

Antioxidant activity of *Piper cubeba* L. berries crude extracts and its fractions

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Article history:

Received: 11 September 2021

Received in revised form: 18 October 2021

Accepted: 19 November 2021

Available Online: 3 August 2022

Keywords:

Antioxidant activity,
DPPH radical scavenging,
Piper cubeba L.,
Total Phenolic Content (TPC)

DOI:

[https://doi.org/10.26656/fr.2017.6\(4\).611](https://doi.org/10.26656/fr.2017.6(4).611)

Abstract

Traditionally, Cubeb (*Piper cubeba* L.) berries have been used as a spice or medicine due to their high phytochemical compounds. An earlier study has reported on the analysis of its pharmacological properties such as anti-inflammatory and antibacterial activity. Nevertheless, the antioxidant activity of *P. cubeba* L. berries extracts and their fractions have not been widely reported. This study aimed to determine the antioxidant activity of *P. cubeba* L. berries crude extracts and their fractions. Ethanol and methanol solvent was chosen to extract crude *P. cubeba* L. berries. Moreover, the active crude extract was fractionated using liquid-liquid partition methods into hexane, chloroform and ethyl acetate fractions. Antioxidant activity was determined by total phenolic content (TPC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The results showed that the TPC of ethanolic and methanolic crude extracts were 0.66±0.02 mg GAE/g and 0.59±0.01 mg GAE/g, respectively. For DPPH radical scavenging, ethanolic and methanolic *P. cubeba* L. berries, crude extracts showed IC₅₀ of 546.14±3.28 µg/mL and 472.43±5.21 µg/mL, respectively. In addition, methanolic crude extracts yield hexane, chloroform and ethyl acetate fractions which have TPC values of 0.45±0.02 mg GAE/g, 0.69±0.12 mg GAE/g and 0.86±0.02 mg GAE/g, respectively. On the other hand, DPPH values IC₅₀ give a result of 3.62±0.24 mg/mL, 197.08±0.36 µg/mL, 98.45±0.57 µg/mL to hexane, chloroform and ethyl acetate fractions, respectively. Based on the findings, *P. cubeba* L. crude extracts and their fractions showed high antioxidant activity. Thus, *P. cubeba* L. crude extracts and their fractions can be potentially developed as a natural antioxidant agent to replace synthetic antioxidants.

1. Introduction

In the last few decades, medicinal plants and herbs have been studied by scientists for their many biological properties (Kumar *et al.*, 2011). Medicinal plants are important sources of food, functional food or drugs and it has promising bioactive compounds to prevent or treat health problems (Newman and Cragg, 2010; Lokhande *et al.*, 2007). Moreover, the use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has been widely observed (Sofowora *et al.*, 2013). Recently, many health-related diseases involve free radicals (Ahmad, Fazal, Ahmad *et al.*, 2011). In the body, free radicals will attack cellular membranes which lead to lipid oxidation, decomposition of biofilms fluidity, forfeit different enzyme activities, forfeit receptor

activity, and cell inactivation with the destroyed proteins in the membranes (Kochhar, 2008; Ahmad, Fazal, Abbasi *et al.*, 2011). Free radicals are the pathogenic factors of cancer where they attack cells' DNA, which causes mutations and eventually induces cancer (Ahmad *et al.*, 2010; Abdullahi *et al.*, 2011). Therefore, natural antioxidants were used to cure cell degeneration due to their low side effects (Obinna *et al.*, 2009; Ahmad, Fazal, and Abbasi, 2011). *P. cubeba* L. or tailed pepper, often called *kemukus* in Indonesia, belongs to Piperaceae family, *Piper* genus. It is mostly grown in Java, Southern Borneo and Sumatra, and other isles in the Indian Ocean. This plant has been used in many countries as a spice, including Europe (in the Middle Ages), Indonesia, Morocco and India. *P. cubeba* L. berries have been used in traditional medicines to treat dysentery, gonorrhoea, abdominal pain, asthma, syphilis, enteritis and diarrhoea

