol/water mixture is often chosen as the extraction solvent due to its widespread use and handling considerations. In Fig. 4, the samples extracted with 50% ethanol have a higher RA content than when extracted with water. This makes sense, given that ethanol has a stronger affinity for phenolic compounds. The polarity of the solvent and the solubility properties of the extracted components can affect the efficiency and performance of the extraction process. When water is used for extraction, many proteins, polysaccharides, and other inorganic compounds can also be extracted from the samples, hindering the solubilization of polyphenols [3,33]. This result was similar to the research results of Teofilović et al. [3] and Aloisio et al. [12]. When using 50% ethanol solvent to extract bioactive compounds from parts of basil, the content of total polyphenols and RA reached the highest levels.

In this experiment, the RA content was highest (11.33 mg/g DW) when dried basil leaves were extracted with 50% ethanol. In contrast, the lowest RA content was 7.04 mg/g DW when using basil stem extracted with water. According to the report of Chaowuttikul et al. [34], RA contents in dried leaves of *Ocimum basilicum* L. (5.97 mg/g DW) in Thailand were lower than in the parts (leaves, stems) of *Ocimum basilicum* L. in this experiment. The findings of the statistical analysis (Fig. 4) showed that the RA content was influenced by the types

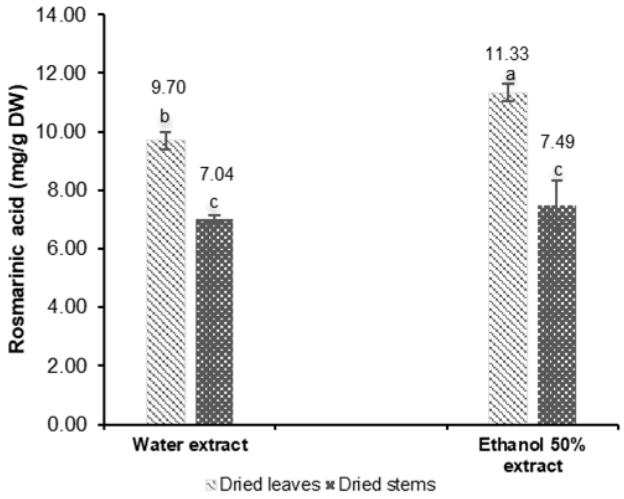

Fig 4. RA content in leaves and stems of basil extract with different solvents

of solvent and the various plant components ($p < 0.05$). Therefore, basil leaves and 50% ethanol solvent were selected for the next experiment.

Effect of Solvent-to-Sample Ratio on RA Content in Basil Extract

The solvent-to-sample ratio is also a factor that significantly affects the extraction process. The minimum amount of solvent must often cover the surface of the raw material layer; then, the raw material will be fully exposed to the solvent. Therefore, the solvent-to-sample ratio selected for investigation in this experiment is 1:20 to 1:70 g/mL. After 60 min of extraction, the filtered extract will be analyzed for RA. The results were shown in Table 4.

In Table 4, the RA content in basil leaf extract gradually increased when the amount of solvent increased from 20 to 40 mL. The results showed when the solvent was small (20–30 mL), it was insufficient to extract all RA from the cells. The ratio of 1:40 g/mL helped the extraction process better. According to the driving force in the mass transfer process, the concentration gradient between solid and liquid will be larger when there is a higher solvent-to-solid ratio. However, when the solvent-to-solid ratio is increased, the extraction efficiency does not grow; it only dilutes the extract [35]. In this experiment, increasing the amount of solvent from 50 to 70 mL, the RA content tended to decrease. Thus, when extracting with too much solvent, while the RA content in the raw material is a fixed number, it will quickly lead to equilibrium between the phases, causing the RA extraction efficiency not to increase but will tend to approach horizontally. Through statistical analysis results (Table 4), there was an influence of solvent-to-sample ratio on RA content ($p < 0.05$). Therefore, the appropriate solvent-to-solid ratio in this experiment was 1:40 g/mL.

Effect of Extract Temperature on RA Content in Basil Extract

The extraction of secondary chemicals from plants is greatly influenced by temperature. The diffusion ability of molecules increases with temperature, and the amount of chemicals acquired is more significant at higher temperatures. RA is a potent antioxidant, so it can be easily oxidized at high temperatures, reducing the yield of RA from basil. Therefore, the extraction temperatures (70, 90, and 110 °C) were investigated by direct heating. The results were reported in Table 5.

RA content increased when the temperature increased from 70 to 90 °C. However, if the temperature continued to grow to 110 °C, the RA content decreased (Table 5). Increasing the temperature would facilitate the extraction by enhancing the solute's solubility and diffusion coefficient. Heating might also soften plant tissue and weaken phenol-protein and phenol-polysaccharide interactions in leaves so that more phenolics would be distributed into the solvent [35]. However, high temperatures probably cause a decrease in the extraction yield due to the possible degradation of phenolic compounds caused by hydrolysis, internal redox reactions, and polymerization [24]. Atanasova et al. [36] found more RA from lemon balm (*Melissa officinalis*) when extracted at 100 °C. Increasing the temperature to 150 °C reduced RA content by up to 20% due to thermal decomposition. Thus, according to statistical analysis results in Table 5 ($p < 0.05$), a temperature of 90 °C should be chosen for the next extraction experiment.

