TOTAL PHENOLIC, TOTAL FLAVONOID CONTENT, AND TOTAL ANTIOXIDANT CAPACITY OF *PIPER LONGUM* L.

Đến tòa soạn 16-01-2024

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TÓM TẮT

HÀM LƯỢNG PHENOLIC TỔNG, FLAVONOID TỔNG VÀ HOẠT TÍNH CHỐNG OXY HÓA CỦA LOÀI TIÊU LỐT PIPER LONGUM L.

Phenolic là hợp chất thiên nhiên được biết đến với khả năng chống oxy hóa hiệu quả. Trong nghiên cứu này hàm lượng flavonoid tổng (TFC), phenolic tổng (TPC), hoạt tính chống oxy hóa tổng (TAC) và khả năng bắt gốc tự do DPPH của phần chiết methanol và các phân đoạn n-hexane, dichloromethane, ethyl acetate và dịch chiết nước của phần trên mặt đất loài tiêu lốt Piper longum đã được đánh giá. TFC, TPC và TAC được xác định bằng phương pháp đo quang với lần lượt các dung dịch thuốc thử aluminum chloride, Follin-Ciocalteu và molybdate. Kết quả cho thấy dịch chiết ethyl acetate có hàm lượng TFC, TPC và hoạt tính chống oxy hóa tổng TAC lớn nhất (1,580 mg RE/100 g, 0,520 mg GAE/100 g và 5,896-18,083 mg AAE/100 g bột dược liệu khô). Thêm vào đó, phần chiết ethyl acetate có khả năng quyét gốc tự do DPPH tốt nhất với giá trị EC_{50} thấp nhất là 0,205 mg/mL. Do đó, tiêu lốt được xem là nguồn dược liệu chống oxy hóa đầy tiềm năng.

Từ khóa: *Tiêu lốt, DPPH, TAC, TFC, TPC, chống oxy hóa.*

1. INTRODUCTION

Free radicals like hydrogen peroxide (H_2O_2) and superoxide (O_2) are byproducts of metabolic processes in the human body. Due to their instability and ability to interact with other molecules by donating or accepting electrons, these radicals contribute to aging, cell damage, tissue degradation, and dysfunction in various organs [1]. As a result, the search for anti-oxidant-rich foods and therapeutic herbs is growing. Numerous studies have highlighted the significance of phenolic compounds in plants for counteracting free radicals and safeguarding cellular health [2]. Phenolic compounds typically feature an aromatic ring fused with a hydroxyl group (HO-) directly attached to the benzene ring [3]. Examples include phenolic acids (gallic, protocatechuic, caffeic, and rosmarinic acids), flavonoids (quercetin and catechin), diterpenes (carnosol, and carnosic acid), and volatile oils (eugenol, carvacrol, and thymol). Several herbs like *P. nigrum, P. umbellatum*, and *P. guineense* have been traditionally used within the Piperaceae family for their antioxidant properties. *Piper longum*, found widely in India, South China, Laos, and Vietnam, has diverse chemical constituents, including lignans, terpenoids, alkaloids, and essential oils [4, 5].

To date, only one study by Akbar et al. [6] has explored the impact of ethanol and water solvents on the total phenolic content, total flavonoids, and DPPH free radical scavenging activity of *Piper longum* fruit. In our research, we assessed the total phenolic and flavonoid contents, total antioxidant activity, and DPPH free radical scavenging potential of methanol extract (PLM) and fractional extracts *n*-hexane (PLH), dichloromethane (PLD),

ethyl acetate (PLE), and water (PLW) from the aerial parts of *P. longum*.

