In vitro Comparative Studies of the Leaf Extracts of Four Different Types of *Calamus* Species

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ABSTRACT: The leaf extracts of *Calamus tenius*, *Calamus viminalis*, *Calamus guruba* and *Calamus erectus* possessed various phytochemicals including alkaloids, carbohydrates, saponins, phenols, tannins, flavonoids, glycosides, fixed oils and steroids. The point of the present investigation was to compare the antioxidant profile of different leaf extracts of these species. In the present study, methanol, ethanol and chloroform extract of leaf of *C. tenius*, *C. viminalis*, *C. guruba* and *C. erectus* were explored for *in-vitro* thrombolytic activity, membrane stabilizing activity by using hypotonic solution-induced & heat-induced hemolysis and antioxidant profile. Ethanol extracts of all plant species demonstrated the highest percentage of clot lysis. During hypotonic solution-induced hemolysis, methanol extract of *C. erectus* inhibited 59.74% whereas during heat induced condition ethanol extracts of all plant species demonstrated the highest percentage inhibition of hemolysis of RBCs. The screenings of the plants revealed that leaf extracts contain phenolic and flavonoid contents in great amount with the high level of antioxidant capacity. The chloroform extract of *C. tenius* (2.45 µg/ml) and methanol extract of *C. erectus* (1.74 µg/ml) leaf showed potent DPPH and H_2O_2 scavenging activity, respectively. The nitric oxide scavenging activity was not significantly different for methanol, ethanol and chloroform extracts. Methanol, ethanol and chloroform extracts of these four plants showed medium to moderate reducing power and cupric reducing capacity in comparison to standard. A strong relationship was found between the antioxidant activity of the extracts with their total phenolic and flavonoid contents.

Key words: Thrombolytic activity, membrane stabilizing activity, antioxidant profile, *Calamus*.

INTRODUCTION

Medicinal plant research has been and keeps on being, thought about a productive methodology in the scrutiny for advanced drugs. The conventional system of medication broadly utilizes plant-derived compounds and formulations to balance the resistant arrangement of the host.[1] Presently a day, in numerous pieces of the world conventional medicine replaces regular medication since natural cures are cost-effective, having minimum toxicity with decreased wellbeing dangers and are effectively accessible in the market when contrasted with synthetic medicines.[2] With different biological actions, numerous medicinal plants have antioxidant activity that is drawing in increasingly more the consideration of a few examination groups for its part in the battle against a few illnesses such as diabetes, cancer, hypertension, atherosclerosis, cerebral cardiovascular events and Alzheimer's disease.[3,4]

It has been recommended that some plants, fruits and vegetables contain an enormous assortment of substances called phytochemicals, which are available in plants.[5-8] These substances are the major source of antioxidants, that can reduce the probable stress triggered by reactive oxygen species.[9] It has been detected that polyphenolic compounds,

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flavonoids, vitamin C, carotenoids, tannins etc. present in plants have antioxidant and free-radical scavenging activities.[10-12]

C. tenuis Roxb. (Family: Arecaceae) is indigenous to India, Bangladesh, Myanmar, Bhutan, Thailand, Vietnam, Cambodia, Laos, Java and Sumatra. *C. viminalis* Willd. is a species of climbing palm, having about 0.5 cm across pale yellow fruits, resembling small, pointed cones and known as Khorkoijjabet in Bangladesh.[2] It is widely distributed in India, Myanmar, Thailand, Cambodia, Vietnam, Malaysia and Indonesia. *C. guruba* Var. is native to India, Bangladesh, Myanmar, Thailand, Peninsular Malaysia and Cambodia and *C. erectus* Var. is widely distributed in Southeast Asia.

