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Research Article

COMPARATIVE STUDY OF THIN-LAYER CHROMATOGRAPHY BIOAUTOGRAPHY AND ANTIOXIDANT ACTIVITIES OF DIFFERENT PARTS OF *CLITORIA TERNATEA* (FABACEAE)

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ABSTRACT

Objective: The present study aims at the investigation of antioxidant activities both by qualitative (thin-layer chromatography [TLC] bioautography) and quantitative (*in vitro*) methods and thereby comparison of the same in different parts of the plants of *Clitoria ternatea* (Fabaceae).

Methods: Leaves, stems, and seeds were macerated with methanol separately and preliminary phytochemical screening was carried out. The extracts were developed using two mobile phase combinations such as Toluene: Ethyl acetate: Formic acid (5:4:0.2) for test 1 and toluene: Ethyl acetate: Methanol (5:3:2) for test 2 on silica gel GF_{254} TLC plates and further sprayed with DPPH. Antioxidant and free radical scavenging activities were assayed by inhibition of lipid peroxidation and DPPH method with the different concentrations of test extracts (25, 50, 100, 150 and 200 μg/ml) and standard ascorbic acid.

Results: The methanolic extract of the leaves, stems, and seeds revealed to possess chiefly alkaloids, phenols, and flavonoids and have shown promising antioxidant activity by all the methods in dose-dependent manner. Leaves were found to be most potent having IC_{50} value of 111.652 μg/ml and 106.683 μg/ml which is almost similar to the stems (IC_{50} of 111.685 μg/ml and 106.26 μg/ml), respectively, in DPPH method and inhibition of lipid peroxidation. Seeds failed to produce any significant activity in any of the methods performed.

Conclusion: The methanolic extract of leaves and stems can act as a potential antioxidant and antiradical, which are highly comparable with standard.

Keywords: *Clitoria ternatea*, Thin-layer chromatography bioautography, DPPH, Lipid peroxidation.

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INTRODUCTION

Reactive oxygen species initiate the oxidation process and affect the metabolism of organisms. Overproduction of these free radicals leads to severe pathological conditions [1] and damage of cellular components by covalent binding and lipid peroxidation leading to tissue injury and in turn to the development and/or progression of various conditions or diseases such as aging, carcinogenesis, physical injury, infection and cardio vascular, liver cirrhosis, diabetes, nephrotoxicity, and degenerative diseases [2,3]. These are potentially very toxic to cells and in the body are usually generated by common metabolic processes [4-6]. Organisms possess antioxidants and antioxidant enzymes to combat these free radicals but external supplementation of antioxidants is equally needed [1], which scavenge or remove these free radicals and thereby terminate the chain reactions and inhibit different oxidative reactions are termed as antioxidants. In simple antioxidants are the molecules that inhibit the oxidation of other molecules [6,7].

Clitoria ternatea (Fabaceae) is a perennial herb which is commonly known as Aparajita [8]. The leaves are pinnate. The deep blue colored flowers are solitary and very short pedicellate and 4–5 cm long. Pods are 6–12 cm long, 0.7–1.2 mm wide with flat, linear shape. The brown or black in colored seeds are 4.5–7 mm long and 3–4 mm wide [9]. It is distributed throughout India [8]. The whole plant and seed extracts are used for stomatitis, hematemesis, insomnia, epilepsy, psychosis, purgative, cathartic [10,11]. The roots and root bark possess anti-inflammatory, analgesic, antipyretic and diuretic, laxative properties [10,12]. The leaves are mainly used for the treatment of nostalgia and eruptions. The seeds have purgative, cathartic activity and useful in visceralgia [10].

Here, in this present study, an attempt has been made to investigate and compare antioxidant activities of methanolic extract of different parts of *C. ternatea* (Fabaceae) by employing both qualitative methods and some suitable *in vitro* models.

METHODS

Reagents and chemicals

Solvents and reagents of analytical grade were used in the study and obtained from Merck, Fisher, and Loba Chemie. Standard was procured from Sigma Aldrich. Precoated thin-layer chromatography (TLC) plates were obtained from Merck.

Collection and identification of plant material

Leaves, stems, and seeds of *C. ternatea* (Fabaceae) were collected from the local area of Ashoknagar, West Bengal, India, in December 2020 and were identified and authenticated from BSI, Howrah, India. A sample specimen is preserved in the laboratory for future reference.

Extraction of plant material and phytochemical screening

The plant materials were shade dried and powdered. 200 g of each powdered material was extracted using methanol by maceration method for 3 days. The extracts were filtered and dried under reduced pressure to get the concentrated extract. Further phytochemical screening and all the *in vitro* tests were performed using these extracts, and they are termed as test extracts [13].

