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IDENTIFICATION OF CHEMICAL CONSTITUENTS AND EVALUATION OF BIOLOGICAL ACTIVITIES OF *Piper cubeba* EXTRACTS

Bihter ŞAHİN*, Department of Chemistry and Chemical Processing Technologies, Susurluk Agriculture and Forest Vocational School, Bandırma Onyedi Eylül University / Balıkesir, Türkiye, bsahin@bandirma.edu.tr
(https:// ORCID: https://orcid.org/0000-0001-8657-052X)
Emine BAŞTÜRK, Department of Chemistry and Chemical Processing Technologies, Susurluk Agriculture and Forest Vocational School, Bandırma Onyedi Eylül University / Balıkesir, Türkiye, eminebasturk12052003@gmail.com
(https:// orcid.org/0009-0001-3599-1065)

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*Corresponding author
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Abstract

Piper cubeba L., commonly known as cubeb, is a medicinal plant known for its essential oil and phenolic compounds with various biological activities. In this study, fruits of P. cubeba collected from Gaziantep were analyzed for their chemical composition and bioactivity. The essential oil, obtained via hexane extraction and analyzed by GC-MS, contained 49 compounds, with Eudesma-3,7(11)-diene (31.00%) as the major component, followed by cubenol, β-cubebene, and others. Sequential solvent extraction (acetone, ethyl acetate, methanol, and water) was performed, and the phenolic profiles of the extracts were determined using HPLC-DAD. The major phenolics identified included 1,4-dichlorobenzene, curcumin, cynarin, and rutin. Antioxidant activities were evaluated using DPPH, ABTS, CUPRAC, and β-carotene-linoleic acid assays. The water extract (PC4) showed the strongest antioxidant capacity among all extracts. Acetylcholinesterase, butyrylcholinesterase, and tyrosinase inhibition assays revealed that the acetone extract (PC1) exhibited the highest enzyme inhibitory activity. This comprehensive study provides new insights into the chemical composition and biological potential of P. cubeba fruits collected from Turkey.

Keywords: *Piper cubeba*, Phenolic compounds, Chemical content, Enzyme inhibition activity

Piper cubeba EKSTRELERININ KIMYASAL BILEŞENLERININ BELIRLENMESI VE BIYOLOJIK AKTIVITELERININ DEĞERLENDIRILMESI

Özet

Piper cubeba L., yaygın olarak kebabe veya kebabiye olarak bilinen, uçucu yağı ve fenolik bileşikleriyle çeşitli biyolojik aktivitelere sahip tıbbi bir bitkidir. Bu çalışmada, Gaziantep'ten toplanan P. cubeba meyvelerinin kimyasal bileşimi ve biyolojik aktiviteleri incelenmiştir. Hekzan ekstraksiyonu ile elde edilen uçucu yağ, GC-MS ile analiz edilmiş ve Eudesma-3,7(11)-dien (%31,00) majör bileşik olarak tespit edilmiştir. Diğer önemli bileşikler arasında cubenol, β-cubebene ve τ-cadinol yer almaktadır. Aseton, etil asetat, metanol ve su ile sırasıyla yapılan ekstraksiyon sonucunda elde edilen ekstrelerin fenolik içerikleri HPLC-DAD ile analiz edilmiştir. Başlıca tespit edilen fenolik bileşikler arasında 1,4-diklorobenzen, kurkumin, sinarin ve rutin yer almaktadır. Antioksidan aktiviteler DPPH, ABTS, CUPRAC ve β-karoten-linoleik asit yöntemleriyle değerlendirilmiş; en yüksek antioksidan aktivite su ekstresinde (PC4) gözlemlenmiştir. Asetilkolinesteraz, bütirilkolinesteraz ve tirozinaz enzim inhibisyon testleri sonucunda ise en yüksek inhibitör etki aseton ekstresinde (PC1) belirlenmiştir. Bu çalışma, Türkiye'den toplanan P. cubeba meyvelerinin kimyasal ve biyolojik özelliklerini kapsamlı bir şekilde ortaya koyan ilk araştırmalardan biridir.