According to Kitukale and Chandewar[13] methanol extract of *C. erectus* has profound anti-hyperglycemic effect. Another literature demonstrated the leaf extract of *C. tenuis* to have antipyretic, anti-nociceptive, gastrointestinal, neuropharmacological and anti-diarrheal activities.[14] Das *et al.*[15] suggested that the leaf extracts of these four plant species have significant anthelmintic activity in a dose dependent way in comparison to its positive control, albendazole. Phytochemical screening of the leaf extracts of these four plants revealed the presence of alkaloids, carbohydrates, saponins, phenols, tannins, flavonoids, glycosides, fixed oils and steroids.[15]

As these plants possess variety of phytochemicals, it is worth to screen other biological functions of these four *Calamus* species. Thus, the present study aimed to determine *in-vitro* antioxidant, thrombolytic and membrane-stabilizing activities of different leaf extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus*.

MATERIALS AND METHODS

Collection, identification and crushing of plant samples. Fresh leaves of *C. tenius, C. viminalis, C. guruba* and *C. erectus* were collected in November 2015 from National Botanical Garden of Bangladesh, Dhaka-1216 and identified with the assistance of the National Herbarium of Bangladesh, where each voucher specimen was kept. The plants were referred to as 42754-DACB, 42755-DACB, 42756-DACB, and 42757-DACB for *C. guruba*, *C. viminalis*, *C. erectus*, and *C. tenuis*, respectively. After thorough washing, the leaves were then sun-dried for several days, after which they were ground into coarse powder using a high-capacity squashing machine (Jaipan originator blender processor, India) and stored in a water/air proof holder with significant markings for ID and kept in a cool, dull, and dry location for the evaluation.

Extraction process. About 30 g of powdered leaf of each plant was extracted using 300 ml of distilled methanol, ethanol and chloroform in a Soxhlet apparatus at increased temperature (45-60°C). The polarity of these organic solvents makes them the most effective solvents for extraction and methanol gives the highest extraction yield as well as the highest content of bioactive compounds when compared to other solvents. All extracts were filtered individually through filter paper and poured on petridishes to evaporate the liquid solvents from the extracts to get dry extracts. After drying, crude extracts were stored in stock vials and kept in refrigerator for further use. Each of the plants was extracted with methanol, ethanol and chloroform and gave a percentage yield of (93.33%, 86.66%, 86.66% and 88.33%), (86.66%, 90%, 83.33% and 80%) and (96.66%, 93.33%, 91.66% and 86.66%) w/v for *C. tenius*, *C. viminalis*, *C. guruba* and *C. erectus* respectively. All extracts were preserved at 4°C after extraction with necessary markings for differentiating proof, for future investigations.

Streptokinase (SK). 5 ml sterile purified water was added to a lyophilized alteplase (streptokinase) vial (Popular Pharmaceutical Ltd.) containing 15,00,000 I.U. This suspension was used to make a stock from which 100 µl (30,000 I.U) was used for testing of thrombolytic activity.

Blood sample. Blood samples (n=6) were taken from healthy human volunteers with no history of oral prophylactic or anticoagulant treatment, and 1-ml of blood was transferred to freshly weighted micro centrifuge tubes and allowed to coagulate.

***In vitro* thrombolytic activity.** The thrombolytic activity of all plant extracts was determined according to Prasad *et al.*[16] using streptokinase (SK) as the standard.[17] From prepared streptokinase (30,000 I.U.) suspension 100 µl was added to the coagulations, followed by incubation at 37°C for 1.5h.

***In vitro* membrane stabilizing activity.** The action of drugs on the stabilization of RBCs may be extrapolated to the stabilization of lysosomal membrane as the erythrocyte membrane resembles to lysosomal membrane.[18] The membrane stabilization by hypotonic solution and heat-induced haemolysis method was used to assess anti-inflammatory activity of the plant extracts by following standard protocol.[19] To prepare the erythrocyte suspension, whole blood was obtained from healthy human volunteer and was taken in syringes (containing anticoagulant 3.1% Na citrate). The blood was centrifuged and blood cells were washed three times with NaCl solution (154 mM) in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 rpm.