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(Graidist et al., 2015). *P. cubeba* L. also is used as a protective and therapeutic agent for various kidney diseases in Unani medicine (Ahmad et al., 2012). *P. cubeba* L. extracts have various biological activities, like anti-type IV allergic (Choi and Hwang, 2003), anti-hepatitis C virus (Januario et al., 2002), anti-leishmanial (Bodiwala et al., 2007), anti-inflammatory (Choi and Hwang, 2003; Perazzo et al., 2013), anti-proliferative (Yam et al., 2008) and anti-genotoxic activities (Junqueira et al., 2007). Lignans, alkaloids and essential oil have been found in dried *P. cubeba* L. berries (Usia et al., 2006). Hence, the study is to determine the antioxidant activity of *P. cubeba* L. berries crude extracts and their active fractions in terms of total phenolic content and DPPH antioxidant assay.

2. Material and methods

2.1 Plant material and extraction

Piper cubeba L. berries were purchased from the traditional herbal market, Bandung, Indonesia and deposited in Natural Medicines and Product Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Malaysia) and deposited at Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia, Malaysia. *P. cubeba* L. extraction procedure was conducted according to the methods described in Rukayadi et al. (2008). Two types of organic solvents with different polarities were used in the extraction of *P. cubeba* L., which were absolute methanol (99.8%) (R and M Chemicals, UK) and absolute ethanol (99.8%) (R and M Chemicals, UK). Summarily, 100 g of dried berries of *P. cubeba* L. was pulverized to a coarse powder. The samples were extracted with 400 mL of each methanol and ethanol solvent for 48 hrs at room temperature with occasional shaking. Whatman filter paper No.2 (Whatman International Ltd., Middlesex, England) was used to filter the plant extracts. A rotary vacuum evaporator (BUCHI Rotavapor R-200, Switzerland) was used to concentrate the extracts at 40°C for three to four hours whereby yielded two crude extracts methanol and ethanol of dried *P. cubeba* L. berries. All the extracts were subjected to freeze-drying for 48 hrs to eliminate water. The yield of crude extracts from *P. cubeba* L. was calculated with the formula below:

$$\text{Yield (\%)} = \frac{W2 - W1}{W0} \times 100$$

Where *W2* is the weight of the extract and container, *W1* is the weight of the empty container and *W0* is the weight of the initial dried sample.

2.2 Liquid-liquid partition of *Piper cubeba* L. berries extract

The liquid-liquid partition was determined using the method reported by Raja Mazlan et al. (2018). The 20 g of selected methanol crude extract of *P. cubeba* L. was mixed with 100 mL of methanol and 200 mL of water. A 1 L separating funnel was used to mix the methanol-water mixture. Subsequently, 500 mL of n-hexane was added and the solution was gently swirled to obtain two layers. The upper layer which was n-hexane was collected while the aqueous methanol layer was left in the funnel. This procedure was repeated several times until the fraction became light in colour. The same process was repeated using chloroform and ethyl acetate. All obtained fractions were evaporated using the rotary vapour, freeze-dried, for further analyses.

2.3 Antioxidant activity determination

2.3.1 Total phenolic content (TPC)

The TPC of the extracts was determined using the method reported by Sembiring et al. (2018). 25 µL of *P. cubeba* L. extract (10 mg/mL) was mixed with 100 µL of 25% diluted Folin–Ciocalteu reagent and shook for 1 min in 96-well flat-bottom microplate. Approximately 75 µL of 10% sodium carbonate was added after 4 mins. The solvents were left to incubate at room temperature for 2 hrs and the absorbance was measured at 750 nm in the spectrophotometer (UV-1650PC, Shimadzu, Tokyo, Japan). The TPC was determined from the linear equation of the standard curve prepared with gallic acid. The amount of the total phenolic content of *P. cubeba* L. was expressed as mg/g gallic acid equivalent (GAE) as shown in the formula below:

$$C = \frac{C1 \times V}{m}$$

Where *C* is the total phenolic content in mg/g, in GAE (Gallic acid equivalent), *C1* is the concentration of Gallic acid established from the calibration curve in mg/mL, *V* is the volume of extract in mL, and *m* is the weight of the plant extract in g.