Anahtar Kelimeler: *Piper cubeba*, Fenolik bileşikler, Kimyasal içerik, Enzim inhibisyon aktivite

Cite

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1. Introduction

The Brazilian Java pepper, commonly known as *Cubeba pepper*, is a plant species that has been widely used since the Middle Ages. It is also a popular medicinal herb in many countries, including Arabia, Morocco, and Indonesia [1]. *Piper cubeba* L. (cubeb pepper) is native to Java, Sumatra, South Borneo, and other islands of the Indian Ocean. It is used in traditional Indonesian medicine to treat asthma, kidney disorders, abdominal pain, diarrhea, enteritis, and venereal diseases. The volatility and aromatic scent of *Piper cubeba* justify its use as a deodorant in the cosmetics, pharmaceutical,

chemical industries, and as a culinary flavoring in the food industry [2].
Some species of *Piper* are known to be used in the traditional medicine of various countries for multiple applications, including the treatment of various diseases such as antioxidant, antimicrobial, anti-inflammatory, and analgesic properties. They contain significant amounts of essential oils, polyphenolic compounds, alkaloids, and others. The phytochemical components with pharmacological activities such as antimicrobial and antioxidant activity have healing properties in many ways, including anti-inflammatory, hepatoprotective, nephroprotective, gastroprotective, and diabetic control features [2].

Several *Piper* species have long been utilized in traditional medicine across various regions of the world for diverse therapeutic purposes, including their antioxidant, antimicrobial, anti-inflammatory, and analgesic effects. These plants are rich sources of bioactive substances such as essential oils, polyphenols, and alkaloids. Due to their phytochemical composition, they demonstrate pharmacological properties associated with antimicrobial and antioxidant activities, contributing to protective roles such as anti-inflammatory, hepatoprotective, nephroprotective, gastroprotective, and antidiabetic effects [2].

Piper cubeba Linn., an aromatic member of the Piperaceae family, is commonly referred to as "cubeba" in Arabic and "piper" in English. The plant is cultivated for both its fruits and essential oil, and its geographical distribution includes Asia, Africa, and parts of North and South America. Aqueous-alcoholic extracts prepared from the seeds of *P. cubeba* exhibit low toxicity while displaying analgesic and anti-inflammatory properties, supporting their use in folk medicine [3].

The extract of *P. cubeba* is characterized by the presence of numerous biologically active compounds. One study investigated the in vitro anthelmintic potential of a hydroalcoholic fruit extract and its main isolated constituents against eggs and larvae of gastrointestinal nematodes from naturally infected livestock. The findings suggested that the observed anthelmintic activity may be more pronounced at higher concentrations of certain extract components [4].

Furthermore, the anti-inflammatory and analgesic effects of seed fractions with varying polarities have been reported. Both aqueous-alcoholic and dichloromethane fractions were shown to reduce edema formation and abdominal constriction [3]. In addition, the anticancer potential of (-)-Cubebin isolated from *P. cubeba* seeds, along with five structurally modified derivatives, was evaluated in vitro against six human cancer cell lines (A549, K562, SiHa, KB, HCT116, and HT29) using the MTT assay. Results demonstrated that Cubebin and its derivatives containing lactone and amide functionalities exhibited notable anticancer activity [5]. In a study including *P. cubeba*, the anti-allergic and analgesic activities of its fruits were examined in mice using the 2,4-dinitrofluorobenzene (DNFB)-induced contact hypersensitivity reaction (type IV) and the hot plate test [6]. In another study, the LC-HRMS/MS profiling and LC-DAD quantification of piperamides were conducted for five different Piper species (*P. longum, P. retrofractum, P. guineense, P. cubeba, and P. borbonense*) and six varieties of *P. nigrum* (black, white, green, red, Tellicherry, and Lampong). No qualitative differences were observed among *P. borbonense, P. guineense, and P. cubeba* [7].

A recent study has found that *P. cubeba* L., *Piperaceae*, and its derivative dibenzylbutyrolactone (-)-6,6′-dinitrohinokinin essential oil exhibited *in vitro* and *in vivo* activity against *Schistosoma mansoni* [8]. In light of these studies, the chemical content analysis of the essential oils obtained from *P. cubeba* (Kebabiye) fruits collected from Gaziantep was performed using GC/MS. Additionally, the phenolic components of the acetone, ethyl acetate, methanol, and water extracts were investigated. Another important aim of the study was to determine the antioxidant activities of the acetone, ethyl acetate, methanol, and water extracts using four different methods: DPPH, ABTS, CUPRAC, and *β*-Carotene linoleic acid, as well as their acetylcholinesterase and butyrylcholinesterase inhibition and tyrosinase inhibition activities. The chemical contents and bioactivities of the extracts with different polarities and the essential oil were compared. Unlike previous studies, this was evaluated within this scope.

