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Phytochemical screening, antioxidant and cytotoxic activity of fruit extracts of *Calamus tenuis* Roxb

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PEER REVIEW

Peer reviewer

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Comments

This is a good study in which authors evaluated the phytochemical screening, antioxidant and cytotoxic activity of *Calamus tenuis* Roxb. Fruit extracts using three different solvents. The paper revealed that the fruit of the plant has some bioactive metabolites which can be useful to prepare herbal medicine as well as modern medicine after more screening using advance tools.

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ABSTRACT

Objective: To investigate the antioxidant and cytotoxic activity of the fruits of *Calamus tenuis* Roxb.

Methods: The preliminary phytochemical group tests were done, which revealed the presence of alkaloid, tannin, flavonoid and steroid. The dried fruit was extracted in soxhlet apparatus using petroleum ether, ethyl acetate and methanol. Antioxidant potential of each extract was evaluated using total phenol content, total flavonoid content, cupric reducing antioxidant capacity, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity, and total antioxidant capacity determinations.

Results: The extracts were found to possess moderate to high amounts of phenolic and flavonoid contents. In cupric reducing antioxidant capacity assay the extracts showed moderate reducing power which increases with concentration. Scavenging of 1,1-diphenyl-2-picrylhydrazyl radical was found to rise with concentration with lowest IC_{50} value for methanol extract, which was confirmed by total antioxidant activity test that shows highest (95 mg/g of extract) in ascorbic acid equivalent for methanol extract. In *Brine shrimp* lethality bioassay the methanol and petroleum ether extracts were found to be toxic to *Brine shrimp* nauplii, with LC_{50} of 25.53 μg/mL and 28.07 μg/mL respectively while the LC_{50} of the reference vincristine sulphate was 1.32 μg/mL. Ethyl acetate extract was found to be moderately cytotoxic showing LC_{50} of 47.79 μg/mL.

Conclusions: The results of the present study suggest that the fruits of *Calamus tenuis* Roxb possess antioxidant and cytotoxic potential. Moreover, phytochemical screening reveals the presence of alkaloid, tannin, flavonoid and steroid, which may be responsible for the observed bioactivities.

KEYWORDS

Calamus tenuis Roxb, Phytochemical screening, Antioxidant, Cytotoxicity

1. Introduction

Calamus tenuis (*C. tenuis*) Roxb locally known as jati bet in Bangladesh occurs from northeast and northern central India east through Bangladesh and Myanmar to Thailand, Cambodia and Lao PDR, as well as in Indonesia[1]. This species fall in the group of rattans or climbing palms (Areaceae) which are important component of tropical forest ecosystems. They are non timber forest products with considerable economic value[2]. About 20% of the known rattan species are of commercial value[3]. The largest rattan genus is *Calamus*, with 370 species which contains most of the best commercial species of rattans[3]. Canes from *Calamus viminalis*, *C. tenuis*, *Calamus inermis*, *Calamus guruba*, and *Daemonorops jenkinsiana* are tough and durable and hence widely used for making baskets,

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furniture, sticks, posts, hats *etc*[4]. Different rattan species have edible parts such as fruits, palm heart. Some are used as traditional medicine such as *Calamus castaneus, Calamus longispathus, Calamus exilis, Calamus javensis, Calamus ornatus, Daemonorops didymophylla, Daemonorops grandis, Korthalsia rigida, Laccosperma secundiflorum, Eremospatha macrocarpa*[3]. Young shoots of *C. tenuis* Roxb are used as vegetables. Traditionally it is used to treat intestinal worm[5]. Hence the antioxidant and cytotoxic activity of *C. tenuis* Roxb has been done for the first time in this experiment till date.

2. Materials and methods

2.1. Plant materials

The fruits of *C. tenuis* Roxb was collected from a village of Kushtia district of Bangladesh at June, 2010. During June to August, fruits were available in plants. The plants were identified by the taxonomist of the national herbarium of Bangladesh (Accession number: 35341) where the voucher specimen has been deposited for future investigation.

2.2. Chemicals

Gallic acid (Sigma Chemicals, USA), quercetin (Sigma Chemicals, USA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Chemicals, USA), ascorbic acid (SD Fine chem. Ltd, Biosar, India), Vincristine sulphate (Sigma Chemicals, USA).