2. EXPERIMENTAL

2.1. Plant materials

The aerial parts of *Piper longum* L. were collected in Vinh Phuc, Vietnam, in November 2022 and authenticated by Dr Nguyen The Cuong. A voucher specimen (NCCT-P136) was kept at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

2.2. Chemicals and Reagents

Equipment: vacuum evaporator (Buchi, Switzerland), ultrasonic cleaner (Japan), UV/Vis spectrophotometer (UV1800, Shimadzu, Japan), 5-digit analytical balance (Ohaus PX2250, USA), Regents and chemicals: $AlCl_3$ 10%, rutin, methanol, CH_3COOK 1M, distilled water, Folin-Ciocalteu phenol 1N reagent, gallic acid (GA), Na_2CO_3 2%, 1,1 diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, ammonium molybdate, sulfuric acid, sodium sulfate.

2.3. Sample extraction

100 grams of dried aerial parts of *P. longum* were finely ground and then ultrasonically extracted with 1 liter of methanol at 50°C for 2 h to yield methanol extract (PLM, 30 grams). The PLM extract was effectively suspended in water and sequentially partitioned with *n*-hexane, dichloromethane, and ethyl acetate to obtain PLH, PLD, and PLE extracts, respectively, and the remaining water layer (PLW). All extracts were then concentrated and dissolved in appropriate solvents to be ready for further use in research.

2.4. Determination of total flavonoid content (TFC)

The total flavonoid content (TFC) of the extracts (PLH, PLD, PLE, PLW, PLM) was quantified using the colorimetric method developed by Chang et al. [7], employing rutin as the standard solution. Firstly, each extract and rutin were dissolved in methanol at 1 mg/mL. Subsequently, 0.5 mL of each sample or rutin solution was combined with methanol to achieve a final volume of 1.5 mL. Different concentrations of rutin (60, 80, 200, 350, and 500 µg/mL) were prepared in methanol to establish a calibration curve. After allowing the solutions to stand for 5 minutes, 0.1 mL of 10% $AlCl_3$ solution was added to each test tube. This was followed by adding 0.1 mL of CH_3COOK 1M and 2.8 mL of distilled water to the reaction mixture. The solution was then incubated at room temperature for 45 minutes, after which the absorbance was measured at 415 nm using a UV/Vis spectrophotometer. A blank solution containing 2 mL of methanol, 0.1 mL of CH_3COOK 1M, and 2.8 mL of distilled water was prepared.

Rutin solutions at 60, 80, 200, 350, and 500 µg/mL concentrations were utilized to construct the calibration curve. Triplicate measurements were conducted for each sample, and the TFC was expressed as milligrams of rutin equivalents (RE) per 100 grams of dry plant material.

2.5. Determination of total phenolic content (TPC)

The total phenolic content (TPC) of the five extracts (PLM, PLH, PLD, PLE, PLW) was assessed using the colorimetric method by Yadav and Agarwala [8], employing gallic acid as the standard. Initially, the extracts and gallic acid were dissolved in methanol to achieve a stock concentration of 1 mg/mL. Subsequently, 1 mL of each sample or gallic acid solution at various concentrations (25, 50, 75, 100, 150, and 300 µg/mL) was mixed with 1 mL of Folin-Ciocalteu phenol 1N. Following a 5-minute incubation period in darkness at room temperature, 5 mL of 2% Na_2CO_3 solution was added to each test tube. The mixture was then incubated for an additional 45 minutes in darkness at room temperature. After incubation, the absorbance of the reaction mixture was measured at 765 nm using a UV/Vis spectrophotometer. A blank solution consisting of 1 mL of methanol and 5 mL of 2% Na_2CO_3 solution was prepared.

A standard calibration curve was constructed using gallic acid to quantify the TPC. The TPC was expressed as milligrams of gallic acid equivalent (GAE) per 100 grams of dry plant material.