2. Material and Method

2.1. Collection of Piper cubeba Fruits and Preparation of Study Materials

P. cubeba fruits were collected from Gaziantep. 200 g of shade-dried fruits were crushed in a grinder and prepared for analysis.

2.2. Preparation of Hexane Extract for GC/MS

After the dried *P. cubeba* fruits were prepared for analysis, they were taken using the maceration method. 4.34% yield was obtained from 200 grams of *P.* cubeba fruits. The obtained hexane extract was then made volatile by derivatization (silylation) to prepare it for analysis.

2.3. Extraction Procedures

195 g of *P. cubeba* residue, which was cut into small pieces, subjected to hexane, separated from the oily parts and removed the solvent, was treated with acetone using the sequential maceration technique and the extraction process was carried out until its color was lightened. From here, acetone extract (PC1) was obtained with a yield of 5.48%. Afterwards, sequential extraction was performed on the remaining plant residue with ethyl acetate, methanol and water solvents,

respectively, until the color was lightened (x3 times). Thus, extracts were obtained. The solvents of the resulting extracts were then removed using a rotary evaporator and stored in the refrigerator until analysis. The ethyl acetate (PC2) obtained here was obtained with a yield of 4.98%, methanol (PC3) with a yield of 5.28% and water extracts (PC4) with a yield of 5.02%.

2.4. GC-MS Analysis

The obtained hexane extract was derivatized and then analyzed using a GC-MS Varian Saturn 2100T equipped with an Rxi-5sil (DB-1 apolar column) capillary column (30m·0.25mm; coating thickness 0.25 µm). The injector temperature was set to 250°C. Helium was used as the carrier gas at a flow rate of 1 mL/min. Injection mode: the split ratio was set to 1:20, and the injected volume was 0.2 µL of oil dissolved in hexane. A method was created with a temperature program ranging from 60°C to 300°C. The pressure was set to 15.0 psi. NIST-Wiley libraries were used for the compounds in the obtained chromatogram (compared with literature).

2.5. Preparation of Samples for HPLC-DAD Analysis

The extracts were prepared by dissolving 8 mg of the substance in 1 mL of methanol. The acetone, ethyl acetate, methanol, and water extracts of *P. cubeba* fruits were homogenized in an ultrasonic bath for 5 minutes at room temperature. Afterward, the extracts were filtered through 0.45 µm PTFE filters and made ready for injection.

2.5.1. Parameters of HPLC-DAD

Phenolic composition of the acetone, ethyl acetate, methanol, and aqueous extracts of *P. cubeba* fruits was determined following the procedure reported by Tokul-Ölmez et al. [2020]. For this purpose, a Shimadzu Prominence-i LC-2030C 3D Plus high-performance liquid chromatography (HPLC) system equipped with a diode array detector (Shimadzu Corporation, Japan) was employed to analyze 42 reference phenolic standards. Chromatographic separation was achieved on a reverse-phase C_{18} column (5 µm, 4.6 mm × 250 mm) with an Inertsil C_{18} guard column, while the column oven was maintained at 35 °C. The mobile phase consisted of methanol and 0.1% aqueous acid solution, applied according to the reported gradient conditions [9]. Detection was carried out at 254 nm.

2.6. Antioxidant Activity Determination Methods

2.6.1. DPPH radical scavenging activity

The DPPH radical scavenging potential of the essential oil and the acetone, methanol, ethyl acetate, and aqueous extracts of *P. cubeba* fruits was assessed following the procedure of Blois [10], with minor modifications. A 0.4 mM DPPH solution was obtained by dissolving 16 mg of DPPH in 100 mL of methanol. Stock solutions of the extracts were first prepared, and subsequently, 40 µL of each extract (12.5-100 µg/mL), diluted in acetone, ethyl acetate, methanol, or water at varying concentrations, was added to 160 µL of freshly prepared DPPH solution in 96-well microplates. The mixtures were incubated at room temperature in the dark for 30 min, after which the absorbance was measured at 517 nm using a microplate spectrophotometer. For control measurements, 40 µL of the respective pure solvents (methanol, or water) replaced the extracts. The radical scavenging activity was then expressed by comparing the absorbance of the samples with that of the controls.