2.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of *P. cubeba* L. extract was evaluated with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. This method was carried out according to Wan et al. (2018) with some modifications. A volume of 200 µL of sample extract and standard with different concentrations (5.00, 2.50, 1.25, 0.63, 0.31 and 0.15 mg/mL) was added to the same volume of DPPH methanolic solution (200 µL). The mixture was shaken and left to be incubated for 30 mins in a dark room at room temperature. The absorbance at

517 nm was measured using TECAN, Infinite F200 Pro. The inhibition percentage of DPPH discolouration was calculated using the equation:

$$\text{Percentage Inhibition} = \frac{(\text{Abs sample} + \text{DPPH}) - (\text{Abs sample blank})}{(\text{Abs DPPH} - \text{Abs solvent})} \times 100$$

Whereby, abs sample + DPPH is the absorbance of the sample with DPPH mixture, abs sample blank is the absorbance of methanolic blank solvent, abs DPPH is the absorbance of blank DPPH solvent and abs solvent is the absorbance of DPPH mixed with a methanolic solvent.

Next, the concentrations of adsorbed DPPH free radical from the extracts were calculated by distinctions in the concentration of DPPH in the presence and absence of the extracts.

$$\text{DPPH adsorption (\%)} = \frac{C1 - C2}{C2} \times 100$$

Where, C1 is the concentration of mixture without DPPH solution and C2 is the concentration of mixture including extract and DPPH solution.

2.4 Statistical analysis

Minitab software (version 16.0) was used to analyse the data for the analysis of variance (one-way ANOVA). Tukey's test was used to determine the significant difference ($P < 0.05$). Data expressed as means of duplicates \pm standard deviations were carried out using Excel software.

3. Results and discussion

3.1 Yield of *Piper cubeba* L. berries extracts

The first step to separating the desired natural products from medicinal plants is extraction. According to the extraction principle, some of the extraction methods include solvent extraction, distillation, pressing and sublimation. Extraction generally yields the desired chemical compounds/components which are then subjected to further separation, purification and characterization (Sasidharan *et al.*, 2011). As the selection of solvent is vital for proper extraction, solvents should be chosen based on the desired end product. Hence, various solvents of different polarities used during maceration should be studied prior to deciding on the appropriate solvents. The extraction method undergoes four stages, first, the solvent penetrates the solid matrix, it then dissolves the solute to be distributed out of the solid matrix and finally ends in the collection of an extract (Zhang *et al.*, 2015). Factors directly affecting the quality and the efficiency of an extraction process include temperature, the particle size of the raw materials, duration of the process and the solvent-to-solid ratio. Whereby the greater the solvent-to-

-solid ratio will produce the greater the yield of extractions (Zhang *et al.*, 2015). In this study, the dried *P. cubeba* L. berries were extracted by using solvent extraction (maceration). The extraction underwent two extraction solvents absolute methanol and ethanol, which displayed crude extracts of dark brown and viscous liquid. The yields of the extraction are shown in Table 1, the highest percentage of yield obtained per 100 g of dried berries was observed in methanol extract with 19.03% followed by 12.95% for ethanol. There are no statistically significant differences in percentage yields of the crude extracts affected by different solvents used.

Generally, methanol is proven to be more effective in the extraction of lower molecular weight polyphenols. In addition, ethanol is a good solvent for the extraction of polyphenol and is safer for human consumption (Dai and Mumper, 2010; Do *et al.*, 2014). Plants extracted with methanol had reports of higher yield obtained compared to those extracted with ethanol (Cvetanović *et al.*, 2017). Methanolic extract depicts potent antimicrobial activities when compared to other aqueous extracts (Mujeeb *et al.*, 2014).