2.6. Evaluation of antioxidant capacity

2.6.1. Determination of total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) of the five extracts (PLM, PLH, PLD, PLE, PLW) was evaluated using the colorimetric method described by Prieto et al. [9], with modifications. Ascorbic acid served as the standard. The extracts and ascorbic acid were dissolved in methanol solvent to obtain a stock concentration of 0.35 mg/mL. Subsequently, 4 mL of each sample or ascorbic acid solution, concentrations from 21 to 77 µg/mL, was mixed with 1 mL of molybdate reagent (prepared by combining 3.24 mL sulfuric acid, 0.397g sodium sulfate and 0.494g ammonium molybdate, and diluting to 100 mL). These mixtures were then incubated for 60 minutes at 95°C, then cooled to room temperature. The absorbance of each reaction mixture was measured at 695 nm using a UV/Vis spectrophotometer. A blank solution comprising 4 mL of methanol and 1 mL of molybdate reagent was prepared. A standard calibration curve was constructed using ascorbic acid to quantify the TAC. The TAC was expressed as milligrams of ascorbic acid equivalent (AAE) per 100 grams of dry plant material.

2.6.2. DPPH free radical scavenging activity

The DPPH free radical scavenging assay was conducted on five extracts (PLH, PLD, PLE, PLW, PLM) with modifications based on the method described by Shirwaikar et al. [10]. The reaction involved mixing DPPH (0.1 mM) with the extracts in a 2:1 ratio, resulting in a color change from purple to yellow, proportional to the concentration of the samples. Ascorbic acid served as the positive control. Both the extracts and ascorbic acid were dissolved in methanol to achieve a stock concentration of 1 mg/mL. Subsequently, 4 mL of 0.1 mM DPPH solution was added to 2 mL of each sample or ascorbic acid solution at various concentrations (ranging from 10 to 100 µg/mL). Following a 40-minute incubation period in darkness at room temperature, the absorbance of the reaction mixture was measured at 517 nm using a UV/Vis spectrophotometer.

A blank sample containing 2 mL of methanol and 4 mL of 0.1 mM DPPH was prepared for background correction. The radical scavenging activity (%RSA) was calculated using the formula:

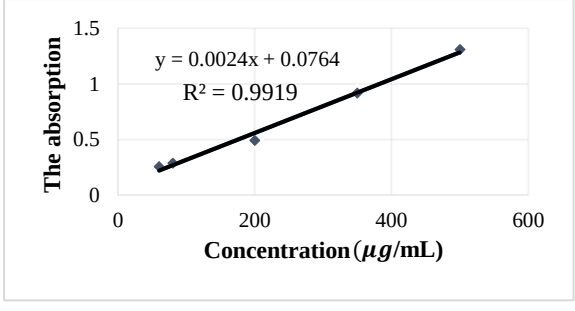
$$\%RSA = \frac{absorbance\ of\ blank - absorbance\ of\ sample}{absorbance\ of\ blank} \times 100$$

The EC_{50} value, representing the concentration of sample required to scavenge 50% of the initial DPPH radical concentration, was determined from the plotted graph of scavenging activity against the concentration of sample extracts. Triplicate measurements were performed, and the scavenging effect was expressed as the percentage of DPPH scavenged.

3. RESULTS AND DISCUSSION

3.1. Total flavonoid content

The total flavonoid content (TFC) of the five extracts was determined utilizing the standard curve equation derived from rutin (*Figure 1a*). The calibration curve for rutin exhibited a linear relationship described by the equation y = 0.0024x+ 0.076, with a coefficient of determination



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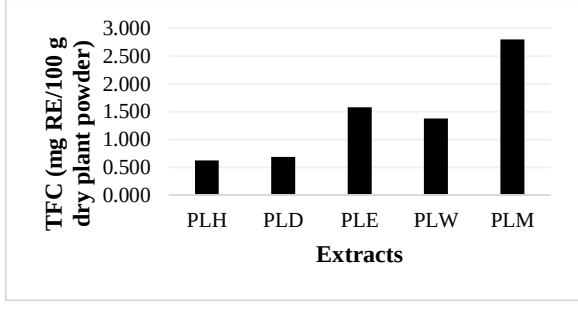

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Figure 1a. Rutin calibration curve, Figure 1b. Total flavonoid content of extracts of the aerial part of P. longum