2.6.2. ABTS cation radical scavenging activity

The ABTS radical cation scavenging activity of the extracts was evaluated according to the method described by Re et al. (1989). A stock solution of $ABTS^{\bullet+}$ was prepared by dissolving 7 mM ABTS and 2.45 mM potassium persulfate in distilled water and allowing the mixture to stand in the dark for approximately 16 hours, enabling the formation of the ABTS radical cation. In this assay, 40 µL of the sample stock solutions prepared at different concentrations (12.5-100 µg/mL) were mixed with 160 µL of ABTS radical solution in a 96-well microplate. After the mixtures were incubated for a specified time at room temperature, the absorbance was recorded at 734 nm using a microplate spectrophotometer. BHA (butylated hydroxyanisole) was used as a positive control for all comparison of antioxidant capacity. The radical scavenging activity was calculated based on the decrease in absorbance compared to the control [11].

$$ABTS\ inhibition\ activity\ (inhibition\%) = A_{Control} - A_{Sample} / A_{Control} \times 100 \quad (1)$$

2.6.3. CUPRAC activity method

The cupric ion (Cu^{2+}) reducing antioxidant capacity of the *P. cubeba* essential oil and its acetone, methanol, ethyl acetate, and water extracts was determined using the CUPRAC method as described by Apak et al. [12], with slight modifications. Briefly, 40 µL of each sample solution at various concentrations (12.5-100 µg/mL) was mixed with 60 µL of ammonium acetate buffer (100 mM, pH 7.0) and 100 µL of the neocuproine–Cu(II) reagent (7.5 mM in absolute ethanol) in a 96-well microplate. The reaction mixtures were incubated at 25°C for 1 hour. After incubation, the absorbance was measured at 450 nm using a microplate reader.

The antioxidant activity was expressed in terms of the concentration required to achieve an absorbance value of 0.5 ($A_{0.5}$). BHA (butylated hydroxyanisole) was used as a positive control for comparison of antioxidant capacity.

2.6.4. β-carotene-linoleic acid decolorization activity

The antioxidant activity of the samples was also evaluated using the *β*-carotene–linoleic acid bleaching method, as described by Miller [13], with slight

modifications. A total of 0.5 mg of β-carotene, previously dissolved in 1 mL of chloroform, was combined with 25 mL of linoleic acid and 200 mg of Tween 40. Following the removal of chloroform by evaporation, 100 mL of oxygen-saturated distilled water was introduced into the mixture under vigorous agitation. For the assay, 40 µL of the sample stock solutions at different concentrations (12.5-100 µg/mL)were mixed with 160 µL of *β*-carotene solution, forming an emulsion. The emulsified mixtures were transferred to 96-well microplates, and the initial absorbance was recorded at 490 nm.

Following the initial measurement, the microplates were incubated at 45°C, and absorbance measurements were repeated every 30 minutes for approximately 120 minutes, until the *β*-carotene in the control sample was fully discolored. The bleaching rate (R) of *β*-carotene was calculated using the following formula:

$$R = \ln(ab)/t \quad (2)$$

where:

a: absorbance value measured at the initial time point (0 min),

b: absorbance value recorded after the incubation period,

t: duration of incubation, expressed in minutes,

ln: denotes the natural logarithm function.

2.7. Anticholinesterase activity

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the acetone, methanol, ethyl acetate, and water extracts of *Piper cubeba* fruits were determined using the spectrophotometric method described by Ellman et al. [14]. AChE (from Electrophorus electricus) and BChE (from horse serum) were used as the enzymes, while acetylthiocholine iodide (AcI) and butyrylthiocholine iodide (BuI) served as the substrates. The chromogenic reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to measure enzymatic activity based on the formation of a yellow color.

In each well of a 96-well microplate, 160 µL of 0.1 M phosphate buffer (pH 8.0), 10 µL of extract solution (2 mL of 2000 ppm stock), and 10 µL of the AChE or BChE (5.32×10^{-3} U) enzyme solution were added. Methanol and hexane (10 µL) were used as controls. The mixtures were incubated at 25°C for 15 minutes. Following incubation, 10 µL of DTNB (0.5 mM) and 10 µL of either AcI or BuI (0.71 mM) were added to each well, and the absorbance was measured kinetically at 412 nm over a 10-minute period.