2.3. Extraction procedure

After washing, fruits were broken into small pieces and dried in hot air oven at 50 °C for 3 d and at 40 °C for the next 4 d. The dried fruits parts were ground to coarse powder with a mechanical grinder. Dried powder of the plant was taken in a bag and placed into the Soxhlet chamber. Hot extraction was carried out with petroleum ether for two times. Similarly other two solvents ethyl acetate and methanol were used. The process was performed for the powders of the aerial parts of the plants. So three extracts which were named as petroleum ether extract, ethyl acetate extract and methanol extract were obtained. The extracts were then poured in a Petri dish and dried by using water bath at a temperature of 50-60 °C until the extract become concentrated and almost all the solvents have evaporated. Then all the three extract containing Petri dishes were labeled with specific information.

2.4. Phytochemical screening of *C. tenuis* Roxb fruits

Qualitative phytochemical tests were performed for the determination of the presence of different class of constituents in the extract using the methods described by Ghani[6].

2.4.1. Tests for alkaloids

A small volume of each extract was neutralized by adding 1 or 2 drops of dilute H_2SO_4. This neutralized solution was treated with a very small amount each of Mayer's reagent, Hager's reagent, Wagner's reagent and Dragendroff's reagent and precipitate of white and cream, yellow crystalline, brownish-black and orange or orange-red color respectively will indicate the presence of alkaloids.

2.4.2. Molisch's test for carbohydrates

Two drops of molisch's reagent were added to about 5 mg of the extract in 5 mL aqueous solution in a test tube. About 1 mL of conc. H_2SO_4 was allowed to flow down the side of the inclined test tube so that the acid formed a layer over the extract solution. At first a red ring will be formed at the common surface of the two liquids. On standing or shaking a dark purple solution will be formed. Finally if shaking and diluting the solution with 5 mL of water produce a dull violet precipitate will indicate the presence of carbohydrate.

2.4.3. Frothing test for saponins

About 0.5 mL of extract was shaken vigorously with water in a test tube. If a frothing was produced and it was stable for 1-2 min and persisted on warming, it may be taken as a preliminary evidence for the presence of saponins.

2.4.4. HCl acid test for flavonoids

A few drops of conc. HCl were added to a small amount of extract. Immediate development of a red color will indicate the presence of flavonoids.

2.4.5. Salkowski's test for steroids

A small amount of extract was added with 2 mL of chloroform and then 1 mL of conc. H_2SO_4 was carefully added from the side of the test tube. In presence of steroids, a red color will be produced in the chloroform layer.

2.4.6. Ferric chloride test for tannins

About 0.5 mL of extract was stirred with 10 mL of distilled water. Production of a blue, blue-black, green or blue-green coloration or precipitation on the addition of $FeCl_3$ (5%) reagent will indicate the presence of tannins.

2.4.7. General test for glycosides

A small amount of extract was dissolved in 1 mL of water, and then few drops of aqueous NaOH solution were added. Development of a yellow color will indicate the presence of glycosides.

2.4.8. Fehling's test for glycosides

A small amount of extract was dissolved in water and alcohol, and then boiled with Fehling's solution. Brick-red precipitation will be noted. Another portion of extract was dissolved in water and alcohol and boiled with a few drops of dilute H_2SO_4. The acid was neutralized with NaOH solution and boiled with Fehling's solution. A brick-red precipitation will indicate the presence of glycosides in the extract.

2.5. Total phenol content determination

The content of total phenolic compounds was determined by Folin-Ciocalteu reagent[7]. A total of 1 mL solution of extracts of 200 μg/mL concentration and gallic acid (standard) of different concentrations was mixed with 500 μL of Folin-Ciocalteu reagent and 4 mL of 7.5% sodium carbonate solution in test tube. The mixture containing the extract was then incubated for 1 h at 20 °C while the mixtures containing

the gallic acid were incubated for 30 min at 20 °C. The absorbance of the solution was measured at 765 nm against blank. The total content of phenolic compounds is expressed as mg per gram of plant extract, in gallic acid equivalents.

2.6. Total flavonoid content determination

A total of 1 mL of extract solution (100 μg/mL) and quercetin (standard) in different concentrations were mixed with 3 mL of methanol, 200 μL (10%) aluminum chloride solution and 200 μL (1 mol/L) potassium acetate solution. Then 5.6 mL of distilled water was added to the mixture and incubated for 30 min at room temperature. The absorbance of the solution was measured at 415 nm against blank[7]. The total flavonoid content is expressed as mg per gram of plant extract, in quercetin equivalents.