(R^2) of 0.9919. By substituting the absorbance values of the five extracts into the equation as 'y', the TFC was calculated in milligrams of rutin equivalents per 100 grams of dry plant powder (mg RE/100 g). The results depicted in *Figure 1b* revealed significant flavonoid content of all extracts, ranging from 0.62 ± 0.00080 to 2.80 ± 0.0029 mg RE/100 g dry plant powder. The methanol extract exhibited the highest TFC value (2.80 ± 0.0029 mg RE/100 g dry plant powder). Among the four fractions, the ethyl acetate extract showed the highest TFC (1.58 ± 0.0021 mg RE/100 g dry plant powder), followed by the water extract (1.38 ± 0.0042 mg RE/100 g dry plant powder), with the *n*-hexane extract exhibiting the lowest content (0.62 ± 0.00080 mg RE/100 g dry plant powder).

This disparity in TFC can be attributed to the composition of *P. longum*, which contains flavonoid compounds with varying polarities. Compounds with strong polarity dissolve well in methanol and water, resulting in higher TFC values in corresponding extracts. Conversely, flavonoids with moderate polarity are also present, allowing for efficient extraction by ethyl acetate despite its medium polarity, thus contributing to the relatively higher TFC observed in this extract compared to the *n*-hexane fraction [11].

The total phenolic content (TPC) of the PLM, PLH, PLD, PLE, and PLW extracts was assessed using the standard curve of gallic acid (*Figure 2a*). The calibration curve for gallic acid followed a linear trend described by the equation y = 0.0121x +0.2746, with a coefficient of determination (R^2) of 0.9957. The TPC values of the extracts (expressed in mg GAE/100 g dry plant powder) are shown in *Figure 2b*. The TPC ranged from 0.01 ± 0.00064 to 1.11 ± 0.00032 mg GAE/100 g dry plant powder. Like the TFC results, the methanol extract exhibited the highest TPC (1.11 ± 0.00032 mg GAE/100 g dry plant powder). Among the four fractions, the ethyl acetate extract (PLE) showed the highest total phenolic content (0.52 ± 0.0016 mg GAE/100 g dry plant powder). The variation in TPC among the five extracts can be attributed to the solubility of phenolic compounds in solvents with different polarities [12, 13]. Previous studies have indicated that the solubility of phenolic compounds is influenced by the polarity of the solvent. Methanol is recognized as one of the most effective solvents for plant phenolic extraction [14, 15]. The higher TPC in the PLE and PLM extracts, which possess moderate polarity (P = 4.4 and P = 5.1, respectively), compared to the water extract with strong polarity (P = 10.2), proved that ethyl acetate is a solvent with reasonable polarity for effective extraction of phenolic compounds.