Hypotonic solution induced haemolysis. The test sample consisted of stock erythrocyte (RBC) suspension (0.5 ml) mixed with 5 mL of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing either the extract (1.0 mg/ml) or acetyl salicylic acid (ASA) (0.1 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm.

The percentage inhibition of either haemolysis or membrane stabilization was calculated using the following equation:

$$\% \text{ Inhibition of haemolysis} = 100 \times (OD_1\text{-}OD_2)/OD_1$$

Where, OD_1 = optical density of hypotonic-buffered saline solution alone (control)

OD_2 = optical density of test sample in hypotonic solution

Heat induced haemolysis. Isotonic buffer containing aliquots (5 ml) of the different extractives were put into two duplicate sets of centrifuge tubes. The vehicle, in the same amount, was added to another tube as control. Erythrocyte suspension was added to each tube and mixed gently by inversion. One pair of the tubes was incubated at 56°C for 30 min in a water bath, while the other pair was maintained at 0-5°C in an ice bath. The reaction mixture was centrifuged for 5 min at 2500 rpm and the absorbance of the supernatant was measured at 560 nm.

The percentage inhibition or acceleration of hemolysis in tests and was calculated according to the equation:

$$\% \textit{ Inhibition of hemolysis} = 100 \times [1\text{-} (OD_1\text{-}OD_2)/ (OD_3\text{-}OD_1)]$$

Where, OD_1 = optical density of unheated test sample, OD_2 = optical density of heated test sample, OD_3 = optical density of heated control sample.

Determination of total phenolic & flavonoid content and antioxidant capacity. Total phenolic content of all extracts of the plants were measured by using the Folin-Ciocalteu reagent[20,21]. For total flavonoid content, the aluminum chloride colorimetric method[22-23] was used. On the other hand, phosphomolybdenum method[24,25] was used for total antioxidant capacity.

Free radical scavenging assays. 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide (H_2O_2), and nitric oxide scavenging assays were performed to assess the antioxidant activity of these four plant extracts.[21, 26-28] The scavenging activity of the plant extracts and its sub-fractions were also assessed by hydrogen peroxide.[29,30] The nitric oxide scavenging assay was performed by using sodium nitroprusside.[25]

Reducing power capacity assessment. The reducing power capacity of all the extracts of the plants were determined by the method of Yildirim *et al.*[31] and Shahriar *et al.*[32]

Cupric reducing antioxidant capacity. This experiment was conducted as described previously by Demiray *et al.*[20]

Statistical analysis. Data was expressed as Mean ± SEM (Standard error of mean).

RESULTS AND DISCUSSION

***In vitro* thrombolytic activity.** Antithrombotic medication is capable of lessening blood vessel apoplexy and venous apoplexy in patients with heart issues. However, these synthetic drugs cause bleeding, confine their use and tend to discover new anticoagulant, antiplatelet and profibrinolytic agents from natural sources (Turmeric, Barberry, Magnolia Dong quai, Ginger). As a part of the revelation of cardio defensive medications from natural resources, the extracts of *C. tenius, C. viminalis, C. erectus* and *C. guruba* were evaluated for thrombolytic activity and the outcomes are introduced in table 1. Following the methodology of the *in-vitro* thrombolytic activity, the expansion of streptokinase

Table 1. Thrombolytic activity of different extracts of *C. tenius, C. viminalis, C. erectus* and *C. guruba.*