2.7. Cupric reducing antioxidant capacity

Cupric reducing antioxidant capacity of the plant extracts was determined following the method described by Resat *et al*[8]. A total of 500 μL of each fraction and standard (ascorbic acid) in different concentrations were taken in test tubes. A total of 1.0 mL of 0.01 mol/L $CuCl_2.2H_2O$ solution was added into the test tubes. Then 1.0 mL of ammonium acetate buffer (pH 7.0) was added into the test tubes. Then 1.0 mL of 0.0075 mol/L of neocaproin solution was added into the test tubes. Finally, after addition of 600 μL of distilled water the final volume of the mixture was adjusted to 4.1 mL. The total mixture was incubated for 1 h at room temperature. Then the absorbance of the solution was measured at 450 nm using a spectrophotometer against blank. The blank solution contained the reagent mixture without test samples or standard and received the same treatment.

2.8. DPPH radical scavenging assay

The DPPH free radical scavenging by the extract was determined. A total of 0.1 mL solution of plant extract of different concentration was added to 3 mL of a 0.004% methanol solution of DPPH. After 30 min the absorbance was determined at 517 nm using a UV spectrophotometer against a blank[9]. The percentage scavenging activity of the extract was calculated.

2.9. Total antioxidant capacity

The total antioxidant activity of the extracts was determined by phosphomolybdenum method[10]. A total of 300 μL solution of each fraction and standard (Ascorbic acid) in different concentrations were taken in test tubes. About 3 mL of reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate) was added into the test tubes. The test tube was incubated at 95 °C for 90 min to complete the reaction. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank containing 3 mL of reagent solution and the appropriate volume (300 μL) of the same solvent after cooling to room temperature. Total antioxidant capacity is expressed as mg per gram of plant extract, in ascorbic acid equivalent.

2.10. Determination of cytotoxic activity by brine shrimp lethality bioassay

Brine shrimp lethality bioassay was used for exploring the potential cytotoxic action of the extracts according to the method described by Meyer *et al*[11]. A total of 38 g of sodium chloride was weighted and then dissolved in 1 L of distilled water. The solution was then filtered to get a clear solution. Two days were given to hatch the shrimp and to be matured as nauplii. A total of 30 μL of different concentration of extract solutions in dimethylsulfoxide (DMSO) is added to test tubes containing 10 nauplii each and adjusted to 5 mL with salt water to get 1 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 500 μg/mL of test solutions. Test were repeated two times. About 5 negative controls were tested for 30 μL of DMSO. Vincristine sulphate was used as the positive control with concentrations same as test solutions. The numbers of surviving nauplii in each test tube for different extract concentrations were counted after 24 h. The percent (%) mortality was calculated to determine the LC_{50} of the extract.

2.11. Statistical analysis

The percent (%) mortality was calculated for each dilution. The effectiveness or the concentration–mortality relationship of plant product is usually expressed as a median lethal concentration (LD_{50}) value. However, LD_{90} values were also calculated in the similar way for all fractions and the reference cytotoxic drug vincristine sulphate.

3. Results

The phytochemical occurrences in the crude extract are summarized in Table 1.

Table 1

Results of phytochemical screening of the different extracts of *C. tenuis* Roxb.

Compound	Name of the test	Result (extracts)		
		Pet ether	Ethyl acetate	Methanol
Alkaloid	Mayer's test	+	+	+
	Hager's test	+	+	+
	Wagner'stest	+	+	+
	Dragendroff's test	+	+	+
Carbohydrate	Molisch's test	−	−	−
Saponin	Frothing test	+	−	−
Flavonoid	HCl acid test	+	−	+
Steroid	Salkowski's test	+	−	+
Tannin	Ferric chloride test	+	+	+
Glycoside	General test	−	−	−
	Fehling's test	−	−	−

'+' showed positive result and '−' showed negative result. Preliminary phytochemical screening of the extracts of *C. tenuis* Roxb revealed the presence of alkaloid, tannin, flavonoid, and steroid.