This study represents the first comprehensive evaluation of *P. cubeba* fruits collected from Gaziantep in terms of their cholinesterase inhibitory potential.

2.8. Tyrosinase enzyme inhibition activity

The tyrosinase inhibitory activity of *Piper cubeba* fruit extracts was determined using a spectrophotometric method [15]. Tyrosinase from mushroom was used as the enzyme, and L-DOPA served as the substrate. The ability of the extracts to inhibit tyrosinase was evaluated using mushroom-derived tyrosinase, with kojic acid serving as reference inhibitor.

In microplate wells, 150 µL of 0.005 M phosphate buffer (50 mM, pH 6.8), 10 µL of stock solutions of *P. cubeba* fruit extracts at various concentrations, and 20 µL of mushroom tyrosinase enzyme (333 units/mL) were added sequentially. The mixtures were mixed for 3 minutes and then incubated at 37°C for 10 minutes.

After incubation, 20 µL of L-DOPA (12 mM) solution was immediately added to the wells, and the kinetic absorbance of the samples was measured at 475 nm for 10 minutes at 37°C.

3. Results and Discussion

A total of 49 compounds were identified based on the obtained GC/MS results. Among these compounds, the concentrations of 13 were calculated to be above 1% in the analysis reporting percentage composition. According to the GC/MS analysis of the essential oil, Eudesma-3,7(11)-diene was identified as the major compound with a concentration of 31.00%. This compound was followed by cubenol (8.23%), *β*-cubebene (5.40%), tau.-cadinol (4.50%), 5*β*,7*β*H,10*α*-Eudesm-11-en-1*α*-ol (3.08%), ɣ-Cadinene (3.09%), *α*-cubebene (2.77%), copaene (1.97%), *β*-caryophyllene (1.65%), ɣ-muurolene (1.59%), *β*-linalool (1.44%), and methyl 4,7-octadecadiynoate (1.39%). There is no clearly demonstrated, potent specific bioactivity for eudesma-3,7(11)-diene. However, it is known as a potentially bioactive sesquiterpene. In this study, the phenolic method was applied, and all extracts were compared with a total of 42 standard compounds. However, variations in the concentrations of phenolics and organic acids present in the acetone, methanol, ethyl acetate, and aqueous extracts were taken into consideration. The obtained results are presented in Table 1.

Table 1. Phenolic content results of acetone (PC1) extract of *P. cubeba* fruits (mg/g)

PC1	Ret Time	Calibration Equation	Concentration
4-hydroxybenzaldehid	32.925	y = 34376x + 4239,6	*tr*
2,4-dihydroxybenzaldehid	41.984	y = 41139x + 16566	*tr*
Protocatechic acid	24.596	y = 65753x - 6932,1	*tr*
Vanilic acid	34.489	y = 66764x + 46508	*tr*
p-coumaric acid	40.477	y = 17265x + 343183	*tr*
Prophylgallate	47.152	y = 29731x - 12781	0.11
Rutin	47.675	y = 47899x + 56096	0.26
Trans-2-hydroxycinnamic acid	48.024	y = 53442x + 104662	*tr*
Trans cinnamic acid	55.388	y = 88190x + 158733	*tr*
4-hydroxyresorcinol	72.753	y = 2389,3x + 109133	11.28
1,4-dichlorobenzene	74.038	y = 7482,7x + 100774	4.93

*_tr_: trace compound

According to the phenolic content results of the PC1 extract analyzed by HPLC-DAD, the main detected compounds were 4-hydroxyresorcinol (11.28 mg/g), 1,4-dichlorobenzene (4.93 mg/g), rutin (0.26 mg/g), and propyl gallate (0.11 mg/g). Additionally, 4-hydroxybenzaldehyde, 2,4-dihydroxybenzaldehyde, protocatechuic acid, vanillic acid, p-coumaric acid, *trans*-2-hydroxycinnamic acid, and *trans*-cinnamic acid were identified as trace components. The HPLC-DAD chromatogram of PC1 is presented in Figure 1.