Table 1. Yield of *P. cubeba* L. berries extract with different solvent extraction

Dried berries (g)	Solvent extraction	Extraction step	Yield (g)	Percentage of yield (%)
100	Methanol	First extraction	19.03	19.03
400	Methanol	Second extraction	79.68	19.92
100	Ethanol	First extraction	12.95	12.95

3.2 Yield of liquid-liquid partition of *Piper cubeba* L. extracts

Liquid-liquid partition was conducted to separate various compounds in two immiscible solvents based on the compounds' polarity. Methanol crude extract of *P. cubeba* L. was selected to further fractionate by liquid-liquid partition since this extract gave consistent yield and is suitable for extracting polyphenols compound in plant-based material (Addai *et al.*, 2013). In addition, a study reported that the phytochemical compounds from plants are more soluble in polar organic solvent (Erlina *et al.*, 2012). In Table 2, 50 g of methanol extract was partitioned into three fractions hexane, chloroform and ethyl acetate. Methanol crude extract partitioned with hexane yielded the highest percentage of 69.78% followed by chloroform 22.51% and ethyl acetate 3.14%. There are no statistically significant differences in percentage yields of the fractions affected by different solvents used.

Table 2. Yield of liquid-liquid partition of methanolic *P. cubeba* L. berries extract

Methanol extract (g)	Solvent	Yield (g)	Percentage of yield (%)
50	n-Hexane	34.89	69.78
	Chloroform	11.25	22.51
	Ethyl acetate	1.57	3.14

3.3 Antioxidant activity of *Piper cubeba* L. berries extracts

3.3.1 Total phenolic content of crude extract

Phenols and polyphenols are secondary metabolites, which are produced by all types of plants. The health benefits derived from the phenolic compounds are attributed to antioxidant activity. In the Folin-Ciocalteu assay, the reducing activity of phenolic was evaluated from its transferring electron ability. The blue compounds formed between phenolic and Folin-Ciocalteu reagent were proportional to the concentration of all phenolics in the extracts and were independent of the structure of phenolic compounds (Kumar *et al.*, 2015). Table 3 shows the determination results of total phenolic content in 5 mg/mL of methanol and ethanol extracts from *P. cubeba* L. berries.

Table 3. Total phenolic content for *P. cubeba* L. berries crude extract

Equation	R ²	Total Phenolic Content (mg GAE/g)	
		Methanol extract	Ethanol extract
$y = 0.0076x + 0.0491$	0.9973	0.59±0.01 ^a	0.66±0.02 ^a

Values are presented as mean±SD of replications (n = 3). Values with different superscript are significantly different at P<0.05 Tukey's range test.

The total phenolic content was reported as gallic acid equivalent and the TPC was varied between extraction processes. The total phenolic contents of ethanolic crude extracts of *P. cubeba* L. berries were 0.66±0.02 mg GAE/g higher compared to methanolic crude extracts at 0.59±0.01 mg GAE/g. It is reported that the cubeb berries contain volatile oil consisting of monoterpenes (sabinene 50%, α -thujene, and carene) and sesquiterpenes (caryophyllene, copaene, α - and β -cubebene, δ -cadinene, germacrene), the sesquiterpenes 1,4- and 1,8-cineole (Ahmed and Hameed, 2017). Long extraction time can affect the release of the tightly bound compounds and improve the efficiency of extraction thus potentially increasing the total phenolic content (Kusumawati *et al.*, 2018). The major factors responsible for the antioxidant activity of phenolic compounds are their chemical structure and redox properties, which allow them to scavenge free radicals, chelate transitional metals, and inhibit lipoxygenase (Park and Kim, 2017).

There are no statistically significant differences in the total phenolic content of the crude extract affected by different solvents used.

3.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of crude extract

Stable free radical DPPH has been widely used to determine primary antioxidant activity (Ahmad *et al.*, 2012; Graidist *et al.*, 2015). DPPH free radical method is a simple, sensitive and rapid method to investigate the antioxidant activity of specific compounds or plant extracts (Choi and Hwang, 2005). It is acceptable that antioxidants scavenge DPPH free radicals because of their ability to supply hydrogen (Perazzo *et al.*, 2013). Antioxidants are reagents that completely stop or delay the oxidation process. The chain reaction of oxidation is blocked by antioxidant compounds (Januario *et al.*, 2002; Bodiwala *et al.*, 2007).