The total phenolic contents of the test extracts were calculated using the standard curve of gallic acid. The total phenolic contents of all the extracts are given in Table 2. Maximum phenolic content of 38 mg equivalent of gallic acid was found per gram of methanol extract. The total flavonoid content was calculated using the standard curve of quercetin and the highest of 146 mg equivalent of quercetin per gram

of extract was found in the methanol extract (Table 2).

Table 2

Total phenol content, total flavonoid content and total antioxidant capacity of the different extracts of fruits of *C. tenuis* Roxb.

Sample	Total phenolic content ±SD (mg/g of extract, gallic acid equivalent)	Total flavonoid content ±SD (mg/g of extract, quercetin equivalent)	Total antioxidant capacity ±SD (mg/g of extract, ascorbic acid equivalent)
Petroleum ether extract	28.060±0.769	94.080±28.860	45.510±8.220
Ethyl acetate extract	19.880±0.769	54.690±0.720	29.620±7.399
Methanol extract	38.110±1.025	146.630±1.020	95.580±6.577

All extracts produced a dose dependent reduction of Cu^{2+} in a way similar to the standard antioxidant ascorbic acid. The extracts showed weak to moderate Cu^{2+} ion reducing capacity. However methanolic extract showed highest Cu^{2+} ion reducing capacity (Figure 1).

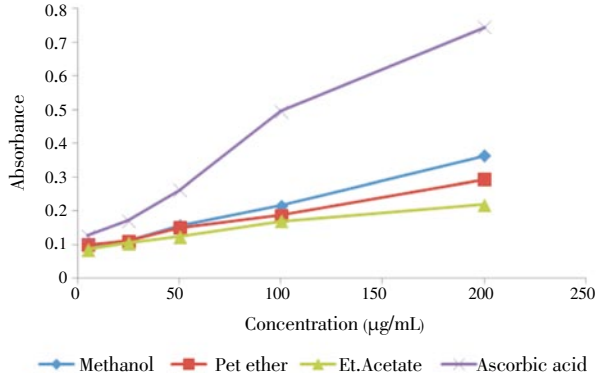

Figure 1. Cupric reducing antioxidant capacity of different extracts of the fruits of *C. tenuis* Roxb.

All extracts showed a dose dependent Cu^{2+} reducing capacity in a way similar to the standard antioxidant ascorbic acid. The extracts showed weak to moderate Cu^{2+} ion reducing capacity. However methanolic extract showed highest Cu^{2+} ion reducing capacity.

Percent scavenging of DPPH radical was found to rise with increasing concentration of the different extracts with highest scavenging displayed by methanol extract of the plant (Figure 2). The IC_{50} values of different extracts were showed in Table 3 and compared with standard antioxidant ascorbic acid.

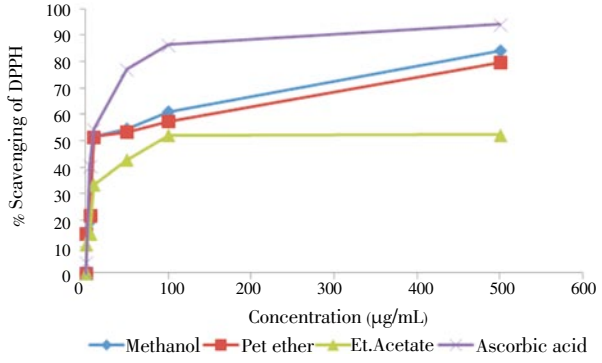

Figure 2. DPPH radical scavenging activity of the different extracts of the fruits of *C. tenuis* Roxb.

Table 3

IC_{50} values of the different extracts of the fruits of *C. tenuis* Roxb.

Sample	IC_{50} (μg/mL)
Petroleum ether extract	185.62
Ethyl acetate extract	384.08
Methanol extract	169.50
Ascorbic acid	88.18

Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid. Maximum antioxidant capacity of 95 mg equivalent of ascorbic acid was found per gram of methanol extract (Table 2). Figure 3 showed correlation of total phenol content, total flavonoid content and total antioxidant capacity with IC_{50}. With lowest amount of total phenol, flavonoid content and antioxidant capacity showed greatest IC_{50} value for ethyl acetate extract, and vice versa for methanol extract.

