Advances in Zoology and Botany 12(1): 26-32, 2024 http://www.hrpub.org
DOI: 10.13189/azb.2024.120103

Phytochemical Composition and Evaluation of Antioxidant Activity in *Canthium angustifolium* Roxb

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Received October 18, 2022; Revised March 20, 2023; Accepted April 19, 2023

Cite This Paper in the Following Citation Styles

(a): *[1] Soja. S, T. Sunilkumar , "Phytochemical Composition and Evaluation of Antioxidant Activity in Canthium angustifolium Roxb," Advances in Zoology and Botany, Vol. 12, No. 1, pp. 26 - 32, 2024. DOI: 10.13189/azb.2024.120103.*

(b): *Soja. S, T. Sunilkumar (2024). Phytochemical Composition and Evaluation of Antioxidant Activity in Canthium angustifolium Roxb. Advances in Zoology and Botany, 12(1), 26 - 32. DOI: 10.13189/azb.2024.120103.*

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Abstract *Canthium angustifolium* Roxb. is used for the preparation of various traditional medicines. Leaf extracts of *Canthium angustifolium* were taken in petroleum ether, chloroform, methanol and water and screened for phytochemicals. For the identification of different phytoconstituents, both qualitative and quantitative phytochemical analysis were done with standard procedures. Antioxidants are substances which scavenge free radicals from the body cell and prevent or reduce the damage caused by oxidation. Active principles such as Alkaloids, Glycosides, Flavonoids, Terpenoids, Phenol, Fatty acids, Quinones, Saponins, Steroids and Tannins were found as a result of qualitative analysis. Total phenols, flavonoids, alkaloids, tannins, saponins and steroids were quantitatively estimated. Antioxidant property of different extract of *Canthium angustifolium* was examined using DPPH and FRAP assay. From the result of the assays, it was clear that water and methanolic extracts show significant inhibitory properties and those combinations can be used as promising antioxidant drug. A higher amount of saponin, tannin and phenol is the reason behind the antioxidant activity. The study reveals the medicinal importance of *Canthium angustifolium* as it contains valuable phytochemicals.

Keywords *Canthium angustifolium*, Antioxidant Activity, Phytochemical Screening, Qualitative Analysis, Quantitative Analysis

1. Introduction

The Rubiaceae family is composed of dicotyledonous herbs, shrubs, trees, and lianas and each genus is well distinguished by leaves simple opposite, stipules interpetiolar and gamopetalous, actinomorphic flower. It comprises of about 611 genera and 13150 species [1]. Most of the species have medicinal properties and economic importance. Therefore, many phytochemical studies have been carried out in most of the genera of the Rubiaceae family. The genus *Canthium* was named by Jean Basptiste Lamark in Encyclopedie Methodique [2]. *Canthium* was a member of the tribe Vanguerieae, a tribe that is monophyletic and easily recognized morphologically [3].

Plants have endless power to produce aromatic substances. The most common ones are phenols or their derivatives [4]. About 12,000 plant-based natural products have been isolated so far. The plant-derived secondary metabolites serve as plant defense mechanisms against microorganisms [5]. From previous studies, the plant has a great role in various common ailments. The leaf paste was applied to swelling part to treat inflammation [6]. The main aim of the present study is the phytochemical analysis of *C. angustifolium* because of its limitless medicinal properties. Phytochemical analysis includes qualitative and quantitative tests of components which are higher in concentration, and also the assessment of antioxidant property. This study leads to the pharmacology of *Canthium angustifolium* with respect to its medicinal properties.

2. Materials and Methods

2.1. Collection of Plant Material

For this study, fresh and healthy leaves of *Canthium angustifolium* were collected from Kottayam District. The plant was identified by visiting the herbaria of TBGRI, KFRI and University of Calicut and authenticated by comparing it with the protologue and several local Floras.

2.2. Preparation of Plant Extract

Soxhlet extraction method is used (extract of 100 mg of powdered leaf in solvents like distilled water, methanol, chloroform and petroleum ether were taken) [7]. Polarity was thus determined based on boiling points and the ability to evaporate. Repeated the process till the solvent got colourless in the extracting chamber, finally the desired compound came out as concentrated mass which was then collected in the distillation flask.

2.3. Determination of Percent Yield

$$\% \text{ Yield} = W1/W2 \times 100 \quad (1)$$

W1 represents weight of extract residue
W2 represents weight of powdered sample taken

2.4. Qualitative Determination of Phytochemicals

Several chemical tests were performed for the presence of phytochemical constituents using standard procedures. The tests for different classes of secondary metabolites are as follows:

2.4.1. Phenolic Compounds

1% of lead acetate solution was added to 2 ml of leaf extract. Presence of phenolic compound is proved as a white precipitate is formed [8].