Plant name	Samples	Percent of clot lysis
	Control	07.20%
	Streptokinase	32.01%
Calamus tenius	Methanol	13.20%
	Ethanol	21.96%
	Chloroform	10.95%
Calamus viminalis	Methanol	12.32%
	Ethanol	14.27%
	Chloroform	9.42%
Calamus erectus	Methanol	15.04%
	Ethanol	16.61%
	Chloroform	15.19%
Calamus guruba	Methanol	18.53%
	Ethanol	27.73%
	Chloroform	11.32%

to the coagulations showed 32.01% of clot lysis. Then again, clots when treated with 100 µl sterile distilled water showed negligible amount of clot lysis (7.2%). All extractives from four plant species showed significant clot lysis activity compared to the control. Ethanol extracts of the four plants studied here exhibited the most noteworthy rate of clot lysis, which stood out from other extraction techniques. It can be expected that distinctive dynamic secondary metabolites are available in this concentrate and may be some of these mixtures may work in a synergistic way. The results showed, for the first time, those leaf extracts of selected four plants possess thrombolytic activity.

***In vitro* membrane stabilizing activity.** Primarily membrane stabilizing activity is identified with anti-inflammatory activity. Hypotonic solution and heat-induced RBC membrane lysis can be used for the *in-vitro* assurance of anti-inflammatory activity of drugs or extracts by stabilizing the erythrocyte membrane. Membrane stabilization prompts the aversion of spillage of serum protein and liquids into the tissues during a time of expanded penetrability brought about by inflammatory mediators.[33] The effect of different extracts of *C. tenius, C. viminalis, C. erectus* and *C. guruba* on hypotonic solution and heat induced hemolysis of erythrocyte were presented in table 2. At a concentration of 1.0 mg/ml, for hypotonic solution induced hemolysis, the methanol extracts of *C. tenius* and *C. erectus* inhibited 41.44% and 59.74%, respectively, whereas the chloroform extracts of *C. viminalis* and *C. guruba* inhibited 54.68% and 38.55% hemolysis of RBCs respectively when contrasted with 43.30% produced by acetylsalicylic acid (0.10 mg/ml). At a concentration of 1.0 mg/ml, for hypotonic solution-induced hemolysis, *C. erectus* inhibited 59.74% in the case of methanol extract which was most noteworthy among the four species and *C. tenius* inhibited 12.96% on account of ethanol extricate which was the most minimal among the four species. Then again, during the heat-induced conditions, ethanol extracts of all plant species exhibited the most elevated rate impediment of hemolysis of RBCs, while acetylsalicylic acid inhibited 40.60%. So, it can be proposed that the inhibition of erythrocyte lysis property of the leaf extracts of selected four plants could be the possible mechanism for their anti-inflammatory activity.

Total phenolic & flavonoid content and antioxidant capacity. Secondary metabolites such as

alkaloids, flavonoids, tannins, essential oils and saponins are by and large created by plants for their protection systems which have been ensnared in the restorative properties of most therapeutic plants.[15, 34] Results of this study showed that the tested leaf extracts have a considerable degree of phenolic content and flavonoids (Table 3). The rich-flavonoid plants could be a fair antioxidant source that would assist with expanding the overall antioxidant capacity of an organism and guard it against lipid peroxidation.[35] Notwithstanding, the total phenolic, flavonoid and antioxidant capacity values were not basically exceptional among all of the concentrates of the four species.

Table 2. *In vitro* membrane-stabilizing activity of different extractives of *C. tenius, C. viminalis, C. erectus* and *C. guruba*.

Plant name	Samples	% Inhibition of hemolysis	
		Hypotonic solution induced	Heat induced
Standard	Acetyl salicylic acid	43.30 ± 1.12	40.60 ± 2.59
Calamus tenius	Methanol ext.	41.44 ± 1.12	18.62 ± 4.34
	Ethanol ext.	12.96 ± 2.29	63.39 ± 2.02
	Chloroform ext.	32.95 ± 2.62	28.93 ± 2.78
Calamus viminalis	Methanol ext.	43.43 ± 1.91	55.71 ± 4.31
	Ethanol ext.	18.74 ± 3.25	57.23 ± 2.09
	Chloroform ext.	54.68 ± 1.74	39.70 ± 1.46
Calamus erectus	Methanol ext.	59.74 ± 1.78	21.29 ± 1.04
	Ethanol ext.	35.26 ± 2.47	66.58 ± 1.15
	Chloroform ext.	14.83 ± 5.11	43.02 ± 2.20
Calamus guruba	Methanol ext.	30.65 ± 1.89	45.22 ± 1.36
	Ethanol ext.	15.87 ± 4.01	56.78 ± 2.87
	Chloroform ext.	38.55 ± 1.86	31.92 ± 1.99