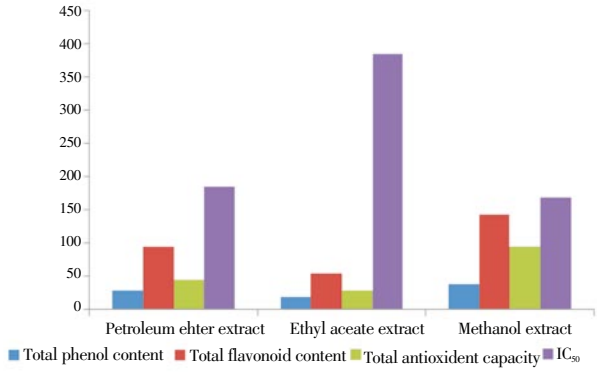

Figure 3. Correlation of total phenol content, total flavonoid content, total antioxidant capacity and IC_{50} value.

With lowest amount of total phenol, total flavonoid content and total antioxidant capacity showed highest IC_{50} value for ethyl acetate extract, and vice versa for methanol extract.

In the brine shrimp lethality bioassay study, methanol and petroleum ether extracts were found to be toxic to brine shrimp nauplii, with LC_{50} of 25.53 μg/mL and 28.07 μg/mL respectively while the LC_{50} of the reference anticancer drug vincristine sulphate was 1.32 μg/mL. Ethyl acetate fraction was also found to moderately cytotoxic showing LC_{50} of 47.79 μg/mL (Table 4). All extracts produced concentration dependent increment in percent mortality of brine shrimp nauplii. Figure 4 shows the comparison of percent mortality different extracts of *C. tenuis* Roxb fruits with standard vincristine sulphate.

Table 4

Results of different extracts of fruits of *C. tenuis* Roxb in *Brine Shrimp* lethality bioassay.

Sample	LC_{50} (μg/mL)	LC_{90} (μg/mL)
Petroleum ether extract	28.0700	230.7600
Ethyl acetate extract	47.7960	469.0400
Methanol extract	25.5340	195.2990
Vincristine sulphate	1.3225	8.2965

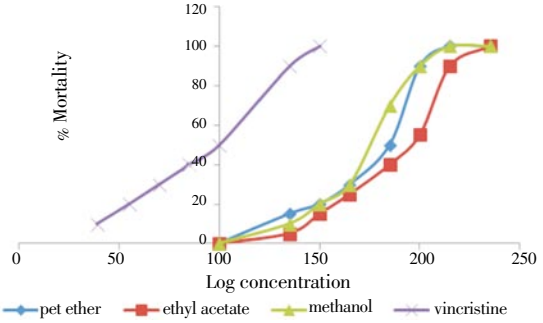

Figure 4. Percent mortality of different extracts of the fruits of *C. tenuis* Roxb

All extracts produced concentration dependent increment in percent mortality of *Brine Shrimp* nauplii.

4. Discussions

Preliminary phytochemical screening of the different extracts of *C. tenuis* Roxb revealed the presence of alkaloid, tannin, flavonoid, and steroid.

Phytochemicals, especially polyphenols, constitute a major group of compounds that act as primary antioxidants[12]. Polyphenols have inhibitory effect on mutagenesis and carcinogenesis in humans when ingested in daily diet[13]. Phenolic compounds have been reported to protect the human body from free radicals, whose formation is associated with the normal natural metabolism of aerobic cells[14]. Natural polyphenols are capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α-tocopherol radicals, and inhibiting oxidases[14]. Phenolic compounds of plants fall into several categories; chief among these are the flavonoids which have potent antioxidant activities[15]. The total flavonoid content range from 54-146 mg/g of extract, calculated as quercetin equivalent. The biological functions of flavonoids include protection against allergies, inflammation, free radicals, platelet aggregation, microbes, ulcer, hepatoxins, viruses and tumors[14]. Natural antioxidants can be phenolic compounds (flavonoids, phenolic acids and tannins), nitrogen containing compounds (alkaloids, chlorophyll, derivative amino acids, peptides and amino acids, peptides and amines), carotenoids, tocopherols orascorbic acids and its derivatives[9]. The antioxidant activities of plant phytochemicals occur by preventing the production of free radicals or by neutralizing or scavenging free radicals produced in the body or reducing and chelating the transition metal composition of foods[14]. Cupric reducing antioxidant activity determines antioxidant activity by hydroxyl radicals where the polyphenol is oxidized to the corresponding quinone[8]. Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, lung damage, inflammation, reperfusion injury, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases. They are also involved in autoimmune disorders like rheumatoid arthritis, *etc*[16]. The prevention of the chain initiation step by scavenging various reactive species such as free radicals is considered to be an important antioxidant mode of action[17]. The DPPH radical scavenging ability of this fruit extracts can be attributed to the high total phenol and flavonoid content of the fruit. Total antioxidant capacity gives the quantitative estimate of antioxidant activity. The antioxidant activity of the three extracts of the fruits of *C. tenuis* Roxb as measured by several *in vitro* tests was found to correlate with total phenol and total flavonoid contents. The total antioxidant capacity is a combination of different antioxidant mechanisms, including free radical scavenging ability, reducing power and Fe (II) chelating ability.