2.4.2. Tannin

0.1% ferric chloride solution was added to 2 ml of leaf extract. Presence of tannin is proved as a brownish green precipitate is formed [9].

2.4.3. Flavonoids

2 ml of leaf extract and 2 ml of 10% Lead acetate were mixed together. Yellowish green colour is the indication of the presence of flavonoids [8].

2.4.4. Saponins

1 ml of leaf extract, 2 ml of distilled water and few drops of olive oil were taken in a test tube and shaken well. It created a foam which persisted. This indicates the presence of saponins [10].

2.4.5. Terpenoids

Mix 2 ml of leaf extract and 2ml of chloroform with 2 ml conc. sulphuric acid and allowed the mixture to evaporate. Then heat it for 2 minutes. The solution turned out grey. The result indicates the presence of terpenoids [8].

2.4.6. Alkaloids

Mix 2 ml of leaf extract with an equal amount of Wagner's reagent. A reddish-brown precipitate indicates the presence of alkaloids [8, 10].

2.4.7. Glycoside

From 5% $FeCl_3$, one drop was taken and added to 5 ml of leaf extract, 2 ml glacial acetic acid and conc. H_2SO_4 was added. There appeared a brown ring. This indicates the presence of glycosides [8].

2.4.8. Quinone

Mix 2 ml of leaf extract with 3-4 drops of concentrated HCl. Presence of quinone is proved as a yellow-coloured precipitate is formed [8].

2.4.9. Fatty Acids

Mix 0.5 ml leaf extract and 5 ml ether and this mixture was allowed to evaporate on a filter paper. There was appearance of transparency on the dried filter paper and this indicates the presence of fatty acids [8,10].

2.4.10. Steroids

1 ml of plant extract was dissolved in 10 ml of chloroform and an equal volume of concentrated sulphuric acid was added to it. Two layers were formed. The top layer turned red and the bottom layer of sulphuric acid appeared yellow with green fluorescence. This result indicates the presence of steroids [11].

2.5. Quantitative Estimation of Phytoconstituents

The quantitative estimation of phytoconstituents was carried out according to the standard procedures [11,12].

2.5.1. Estimation of Total Phenol

Extracts in different solvents (in separate test tubes) made up to 3 ml by adding distilled water. To each test tubes, 0.5 ml Folin-Ciocalteau reagent and 2 ml of 20% Na_2CO_3 were added, which was placed in a boiling water bath (one minute). OD at 750 nm was taken, against a reagent blank, after cooling down it. The same process was repeated using gallic acid (as standard) solutions (2.5-100 µg/ml) [12].

2.5.2. Estimation of Tannin

Folin-Ciocalteu method is followed. 1 ml of leaf extract was made up to 7.5 ml using distilled water, and added to this 0.5 ml of Folin-Ciocalteu reagent and 1 ml sodium carbonate solution (from 35%) were added. The volume of the solution was made up to 10 ml using distilled water and the absorbance was taken at 700nm [11].

2.5.3. Estimation of Flavonoid

Aluminum chloride colorimetric assay method is applied. 0.30 ml of 5 % sodium nitrite was added to the reaction mixture (1mg of leaf extract + 4 ml of distilled water). Then added 0.3 ml of 10 % aluminum chloride after 5 minutes. 2 ml of 1M Sodium hydroxide was added to the mixture and then total volume made to 10 ml by adding distilled water. Quercetin in different concentration (20, 40, 60, 80 and 100 µg/ml) was used as standard. Using UV/Visible spectrophotometer the absorbance of solutions (both test and standard) was measured against the reagent blank at 510 nm [11].

2.5.4. Estimation of Saponins

The vanillin-sulphuric acid assay method was used to determine the total saponin content. To 1 ml leaf extract 0.25 ml of vanillin in ethanol solution (0.8% (w/v)) was added. Then 2.5 ml of Sulphuric acid (72% (v/v)) was added. The prepared solution was placed in a shaking water bath for 15 min at 60℃. Diosgenin was used as standard. In reagent blank, extraction solvent was used instead of leaf extract. The solution was cooled down and the absorbance was measured at 544 nm [11].