[Values represent Mean ± SEM]

Table 3. Comparative total phenolic, flavonoid and antioxidant capacity of different extracts of *C. tenius, C. viminalis, C. erectus* and *C. guruba*.

Plant name	Solvents	Total phenolic content (mg/g, gallic acid equivalents)	Total flavonoid content (mg/g, Quercetin equivalents)	Total antioxidant (mg/g, L-ascorbic acid equivalents)
Calamus tenius	Methanol	1.37 ± 0.12	0.149 ± 0.0010	1.26 ± 0.027
	Ethanol	1.29 ± 0.03	0.147± 0.0004	1.23 ± 0.01
	Chloroform	1.33± 0.08	0.146 ± 0.001	1.32 ± 0.042
Calamus viminalis	Methanol	1.62 ± 0.22	0.146 ± 0.0003	1.19 ± 0.02
	Ethanol	1.54 ± 0.13	0.147± 0.0002	1.25 ± 0.06
	Chloroform	1.55 ± 0.60	0.147 ± 0.0005	1.19 ± 0.014
Calamus erectus	Methanol	1.28 ± 0.02	0.148 ± 0.0003	1.26± 0.22
	Ethanol	1.30 ± 0.01	0.149± 0.0007	1.28 ± 0.01
	Chloroform	1.27 ± 0.01	0.148± 0.0004	1.25 ± 0.03
Calamus guruba	Methanol	1.28± 0.01	0.147 ± 0.0003	1.15 ± 0.02
	Ethanol	1.29 ± 0.02	0.147 ± 0.0003	1.17 ± 0.02
	Chloroform	1.32± 0.07	0.146 ± 0.0002	1.16 ± 0.017

[Values represent Mean ± SEM]

DPPH free radical scavenging activity. The DPPH radical is frequently employed as a model system to investigate the scavenging activities of a variety of natural substances, such as phenolic or crude plant extracts. The scavenging abilities of various solvent extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus* were concentration dependent and given IC_{50} values, as shown in table 4. In contrast to IC_{50} values of methanol, ethanol and chloroform extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus*, ascorbic acid had an IC_{50} of 4.77g/ml. The chloroform extracts of *C. viminalis* had the lowest IC_{50} of DPPH radical scavenging (7.93 µg/ml) among the four *Calamus* species, whereas the chloroform extracts of *C. tenius* had the highest IC_{50} (2.45 µg/ml).

Antioxidants, with DPPH radical scavenging activity usually donate hydrogen to free radicals, mainly to the lipid peroxides or hydro peroxide radicals that are the main propagators of the chain autoxidation of lipids and to form non-radical species which results in the inhibition of promulgating phase of lipid peroxidation.[36]

H_2O_2 scavenging assay. Hydrogen peroxide, albeit not a radical species, plays a role to contribute oxidative stress. Hydrogen peroxide is a frail oxidizing agent that inactivates a couple of proteins straightforwardly and can cross cell layers quickly; once inside the cell, it can likely respond with Fe^{2+} and conceivably Cu^{2+} particles to form hydroxyl radicals and this might be the beginning of a large number of its harmful impacts.[17] The H_2O_2 scavenging activity of methanol, ethanol and chloroform extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus* were recognized and contrasted with ascorbic acid and the outcomes are given in table 4. IC_{50} values of ascorbic acid were 1.77 µg/mL in contrast with IC_{50} values of methanol, ethanol and chloroform extracts of four plants. The outcomes show that every one of the concentrates had potent H_2O_2 scavenging activity which might be because of the cell reinforcement compounds.