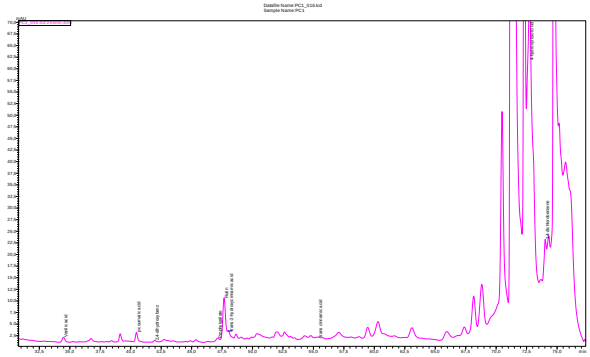

Figure 1. HPLC-DAD chromatogram of PC1 extract at 254 nm wavelength

The results obtained are included in Table 1 and the chromatogram given in Figure 1.

Table 2. Phenolic content results of ethyl acetate (PC2) extract of *P. cubeba* fruits (mg/g)

PC2	Ret. time	Calibration Equation	Concentration
Pyrocatechol	24.601	y = 3772,8x + 23692	*tr*
Theophylline	29.811	y = 36694x + 68674	*tr*
Catechin	30.653	y = 2611,2x + 74392	*tr*
Epicatechin	34.491	y = 2097,6x + 7998,2	0.21
p-coumaric acid	40.470	y = 17265x + 343183	*tr*
Coumarin	44.886	y = 81802x + 153471	*tr*
Prophylgallate	47.131	y = 29731x - 12781	0.10
Rutin	47.659	y = 47899x + 56096	0.17
Trans cinnamic acid	57.088	y = 88190x + 158733	*tr*
Curcumin	72.746	y = 15850x + 140009	16.80
1,4-dichlorobenzene	74.040	y = 7482,7x + 100774	4.92

**tr*: trace compound

The data related to the HPLC-DAD chromatogram of PC2 are presented in Table 2. According to the results obtained from this extract, curcumin (16.80 mg/g) was the predominant compound, followed by 1,4-dichlorobenzene (4.92 mg/g), epicatechin (0.21 mg/g), rutin (0.17 mg/g), and propyl gallate (0.10 mg/g). Additionally, pyrocatechol, theophylline, catechin, p-coumaric acid, coumarin, and *trans*-cinnamic acid were identified as trace components. The HPLC-DAD chromatogram of the PC2 extract is shown in Figure 2.

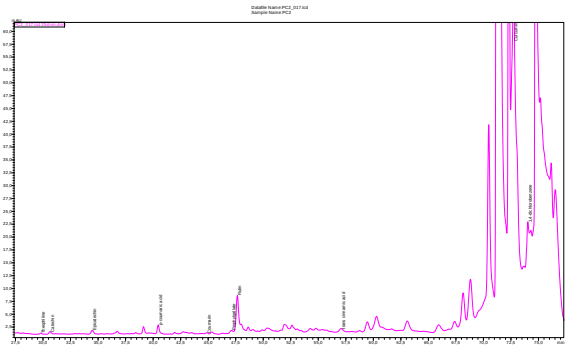

Figure 2. HPLC-DAD chromatogram of PC2 extract at 254 nm wavelength

According to the HPLC-DAD results of the PC3 extract presented in Table 3, 1,4-dichlorobenzene (8.45 mg/g) was identified as the major compound. This was followed by cynarin (5.88 mg/g), rutin (0.31 mg/g), and propyl gallate (0.14 mg/g), respectively.

Table 3. Phenolic content results of methanol (PC3) extract of *P. cubeba* fruits (mg/g)

PC3	Retention time	Calibration Equation	Concentration
Protocatechuic acid	24.622	y = 65753x - 6932,1	0.06
4-hydroxybenzoic acid	30.645	y = 123758x + 75779	*tr*
4-hydroxy benzaldehid	32.921	y = 34376x + 4239,6	*tr*
Vanilic acid	34.481	y = 66764x + 46508	0.03
2,4-dihydroxybenzaldehid	39.120	y = 41139x + 16566	0.03
p-coumaric acid	40.455	y = 17265x + 343183	*tr*
Ferrulic acid	42.714	y = 42245x + 110701	*tr*
Cynarin	44.490	y = 42559x - 2E+06	5.88
Coumarin	44.868	y = 81802x + 153471	*tr*
Prophylgallate	47.117	y = 29731x - 12781	0.14
Rutin	47.639	y = 47899x + 56096	0.31
Trans-2-hydroxycinnamic acid	47.996	y = 53442x + 104662	*tr*
Trans cinnamic acid	55.808	y = 88190x + 158733	*tr*
1,4-dichlorobenzene	74.023	y = 7482,7x + 100774	8.45

**tr*: trace compound

4-Hydroxybenzoic acid, 4-hydroxybenzaldehyde, *p*-coumaric acid, ferulic acid, coumarin, *trans*-2-hydroxycinnamic acid, and *trans*-cinnamic acid were identified as trace components. The HPLC-DAD chromatogram of the PC3 extract is presented in Figure 3.