2.5.5. Alkaloid Estimation

1 ml dimethyl sulphoxide (DMSO) was added to 1 ml leaf extract, to this 1ml of 2N HCl was added and filtered. 5 ml of bromocresol green solution and 5 ml of phosphate buffer were added to a separating funnel after transferring this solution to it. This mixture was subjected to vigorous shaking with 1- 4 ml of chloroform, which was collected in a 10 ml volumetric flask, which was then diluted with chloroform. Atropine was used as standard. Then absorbance was tested against the reagent blank at 470 nm [11].

2.5.6. Estimation of Steroids

Took 1 ml of leaf extract, 5 ml of ferric chloride and 4 ml conc. sulphuric acid together in a test tube. Cholesterol in varying concentrations was used as standard. 5 ml of ferric chloride reagent and 4 ml of concentrated sulphuric acid were added to the standard. OD of the reaction mixture was measured at 540nm after incubating it for 30 minutes. To find out unknown value of steroid in the test sample, a standard graph was plotted [11].

2.6. Determination of Antioxidant Activity

2.6.1. FRAP Assay Method

The FRAP reagent consisting 2.5 ml of 10 mM 2,4,6-tris(2pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3$ in 25 mL of 0.1 M acetate buffer pH 3.6, was prepared. This was then incubated at 37℃ for 10 minutes. Adding varying concentrations of leaf extract with 2 ml of the FRAP reagent and 1 ml distilled water. A blank solution was prepared by the same method but using distilled water instead of the leaf sample. This solution was incubated for 30 minutes and absorbance was measured at 593 nm against blank [13].

2.6.2. DPPH Radical Scavenging Assay

Radical scavenging activity of the leaf extract was tested against DPPH [14]. Ascorbic acid was used as the standard (prepared in distilled water (1 mg/ ml; w/v)). 1 ml of leaf extract at various concentrations (1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200, 400,800, 1000µg/ml) was mixed with 3.9 ml, 60µM solution of DPPH in methanol. The absorbance was measured at 515 nm after incubating for 15 minutes at room temperature. Control used here consists of only DPPH solution. A blank solution used here consists of only 95% methanol.

For the calculation of radical scavenging activity, the following formula (2) was used:

$$\% \text{ inhibition} = \frac{ABS^{control} - ABS^{test} \times 100}{ABS^{control}} \quad (2)$$

3. Results

Secondary metabolites are responsible for the therapeutic effect of plants. The present study performed on the plant *Canthium angustifolium* reveals the presence of bioactive compounds. The physical characteristics and percentage yield of different extract are described in table 1 and table 2 respectively. Qualitative phytochemical screening shows the presence of alkaloids and saponins in all solvents. Flavonoids, steroids, phenols and tannins were present in all solvent systems except in petroleum ether (Table 3).

3.1. The Percent Yield

Table 1. Physical characteristic of different extract of *C. angustifolium*

Sl. No	Extracts	Consistency	Colour
1	Petroleum ether	Powdery	Yellowish green
2	Chloroform	Sticky	Greenish black
3	Methanol	Sticky	Yellowish green
4	Water	Powdery	Brown

Table 2. Percentage yield of different extracts of *C. angustifolium*

Sl. No	Extracts	Weight of sample (g)	Weight of extract (g)	% Yield
1	Petroleum ether	25.033	0.322	1.29
2	Chloroform	25.033	1.264	5.05
3	Methanol	25.033	1.026	4.10
4	Water	25.033	1.367	5.46

3.2. Qualitative Analysis of Phytochemicals

Table 3. Phytochemical composition of the leaves of *C. angustifolium*

Sl. No.	Name of test	Extracts			
		PE	Chl	MeOH	H_2O
i	Phenols	-	++	+	+++
ii	Tannins	-	++	+	++
iii	Flavonoids	-	++	++	+++
iv	Saponins	++	+++	+++	++
v	Terpenoids	-	+++	+	-
vi	Alkaloids	++	+	+	++
vii	Glycosides	-	++	+	-
viii	Quinones	-	+++	+	-
ix	Fatty acids	-	+	+	-
x	Steroids	-	++	++	+

'+' indicates presence and '-' indicates absence, PE- Petroleum ether, Chl- Chloroform, MeOH- Methanol, H_2O- Water