Table 4. Comparative DPPH, H_2O_2 and nitric oxide - scavenging assay of different extracts of *C. tenius, C. viminalis, C. erectus* and *C. guruba* with standard.

Plant name	Solvents for extraction	DPPH radical scavenging activity	H_2O_2 scavenging assay	Nitric oxide scavenging assay
		IC_{50} value (μg/mlL)		
	Ascorbic acid	4.77	1.77	2.34
Calamus tenius	Methanol	6.21	1.77	2.19
	Ethanol	3.11	1.76	2.20
	Chloroform	2.45	1.75	2.22
Calamus viminalis	Methanol	4.44	2.00	2.21
	Ethanol	5.06	1.71	2.11
	Chloroform	7.93	1.82	2.23
Calamus erectus	Methanol	3.88	1.74	2.21
	Ethanol	5.32	1.75	2.22
	Chloroform	3.34	1.76	2.28
Calamus guruba	Methanol	4.44	2.00	2.18
	Ethanol	5.09	1.93	2.21
	Chloroform	5.12	2.21	2.17

Nitric oxide radical scavenging assay. Nitric oxide is a powerful pleotropic arbiter of different physiological interaction, for example, smooth muscle relaxation, inhibition of platelet aggregation, neuronal signaling and regulation of cell mediated toxicity. Notwithstanding responsive oxygen species, nitric oxide likewise embroiled in inflammation, cancer and other pathological conditions.[37-39] The methanol, ethanol and chloroform extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus* effectively reduced the generation of nitric oxide from sodium nitroprusside. However, the scavenging

activities were not significantly different among all the extracts of the four species. IC_{50} values of ascorbic acid were found 2.34 µg/ml in contrast, IC_{50} values of methanol, ethanol, and chloroform extracts of four plants (Table 4).

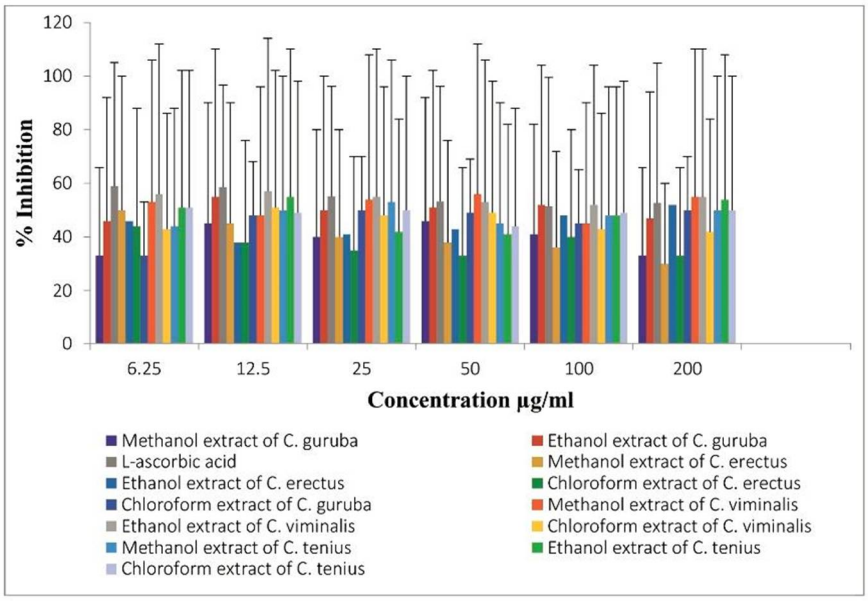

Figure 1. Comparative study of percent inhibition of reducing power capacity of different extracts of *C. tenius*, *C. viminalis*, *C. erectus* and *C. guruba* with standard (L-ascorbic acid).