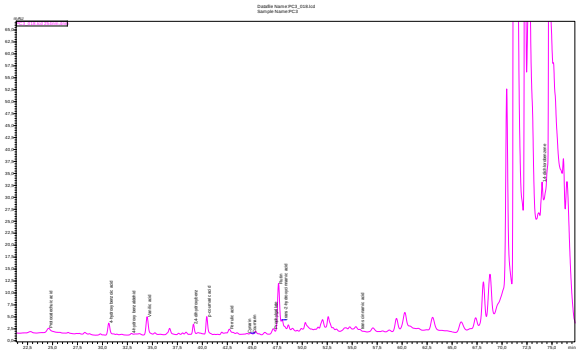

Figure 3. HPLC-DAD chromatogram of PC3 extract at 254 nm wavelength

The results of the PC4 extract analyzed using the HPLC-DAD system to determine its phenolic content are presented in detail in Table 4. According to the findings,

1,4-dichlorobenzene (12.38 mg/g) was identified as the major compound, followed by cynarin (6.00 mg/g), vanillic acid (0.44 mg/g), and protocatechuic acid (0.30 mg/g).

Table 4. Phenolic content results of water (PC4) extract of *P. cubeba* fruits (mg/g)

PC4	Retention time	Calibration Equation	Concentration
Protocatechuic acid	24.602	y = 65753x - 6932,1	0.30
Theophylline	29.257	y = 36694x + 68674	*tr*
Catechin	29.822	y = 2611,2x + 74392	*tr*
4-hydroxy benzaldehid	33.760	y = 34376x + 4239,6	0.01
Vanilic acid	34.481	y = 66764x + 46508	0.44
p-coumaric acid	40.466	y = 17265x + 343183	*tr*
Ferrulic acid	42.742	y = 42245x + 110701	0.08
Cynarin	44.472	y = 42559x - 2E+06	6.00
Prophylgallate	47.093	y = 29731x - 12781	0.19
Trans-2-hydroxy cinnamic acid	48.005	y = 53442x + 104662	*tr*
Trans cinnamic acid	55.787	y = 88190x + 158733	*tr*
Apigenin	63.876	y = 71990x + 62472	0.19
1,4-dichlorobenzene	74.587	y = 7482,7x + 100774	12.38

**tr*: trace compound

Additionally, theophylline, catechin, *p*-coumaric acid, *trans*-2-hydroxycinnamic acid, and *trans*-cinnamic acid were identified as trace components. The HPLC-DAD chromatogram of the PC4 extract is presented in Figure 4. The phenolic compounds detected as minor constituents have also been shown in previous studies to possess significant bioactive potential [16,17,18].

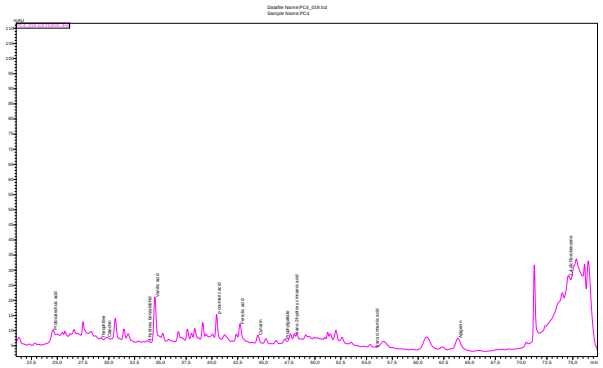

Figure 4. HPLC-DAD chromatogram of PC4 extract at 254 nm wavelength

The antioxidant activity was evaluated using four complimentary tests, namely, *β*-carotene-linoleic acid, ABTS, DPPH, and CUPRAC assays. As shown in Table 5, the results of antioxidant activity test are compared with the BHA. The analysis of variance revealed significant difference between tested samples and assays. Antioxidant activity results of all extracts obtained are shown in Table 5.