3.2. Total phenolic content

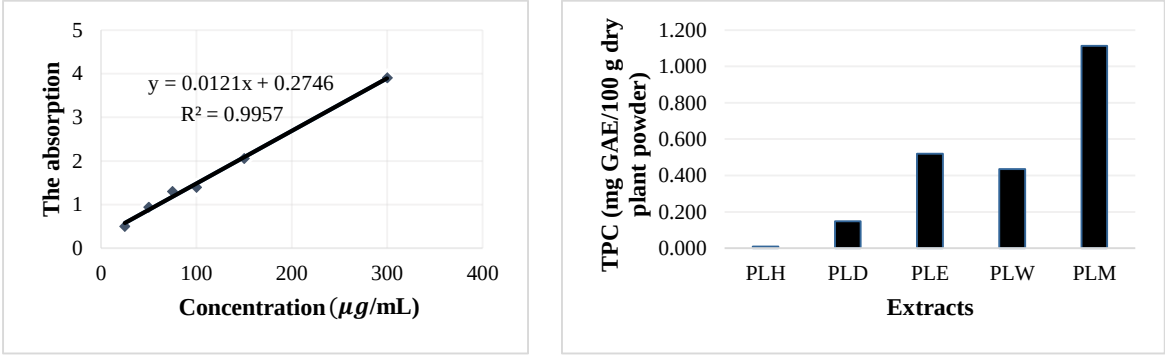

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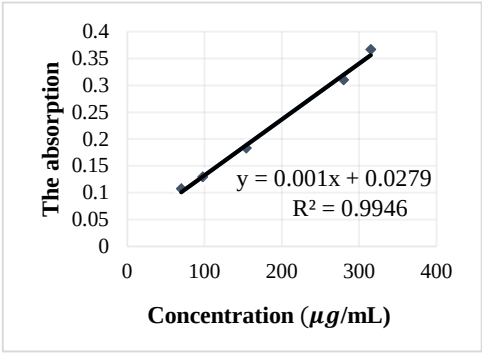
Figure 2a. Gallic acid calibration curve, Figure 2b. Total phenolic content of extracts of the aerial part of P. longum

3.3. Antioxidant capacity

3.3.1. Total antioxidant capacity

The total antioxidant capacity (TAC) of the five extracts was determined utilizing the standard curve derived from ascorbic acid (*Figure 3a*). The curve equation for ascorbic acid was y = 0.001x +

0.0279, with a coefficient of determination (R^2) of 0.9946. By substituting the absorbance values of the five fractional extracts into 'y', the TAC was calculated in milligrams of ascorbic acid equivalents per 100 grams of dry plant powder (mg AAE/100 g). The results in *Figure 3b* and *Table 1* indicated varying antioxidant capacity levels of all extracts, with the methanol extract exhibiting the highest antioxidant capacity. The TAC ranged from 10.70 ± 0.096 to 20.68± 0.042 mg AAE/100 g dry plant powder. Among the four fractions, the ethyl acetate extract (PLE) demonstrated the highest total antioxidant capacity, ranging from 5.82 ± 0.081 to 18.11± 0.071 mg AAE/100 g dry plant powder (*Table 1*). These findings corroborated the results of total phenolic and flavonoid content analyses (*Figure 1* and *Figure 2*).



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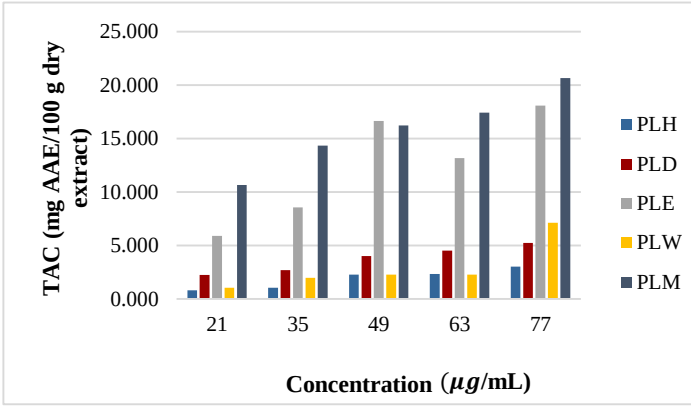

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Figure 3a. Ascorbic acid calibration curve, Figure 3b. Total antioxidant capacity of extracts of the aerial part of P. longum

Table 1. Total antioxidant capacity of the extracts of the aerial part of P. longum

Extraction part	TAC (mg AAE/100 g dry plant powder)				
	21 μg/mL	35 μg/mL	49 μg/mL	63 μg/mL	77 μg/mL
PLH	0.89± 0.094	1.08± 0.041	2.32± 0.027	2.39± 0.056	3.01± 0.041
PLD	2.27± 0.046	2.76± 0.056	4.05± 0.031	4.08± 0.092	5.25± 0.041
PLE	5.82 ± 0.081	8.64 ± 0.080	16.74 ± 0.086	13.25 ± 0.081	18.11± 0.071
PLW	1.11± 0.077	2.00± 0.046	2.30± 0.062	2.35± 0.092	7.19± 0.067
PLM	10.70 ± 0.096	14.36 ± 0.041	16.26 ± 0.015	17.49 ± 0.056	20.68 ± 0.042