Table 4. The effect of solvent-to-sample ratio on RA content in basil extract

Solvent-to-sample ratio (g/mL)	RA content (mg/g DW)
1:20	9.32 ± 0.09^{cd}
1:30	10.10 ± 0.09^{b}
1:40	10.83 ± 0.63^{a}
1:50	9.90 ± 0.30^{bc}
1:60	9.07 ± 0.36^{d}
1:70	9.40 ± 0.27^{cd}

*The means with different letters indicate a significant difference at the 5% level, according to the LSD test

Table 5. The effect of the extraction temperature on RA content in basil extract

Temperature (°C)	RA content (mg/g DW)
70	11.56 ± 0.24^{b}
90	14.42 ± 0.16^{a}
110	10.10 ± 0.32^{c}

*The means with different letters indicate a significant difference at the 5% level, according to the LSD test

Effect of Extraction Time on RA Content in Basil Extract

Not only the plant part, solvent type, solvent-to-sample ratio, and temperature but also the time factor is essential to pay attention to in the extraction of secondary compounds from plants. Controlling the extraction time will help increase the efficiency of the extraction process, saving extraction time and energy costs. Usually, the extraction time will be different depending on the type of raw material and extraction factors. Therefore, the extraction time (30, 60, 90, and 120 min) was investigated in this experiment. The results are reported in Table 6.

In Table 6, RA content increases when the extraction time increases from 30 to 60 min, reaching 11.35 to 13.38 mg/g. Next, RA content decreased by increasing the extraction time (90 and 110 min). The RA content at the extraction time of 110 min was the lowest, reaching only 7.40 mg/g. That may be because increased extraction time can cause some oxidants to be oxidized, so RA content decreases [36-37]. Through statistical analysis results (Table 6), there was an influence of extraction time on RA content ($p < 0.05$). Therefore, the most appropriate RA extraction time from basil leaves was 60 min.

Table 6. The effect of extraction time on RA content in basil extract

Time (min)	RA content (mg/g DW)
30	11.35 ± 0.39^{b}
60	13.38 ± 0.09^{a}
90	8.91 ± 0.36^{c}
120	7.40 ± 0.10^{d}

*The means with different letters indicate a significant difference at the 5% level, according to the LSD test

Antioxidant Activity of Basil Extract

DPPH is a widely used method in assessing antioxidant activity to test the ability to scavenge free radicals and hydrogen-donating groups. The unpaired electrons in the DPPH free radical (the purple compound) give the most vital absorption at 517 nm. These unpaired electrons can combine with the hydrogen of the antioxidant to form DPPH-H and the compound's color changes from purple to yellow. Therefore, the higher a substance's free radical scavenging ability, the lower the spectral absorption measured at 517 nm of the DPPH reaction and vice versa [38]. The results of testing the antioxidant activity of the extract of basil are presented in Table 7.

Based on the results in Table 7, a linear equation shows the correlation between antioxidant activity and high concentration of basil extract ($y = 0.0148x + 4.073$). The higher the concentration of the extract, the higher the antioxidant capacity. Therefore, basil extract had antioxidant activity against DPPH free radicals with an IC_{50} value of 3103.18 µg/mL. Compared with the IC_{50} value of ascorbic acid of 26.02 µg/mL, it was concluded that the antioxidant activity of the extract of basil leaves was lower. This result showed that the antioxidant capacity of the extract depended on the type of solvent, plant type, and extract concentration [26]. Compared with other plant species, such as ethanol extract of *Streptocaulon juventas* (Lour.) Merr (IC_{50} = 2571 µg/mL), *Pseuderanthemum palatiferum* (Nees) radlk (IC_{50} = 3429 µg/mL) [39], and *Ocimum basilicum* L. in Portugal (IC_{50} = 3990 µg/mL) [40], basil extract had equivalent antioxidant activity. Thus, this study has shown that basil leaf extract can inhibit free radicals and enhance antioxidant capacity when increasing the extract content. Basil can be a new source of raw materials, rich in RA, for food and pharmaceutical production.

Table 7. IC_{50} values of plant extracts for DPPH free radical scavenging activity

Sample	Linear regression equation	IC_{50} (µg/mL)
Ascorbic acid	$y = 1.9136x + 0.2129$ ($R^2 = 0.9811$)	26.02
Basil extract	$y = 0.0148x + 4.073$ ($R^2 = 0.9637$)	3103.18

■ CONCLUSION

Basil (*Ocimum basilicum* L.) belongs to the genus *Ocimum*, family Lamiaceae, and is both a spice and a medicinal plant. Basil contains many natural compounds with high antioxidant activity, such as polyphenols and flavonoids. This study evaluated different plant parts and solvent types, solvent-to-sample ratio, extraction temperature, and time to identify optimum extraction conditions for RA in basil extract. The results showed that RA content could be

achieved using leaves with the solvent-to-sample ratio of 1:40 g/mL at 90 °C for 60 min. Basil leaf extract has a high RA content (14.42 mg/g DW). Furthermore, the extract had antioxidant activity (IC_{50} = 3103.18 µg/mL), possibly due to bioactive compounds in basil leaves, such as polyphenols and flavonoids, especially RA. When compared with several other plant species, basil extract had comparable antioxidant activity. It may be a new source of raw materials in food and pharmaceutical production.

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■ CONFLICT OF INTEREST

Authors declare no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Anh Giang Que Pham did experiments, guided and supervised by Tram Thi My Pham. Anh Giang Que Pham and Tram Thi My Pham carried out the calculations. The paper was written by Anh Giang Que Pham and corrected by Tram Thi My Pham. All authors agreed to the final version of this manuscript.

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