Table 5. IC_{50} results of antioxidant activities of *P. cubeba* extracts (µg/mL)

Codes	DPPH$^{\cdot}$	ABTS$^{\cdot+}$	*β*-carotene-linoleic acid	CUPRAC ($A_{0.5}$)
PC1	> 200	> 200	100.56±0.10	> 200
PC2	> 200	193.31±0.28	> 200	> 200
PC3	> 200	173.47±0.05	125.06±0.84	> 200
PC4	149.19±1.03	68.75±0.29	83.53±0.39	153.69±0.88
BHA*	5.09±0.22	3.46±0.34	22.12±0.79	6.14±0.13

*Standard compound.

Based on the antioxidant activity results, the PC4 extract demonstrated the highest all antioxidant activity compared to the other extracts (DPPH radical scavenging; IC_{50}: 149.19 ± 1.03 µg/mL, ABTS cation radical scavenging; IC_{50}: 68.75 ± 0.29 µg/mL, *β*-carotene-linoleic acid assay; IC_{50}: 83.53 ± 0.39 µg/mL, CUPRAC; $A_{0.5}$: 153.69 ± 0.88 µg/mL). It is observed that PC3 extract, following PC4 extract, gives better antioxidant (especially ABTS$^{\cdot+}$ and *β*-carotene-linoleic acid) activity than other extracts.

Acetylcholinesterase and butyrylcholinesterase enzymes are closely linked to the progression of Alzheimer's disease. Plants may serve as valuable natural reservoirs for novel cholinesterase-inhibiting compounds that could contribute to the development of therapeutic agents for neurodegenerative disorders [19]. Enzyme inhibition activity results are given in Table 6.

Table 6. Anticholinesterase inhibition and tyrosinase inhibition activity IC_{50} results of *P. cubeba* extracts (µg/mL)

Codes	AChE	BChE	Tyrosinase
PC1	94.16± 2.35	31.67±0.79	84.18±2.10
PC2	169.74±4.24	27.70±0.69	190.03±4.88
PC3	123.74±3.09	35.26±0.88	141.42±3.51
PC4	*NA*	*NA*	> 200
Galantamin*	9.02±0.81	3.62±0.34	*NT*
Kojic acid*	*NT*	*NT*	24.99±0.13

*Standard compound. *NT*: not tested.

Anticholinesterase activity assays of *P. cubeba* extracts were conducted. According to the acetylcholinesterase (AChE) inhibition results, the acetone extract (PC1) exhibited the highest activity compared to the other extracts. In terms of butyrylcholinesterase (BChE) inhibition activity, it was determined that PC1, PC2, and PC3 extracts showed comparable levels of activity. Regarding the tyrosinase inhibition activity of *P. cubeba* extracts, PC1 again exhibited superior inhibitory activity compared to the other extracts.

4. Conclusion

Piper cubeba is a widely used culinary spice in Europe and is known to contain numerous bioactive constituents. The chemical compositions of *P. cubeba* extracts were identified. GC/MS and HPLC-DAD instruments were used to detect apolar and polar

compounds, respectively, and the concentrations of the identified compounds were calculated. Except for Hegzan, the antioxidant activities of all obtained extracts were evaluated in vitro using four different spectroscopic methods. Analysis of the results indicated that the PC4 extract exhibited superior activity compared to the other extracts across all antioxidant activity parameters. The concentrations of 1,4-dichlorobenzene and Cynarin were found to be higher in PC4 than in the other extracts. Notably, Cynarin, which has been previously reported to possess strong antioxidant properties, was most abundant in the PC4 extract. Additionally, anticholinesterase and tyrosinase enzyme inhibition activities were also assessed. Enzyme activity assay results revealed that the PC1 extract exhibited superior activity compared to the other extracts. Rutin is known to exhibit significant inhibitory effects on AChE and BChE. The ability of 4-hydroxyresorcinol to suppress melanin synthesis has been confirmed in previous studies. Therefore, the strong enzyme inhibition observed in the PC1 extract is a natural consequence of its constituent compounds. It can be inferred that the *P. cubeba* fruits collected from Gaziantep, which were used to obtain the extracts in this study, predominantly contain more apolar compounds. Currently, there is no definitive cure for diseases such as Alzheimer's and various dermatological disorders. Existing treatments are mostly aimed at slowing the progression or alleviating symptoms. The findings of this study suggest that the potential of newly obtained bioactive extracts and factors such as the extraction method may enhance the efficacy of treatments for such disorders.

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