3.3.2. DPPH free radical scavenging activity

The antioxidant capacity evaluated using DPPH reagents is based on the single electron transfer proton transfer (SET-PT) mechanism, in which electrons are transferred from polyphenolic compounds to free radicals, followed by proton transfer [16]. This mechanism exhibits the ability to transfer electrons and is influenced by factors such as ionization energy and solvent polarity. Therefore, to effectively mimic the properties of polyphenol oxidation reactions, we investigated the antioxidant activity using solvents of different polarities. The ability of DPPH to scavenge free radicals via the SET-PT mechanism is demonstrated by the color change from purple to yellow, facilitating rapid evaluation of antioxidant activity. The DPPH radical scavenging ability of five extracts, PLM, PLH, PLD, PLE, and PLW, was evaluated. The EC_{50} values of all extracts are shown in Table 1. As observed, all extracts exhibited DPPH radical scavenging ability with EC_{50} values ranging from 0.205 to 0.388 mg/mL. In particular, PLE extract showed the most potent antioxidant activity, with an EC_{50} value of 0.205 mg/mL, approximately 3.6 times that of the

positive control ascorbic acid (0.0566 mg/mL). PLH showed the lowest antioxidant activity with an EC_{50} value of 0.388 mg/mL. These results are consistent with previous studies highlighting the influence of solvent polarity on antioxidant capacity [17].

Table 2. DPPH radical scavenging activity of the extracts of the aerial part of P. longum

Extraction part	DPPH radical scavenging activity (%)					EC_{50} (mg/mL)
	0.05 mg/mL	0.075 mg/mL	0.1 mg/mL	0.2 mg/mL	0.3 mg/mL	
PLH	6.738	10.550	12.891	25.781	38.671	0.388
PLD	10.547	15.527	19.922	37.598	57.324	0.268
PLE	15.039	21.289	26.758	48.926	70.996	0.205
PLW	13.281	19.922	24.512	45.313	59.570	0.246
PLM	16.699	22.949	21.680	33.105	44.434	0.348

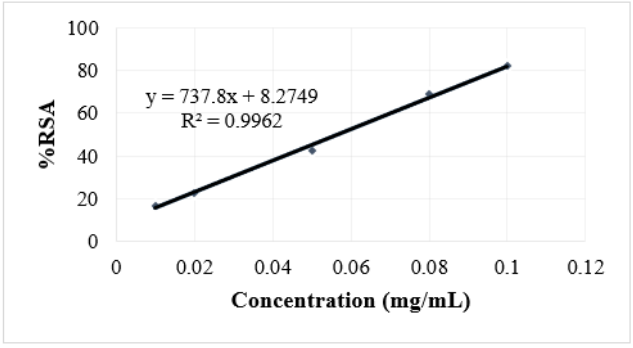

Figure 4. DPPH radical scavenging activity of ascorbic acid

4. CONCLUSION

The methanol extract and all fractions of *P. longum S*were found to contain flavonoids and phenolics, showing significant antioxidant activity. There was a close correlation between the total phenolic and total flavonoid content and the antioxidant activity of the extracts. The PLE extract exhibited the highest content of total flavonoids (1.58 ± 0.0021 mg RE/100 g dry plant powder) and total phenolics (0.52 ± 0.0016 mg GAE/100 g dry plant powder). It possessed the most potent antioxidant capacity among the four fractions, with the total antioxidant capacity ranging from 5.82 ± 0.081 to 18.11 ± 0.071 mg AAE/100 g dry plant powder and an EC_{50} value of 0.205 mg/mL. Thus, ethyl acetate emerged as the most suitable solvent for extracting phenolics and flavonoids from *P. longum*.

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