The degree of lethality shown by the extracts was found to be directly proportional to the concentration of the extractives ranging from the lowest concentration (1 μg/mL) to the highest concentration (320 μg/mL). This concentration dependent increment in percent mortality of brine shrimp nauplii produced by the *C. tenuis* Roxb extracts indicates the presence of cytotoxic principles in these extractives. According to Meyer *et al.* several naturally extracted products which had LC_{50}<1 000 μg/mL using brine shrimp bioassay were known to contain physiologically active principles[11]. It has been established that the cytotoxic compounds generally exhibit significant activity in the brine shrimp lethality bioassay, and this assay can be recommended as a guide for the detection of antitumour and pesticidal compounds because of its simplicity and low cost[18]. The plant is reported to contain several phytochemical constituents most notably alkaloid, tannin, flavonoid, and steroid. So the observed cytotoxic action may be due to the presence of such compounds. All phenolic compounds showed cytotoxicity in Hoechst 33258 fluorescence assay by inhibiting cellular DNA in a concentration-dependent manner[19]. Again, alkaloids from *Prosopis juliflora* Sw. D.C. (Algaroba) pods showed cytotoxicity on GL-15 cell line[20]. Steroids and tannins also reported to show cytotoxic activity[21,22].

Based on the results of the present study, it can be proposed that the fruits of *C. tenuis* Roxb in general, methanol and petroleum ether fractions in particular, have antioxidant and cytotoxic activity. These results also support to the preliminary identified phytochemical groups. Various phytochemical constituents like alkaloid, flavonoid, tannin and steroid present in the plant. However, further studies are suggested to be undertaken to understand the mechanism of the observed activities and to isolate, purify and characterize active phytochemical ingredients responsible for the bioactivities.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are grateful to Department of Pharmacy, Jahangirnagar University, Bangladesh for their support.

Comments

Background

This research work detailed the antioxidant and cytotoxic activity of fruit extracts of *C. tenuis* Roxb. Very little is known about the medicinal properties of *C. tenuis* Roxb. Present investigation will add the scientific information on this plant species.

Research frontiers

Studies are being performed to determine the phytochemical properties, antioxidant activity and cytotoxic activity of different solvent extracts of fruit

of *C. tenuis* Roxb. Antioxidant potential of each extract was evaluated using total phenol content, total flavonoid content, cupric reducing antioxidant capacity, DPPH radical scavenging activity, and total antioxidant capacity determinations.

Related reports

Traditionally *C. tenuis* Roxb is used to treat intestinal worm (Saikia P and Khan ML, 2011). Some are used as traditional medicine such as *Calamus castaneus*, *Calamus longispathus*, *Calamus exilis*, *Calamus javensis*, *Calamus ornatus* (Dransfield *et al.*, 2002).

Innovations & breakthroughs

It has been demonstrated successfully that the methanolic extract of this plant showed good antioxidant activity as compared to standard, which has been confirmed by the highest phenolic and flavanoid content and to the antioxidant activity of the methanolic extracts.

Applications

The results of the present study suggest that the fruits of *C. tenuis* Roxb possess antioxidant and cytotoxic potential. So, considering the potential bioactivity, the plant can be further studied extensively to find out their unexplored efficacy and to rationalize their uses as traditional medicines.

Peer review

This is a good study in which authors evaluated the phytochemical screening, antioxidant and cytotoxic activity of *C. tenuis* Roxb. fruit extracts using three different solvents. The paper revealed that the fruit of the plant has some bioactive metabolites which can be useful to prepare herbal medicine as well as modern medicine after more screening using advance tools.

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