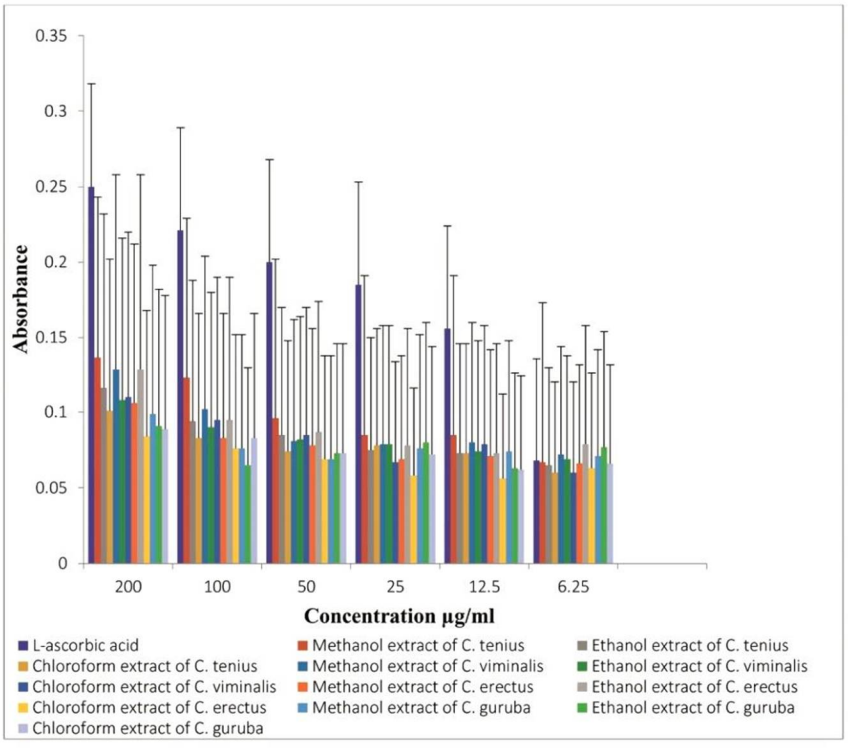

Figure 2. Comparative study of cupric reducing antioxidant capacity of different extracts of *C. tenius*, *C. viminalis*, *C. erectus* and *C. guruba* with standard (L-ascorbic acid).

Cupric reducing antioxidant capacity. Leaf extracts of *C. tenius, C. viminalis, C. guruba* and *C. erectus* plants were oppressed in various concentrations (200, 100, 50, 25, 12.5, 6.25 µg/ml) to conduct cupric lowering antioxidant capacity. The standard L-ascorbic acid shows the most noteworthy lessening limit at the greatest concentration 200 µg/ml. In comparison to the reference standard, methanol, ethanol and chloroform extracts of four plants demonstrated medium cupric lowering capability at the highest concentrations (Figure 2). The presence of a sufficient number of polyphenolic compounds and flavonoids, as well as the responsive hydroxyl groups of polyphenols and oligomeric flavonoids, which are oxidized with the CUPRAC reagent to the corresponding quinines, is thought to be the mechanism of cupric reducing power of the extracts.[26]

CONCLUSION

So far, compound examinations on *C. tenius, C. viminalis, C. erectus, and C. guruba* have been done in this inquiry, with the focus mostly on the plant's leaf. The methanol, ethanol and chloroform leaf extracts of all four *Calamus* species displayed significant *in-vitro* antioxidant, thrombolytic and membrane-stabilizing action according to the results of this study and available studies. Isolating novel bioactive compounds and studying the activities as well as the toxicity profile may be the next steps in the process of discovering new medicinal compounds. The plant can also be evaluated against a variety of diseases to determine its unsuitability, and it can be a common source of deceptively appealing and typically large drug competitors.

CONFLICT OF INTERESTS

The authors announce that there is no conflict of interest about the publication of this paper.

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