Table 4. Quantitative estimation of phytoconstituents of *C. angustifolium*

Phytochemical	Chloroform	Methanol	Water	Petroleum ether
Phenols (mg GAE/g extract)	59.08±0.44	12.09±0.37	83.56±0.25	-
Tannins (Tannic acid mg/ml)	89.16±0.30	49.22±0.29	90.86±0.30	-
Flavonoids (QE/g)	36.50±0.33	25.63±0.29	45.41±0.24	-
Saponins (µg/mg)	239.85±0.24	235.6±0.38	115.6±0.71	112.1±0.29
Alkaloids(µg/mg)	25.11±0.25	22.33±0.27	72.33±0.31	98.44±0.34
Steroids (mg/100g)	70.13±0.31	24.85±0.40	10.82±0.18	-

3.3. Quantitative Analysis of Phytoconstituents

Quantitative estimation was carried out only for selected phytochemicals according to previous literature. Thus, six of them were selected for quantitative analysis. They were phenols, tannins, flavonoids, saponins, alkaloids and steroids as this plant (*Canthium angustifolium*) was rich in these constituents. In *Canthium angustifolium* the water extract contained higher levels of total phenols, tannins, flavonoids. The highest saponin content was found in chloroform extract followed by methanol extract. The highest alkaloid content was in the petroleum ether extract, which was also followed by water extract. The highest flavonoid content was found in the water extract which was also followed by chloroform extract. From the quantification results it was found out that flavonoid, phenol and tannin contents in the order of extracts water>chloroform>methanol. Saponin content in the order chloroform>methanol> water>petroleum ether. Alkaloid content was in the order petroleum ether>water>chloroform>methanol and the steroid content was in the order chloroform>methanol>water (Table 4).

3.4. Antioxidant Analysis

3.4.1. Result of FRAP Assay

The present values occur in the range of 5.21±0.29 mg to 55.02±0.68 mg. Higher reducing power is found in higher concentration of the sample (Table 5 and 6). Water extract shows the highest reducing power out of the four.

Table 5. Absorbance of Ferrous Sulphate as standard

Sample code	Concentration (mg/ml)	OD at 593nm
FeSO4 (Standard 3.6µM/ ml)	10	0.231
	20	0.410
	40	0.822
	60	1.364
	80	1.831
	100	2.090

Table 6. Reducing power of different extracts of *Canthium angustifolium* in different concentration (FRAP assay)

Sl. No.	Sample code	Concentration (mg/ml)	OD at 593nm	Mm Fe2+/ (mg)
1	Petroleum ether	20	0.116	5.21 ±0.29
		40	0.175	7.93 ±0.33
		60	0.195	8.85 ±0.38
		80	0.212	9.63 ±0.58
		100	0.217	9.86 ±0.08
2	Chloroform	20	0.148	6.68 ±0.24
		40	0.185	8.39 ±0.21
		60	0.205	9.31 ±0.37
		80	0.289	13.18 ±0.41
		100	0.327	14.93 ±0.73
3	Methanol	20	0.244	11.11 ±0.39
		40	0.307	14.01 ±0.54
		60	0.504	23.09 ±0.65
		80	0.637	29.22 ±0.61
		100	0.756	34.70 ±0.56
4	Water	20	0.473	21.66 ±0.61
		40	0.634	29.08 ±0.09
		60	0.879	40.37 ±0.71
		80	1.011	46.45 ±0.46
		100	1.197	55.02 ±0.68

3.4.2. Result of DPPH Assay

The leaf extract exhibited a dose dependent inhibition of DPPH activity. The absorbance of the sample was lower at higher concentration. The radical scavenging activity was higher (higher % of inhibition) in higher concentration (Tables 7 and 8).

Table 7. Absorbance of different extract of *Canthium angustifolium* at 515 nm by UV-Visible Spectrophotometer (DPPH assay)

	OD at 515 nm				
Conc. (µg/ml)	Ascorbic acid	PE	Chl	MeOH	H_2O
Control	1.28	0.8716	0.8716	0.8703	0.8703
1.56	1.20	0.8625	0.8518	0.8548	0.8854
3.12	1.19	0.7812	0.8174	0.8444	0.8033
6.25	1.12	0.7532	0.7937	0.8214	0.7574
12.5	0.96	0.7233	0.7510	0.7867	0.7625
25	0.65	0.7032	0.7074	0.7020	0.7041
50	0.09	0.6945	0.6866	0.6494	0.6647
100	0.09	0.6607	0.6713	0.5763	0.5136
200	0.08	0.6564	0.6552	0.4686	0.3569
400	0.08	0.6213	0.6243	0.3026	0.3025
800	0.09	0.5845	0.5904	0.2706	0.2706
1000	0.09	0.5506	0.5533	0.1544	0.1906