DPPH assay is considered a valid method to evaluate the free radical scavenging activity of antioxidants in the terms of hydrogen donating or radical scavenging ability since the radical compound is very stable and does not have to generate free radicals as in other radical assays. DPPH radicals react with suitable reducing agents, then electrons become paired off, and the solutions lose colour stoichiometrically with the number of electrons taken up. Such reactivity has been widely used to test the ability of plant extract to act as free radical scavengers (Subha *et al.*, 2018). The activity was evaluated by the decrease in absorbance as the result of the DPPH colour change from purple to yellow. The higher the sample concentration used, the stronger the free radical-scavenging effect.

Piper cubeba L. extracts collected exhibited significant DPPH free radical scavenging activity at different concentrations. Thus, the inhibiting concentration and IC₅₀ were calculated and the results are displayed in Table 4. The crude extracts of methanol and ethanol showed significant DPPH radical scavenging activity, with inhibition of 79.4% and 70.3% at 1.25 mg/mL, respectively. With regard to IC₅₀ value, ethanol crude extract (546.14 μ g/mL) showed a lower value than crude methanol extract (472.43 μ g/mL). There are no statistically significant differences in the percentage of inhibition and IC₅₀ of the crude extract with different solvents used.

In comparison, it implies that the fractions possessed radical scavenging activity and are capable of inhibiting and mopping up free radicals in the system. The reducing power assay with an increase in absorbance indicates an increase in antioxidant potential. Moreover, the reducing capacity is attributed to the reductants' availability and is

therefore based on its ability to break radical chains free by collectively donating hydrogen atoms (Subramanian *et al.*, 2013). A correlation was found between TPC and IC₅₀, when the TPC level was high, the IC₅₀ value was low.

Table 4. DPPH free radical scavenging for *P. cubeba* L. berries crude extract

Sample	DPPH free radical scavenging activity		
	R ²	Inhibition (%)	IC ₅₀ (µg/mL)
Methanol crude	0.992	79.4±2.34 ^a	472.43±5.21 ^a
Ethanol crude	0.991	70.3±4.67 ^a	546.14±3.28 ^a
Gallic acid	0.988	58.28±0.86	0.42±0.48

Values are presented as mean±SD of replications (n = 3). Values with different superscript within the same row are significantly different at P<0.05 Tukey's range test.

Several studies reported the ability of essential oils from rhizomes to inhibit free radicals is directly proportional to their contribution to their major compounds such as phenolic acids and flavonoids (Chryssavgi *et al.*, 2008). High antioxidant potency has been directly related to phenolic compounds as they are effective in inhibiting and reducing the formation of free radicals (Rezaie *et al.*, 2015). However, some plants were reported to have other compounds attributed to their antioxidant abilities such as monoterpenes, and sesquiterpenes (Aissi *et al.*, 2016). Besides that, synergistic effects from the presence of multiple compounds namely are limonene, α-pinene, β-elemene, γ-gurjunene, germacrene-B and spathulenol were associated with the antioxidant activities of *P. alantica* (Labe-Zouad *et al.*, 2017). As described by Hasheminya and Dehghannya (2020), phenolic compounds with higher molecular weight have more potential in inhibiting free radicals, as it is highly dependent on the number of aromatic rings and the nature of the hydroxyl group.

3.4 Antioxidant activity of *Piper cubeba* L. fractions

3.4.1 Total phenolic content of fractions

Total phenolic content activity is the method to figure out the amount of phenolic content in the samples. Phenolic compounds contained in the plants have redox properties, and the properties allow them to act as antioxidants. The total polyphenols in the solvent fractions were determined using Folin-Ciocalteu's reagent. The total phenolic contents of *P. cubeba* L. fractions varied between liquid-liquid partitions. The total phenolic contents of 5 mg/mL *P. cubeba* L. berries fractions were in the range between 0.45±0.02 mg GAE/g and 0.86±0.02 mg GAE/g (Table 5). Based on the data collected, the highest total phenolic content was observed to be in the ethyl acetate fraction with 0.86±0.02 mg GAE/g, followed by the chloroform

fraction which is 0.69±0.12 mg GAE/g, and the lowest total phenolic content among the other fraction was found to be in hexane fraction which is 0.45±0.02 mg GAE/g. There are no statistically significant differences in the total phenolic content of the fractions with different solvents used. The major factors responsible for the antioxidant activity of phenolic compounds are their chemical structure and redox properties, which allow them to scavenge free radicals, chelate transitional metals, and inhibit lipoxygenase (Park and Kim, 2017).