Table 8. Percentage inhibition of different extracts of *Canthium angustifolium* with ascorbic acid as standard

	Percentage inhibition				
Conc. (µg/ml)	Ascorbic acid	PE	Chl	MeOH	H_2O
1.56	6.0	1.05	2.28	1.78	1.74
3.12	6.8	10.38	6.21	2.98	7.70
6.25	12.2	13.58	8.94	5.61	12.97
12.5	25.0	17.01	13.83	9.60	12.38
25	48.7	19.32	18.84	19.34	19.09
50	93.0	20.32	21.22	25.38	23.63
100	93.2	24.20	22.98	33.78	40.99
200	93.5	24.69	24.83	46.16	58.99
400	93.2	28.72	28.37	65.23	65.25
800	93.2	32.94	32.27	68.91	68.91
1000	93.2	36.82	36.52	82.26	78.10

The methanolic extract inhibited DPPH with a half maximal inhibitory concentration (IC 50) value of 253.61 µg/ml and which is 155.10 µg/ml in water extract.

4. Discussion

Alkaloids, Glycosides, Flavonoids, Terpenoids, Phenol, Fatty acids, Quinones, Saponins, Steroids and Tannins were present as a result of phytochemical analysis of the leaf extract of *Canthium angustifolium*. Phytochemical analysis of *Canthium parviflorum* in various extracts shows the presence of alkaloids, flavonoids, steroids, tannin, saponins [15]. Alkaloids in plants regulate plant growth and give protection from predators [16]. Therapeutically, alkaloids were important for their anesthetic, cardioprotective and anti-inflammatory activities [17]. Glycosides were important in the treatment of congestive heart failure [18]. Flavonoids are potential active principles, which is anticancerous, antioxidant, anti-inflammatory and antiviral in property. They also have neuroprotective and cardioprotective effects [19]. Terpenoids were reported as good antiulcer and antioxidant compound. Tannin acts as a diuretic agent [20]. Phenolic compounds are chemo preventive [21]. According to modern clinical studies, steroids are anti-inflammatory and analgesic agents. Saponins have various medicinal applications like anti-inflammatory, antimicrobial, antioxidant, antidiabetic etc. as well as emulsifiers or sweeteners in food and cosmetic industries [22,23,24,25]. According to another study, they noticed that saponins also have strong antioxidant activity which depends on their free radical scavenging abilities [26,27]. According to a study [28], saponins have strong antioxidant activity in vitro than in vivo condition. This result also supports the present study.

From the quantitative analysis, it was observed that many of the phytochemicals were abundant in methanolic and aqueous extracts. As successive serial extraction was done, the phytochemicals were dissolved maximum in polar solvents such as chloroform and methanol than non-

polar solvents. These results were in accordance with the study conducted by Sasikala [29] that phenols and flavonoids are higher in methanolic extract than others. In most of the studies ethanolic extract was more preferable than other solvents. But unlike the studies conducted so far, phytoconstituents in the present study were highly concentrated in methanol and chloroform extracts. As a result, it is clear that methanolic and chloroform extracts are also a good option economically (instead of ethanolic extract). The value of percentage yield of *Canthium angustifol*ium was higher than that in *Tabernaemontana divaricata* [30].

The antioxidant activity of the leaf extract of *Canthium coromandelicum* is reported as it contains flavonoids and tannins [31]. According to a study, only the water soluble phenolics was important as antioxidant compound [32]. Both the methanolic and water extract show higher free radical scavenging activity. The study conducted by Amalraj reported that methanolic and aqueous extracts showed significant free radical scavenging abilities [33] and it also supports the present study.

5. Conclusions

This study provides useful knowledge about the antioxidant properties and responsible active principle in *Canthium angustifolium.* The qualitative analysis was very useful to find out the presence of various chemical compounds which leads to their quantitative estimation. The result of the present work may vivify the standardization process of phytochemicals from the medicinally important plant, *Canthium angustifolium*. The abundance of saponins, tannins and phenols may be the reason for the antioxidant activity. The plant is a promising source of potential antioxidants. As saponins are present in a higher amount, this study also leads to the provision for the isolation of the specific compound responsible for the antioxidant activity.

Acknowledgements

The authors would like to thank the Head of the Department and staff of the Department of Botany, Manager, Principal of S D College, Alappuzha for the valuable support. We would also like to convey our sincere thanks to the Head of the Department and staff of University Department, Karyavattom. We would also like to thank Dr. N. Sasidharan, KFRI, Peechi to the support and valuable instructions to this study.

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