Table 5. Total phenolic content for *P. cubeba* L. berries fractions

Equation	R ²	Total Phenolic Content (mg GAE/g)		
		Hexane Fraction	Chloroform Fraction	Ethyl Acetate Fraction
y = 0.0071x + 0.9979	0.9979	0.45±0.02 ^a	0.69±0.12 ^a	0.86±0.02 ^a

Values are presented as mean±SD of replications (n = 3). Values with different superscript are significantly different at P<0.05 Tukey's range test.

3.4.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay of fractions

The DPPH (2,2-diphenyl-1-picrylhydrazyl radical) assays are used as a tool for the *in vitro* evaluation of fractions and have been widely used to determine the free radical-scavenging activity of various plants where its results can indicate the presence of phenolic and flavonoids compounds in plant extracts. The DPPH IC₅₀ value is the concentration of the sample required to inhibit 50% of radical for each fraction of methanol extract *P. cubeba* L. Table 6 shows the percentage of inhibition of methanolic crude extracts *P. cubeba* L. berries which were fractionated into three fractions, hexane, chloroform and ethyl acetate fraction by using DPPH radical scavenging ability where each exhibited antioxidant activities at different magnitudes of potency.

Table 6. DPPH scavenging for *P. cubeba* L. berries fractions

Sample	DPPH free radical scavenging activity		
	R ²	Inhibition (%) (1.25 mg/mL)	IC ₅₀ (µg/mL)
Hexane fraction	0.992	21.65±0.52 ^a	3.614±0.24 ^a
Chloroform fraction	0.992	87.07±0.67 ^b	0.197±0.36 ^b
Ethyl acetate fraction	0.952	93.84±0.84 ^b	0.098±0.57 ^b
Gallic acid	0.988	58.28±0.86	0.42±0.48

Values are presented as mean±SD of replications (n = 3). Values with different superscript within the same row are significantly different at P<0.05 Tukey's range test.

Piper cubeba L. fractions exhibited significant DPPH free radical scavenging activity at different concentrations. Therefore, the inhibiting concentration and IC₅₀ were calculated. The results are displayed in Table 6. The most active fraction among the three

fractions was ethyl acetate with the inhibition of 93.84% at 1.25 mg/mL of DPPH radical scavenging activity. In addition, the values of hexane and chloroform DPPH free radical inhibition were 21.65% and 87.07%, respectively. As for the IC₅₀, hexane, chloroform and ethyl acetate with the values of 3.614±0.24 mg/mL, 0.197±0.36 mg/mL and 0.098±0.57 mg/mL, respectively. There are no statistically significant differences between the percentage of inhibition and IC₅₀ of hexane, chloroform and ethyl acetate fraction whereas there are significant differences between the percentage of inhibition and IC₅₀ of different solvents used between hexane and chloroform and ethyl acetate. This implies that chloroform and ethyl acetate fractions are better than hexane fractions. The DPPH assay provided an easy and rapid way to determine the antioxidant activity of most of the substances tested in this study. It was found the ethyl acetate fraction has the highest percentage of inhibition and lowest IC₅₀ compared to another fraction.

4. Conclusion

Cubeb (*P. cubeba* L.) berries extracts and active fractions were scientifically reported to have numerous beneficial phytochemicals that proved to have high antioxidant activity. The results of antioxidant activity indicate higher free radical scavenging activity in methanolic crude extracts and active fraction ethyl acetate of *P. cubeba* L. due to the presence of phytochemical constituents, especially polyphenols. It's recommended that *P. cubeba* L. berries extract could be further exploited for food preservation. This experiment also supports that *P. cubeba* L. berries can be used in pharmaceutical industries as natural antioxidants.

Conflict of interest

The authors declare no conflict of interest.

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