Optimization of thin-layer chromatography system and antioxidant activity by bioautography

Different solvent combinations were tried for best separation, and finally, TLC chromatogram of methanol extracts was developed using the two mobile phase combinations such as Toluene: Ethyl acetate: Formic acid (5:4:0.2) for test 1 and Toluene: Ethyl acetate: Methanol (5:3:2) for test 2 on silica gel GF_{254} TLC plates were observed at UV 254 nm and 366 nm and sprayed with DPPH (0.2% in methanol) solution. Development of yellow-colored bands was observed on pink

or purple background on TLC plates which were indicated the presence of antioxidant substances [6,14].

DPPH radical scavenging activity
To the test extracts and standard ascorbic acid of different concentrations (25, 50, 100, 150, and 200 μg/ml in methanol), a solution of DPPH (0.2 mM in methanol) was added in the test tube to remain 30 min. To prepare the blank, a similar way was followed which contain same reagents but no test or standard substances. The absorbance was taken at 517 nm. The whole things were done in triplicates. The following formula is involved to calculate the result [6,15].

% Inhibition = (ABC − ABS)/ABC × 100.

In this, ABC is the absorbance of the control, and ABS is the absorbance of the standard/test.

Inhibition of lipid peroxidation
Inhibition of lipid peroxidation was done by thiobarbituric acid reactive species assay as described by Ruberto *et al.* with slight modifications. In this egg yolk, homogenate was employed as lipid-rich source. Egg yolk homogenate (0.5 ml, 10% v/v in 1.15% of KCl) was added to the different concentrations of test extract and standard ascorbic acid (25, 50, 100, 150, and 200 μg/ml) separately up to 1 ml using distilled water. For lipid peroxidation, 0.5 ml of $FeSO_4$ (0.07 M) was added and incubated for 30 min. After completing incubation for 30 min, 1.5 ml each of 20% acetic acid, thiobarbituric acid (0.8% w/v in 1.1% SDS) and 0.05 ml of 20% TCA were added with it and vortexed. The tubes were cooled in boiling water bath for 1 hour and butanol (5 ml) was added and centrifugation was done for 10 min at 3000 rpm. The upper organic layer was measured at 532 nm and % inhibition of lipid peroxidation was calculated using the following formula [6,16].

% Inhibition of lipid peroxidation = (1 − A. sample/A. control) × 100.

RESULTS AND DISCUSSION

Leaves and stem extract contain high number of alkaloids, phenols, and flavonoids. The result of phytochemical screening is given in Table 1.

Thin-layer chromatography bioautography study of antioxidant activity
The TLC chromatogram shows some major bands at 366 nm. The major R_f values of the bands were found for leaves extract to be 0.76, 0.54, and 0.40, for seed extract values 0.46 and for stem extract 0.76, 0.54, and 0.40 using mobile phase Toluene, Ethyl Acetate and Formic Acid at the ratio of 5:4:0.2.

The major R_f values of the bands in leaves extract were found to be 0.93, 0.84, and 0.64, for seed extract values 0.84, 0.69, and 0.57 and for stem extract 0.93, 0.84, 0.72, and 0.69 using mobile phase Toluene: Ethyl acetate: Methanol (5:3:2).

Table 1: Qualitative phytochemical analysis of methanolic extract of leaves, stems and seeds of *Clitorea ternatea*

Secondary metabolites	Leaves extract	Stems extract	Seeds extract
Alkaloids	+++	+++	+
Glycosides	−	−	+
Flavonoids	+++	+++	+
Carbohydrates	++	+	++
Phenols	+++	+++	+
Tannins	++	+	++
Resins	−	−	−
Amino acids and proteins	+	−	−
Terpenoids	+	+	−
Saponins	−	−	+

+, ++, +++ represent degree of intensity of colour change i.e., presence of phytochemical groups and - represents absence of phytochemical groups

Most of the bands turned into yellow color when the plate was sprayed with DPPH (0.2% in methanol), which indicates the presence of antioxidant substances. Detailed findings have been given in Tables 2 and 3 and the TLC chromatograms are given in Fig. 1.

The phytochemical screening may be called as the very first procedure that is applied to the sample under consideration to establish an idea for the presence or absence of certain compounds or class of compounds basically it is a simple measurement which gives a direct qualitative response only [17]. However, it is very difficult to relate this information directly with that of the biological properties. Effect-directed analysis approach solves the problem. It can provide information of biologically active substances even if it is present in very small concentration and in complex environment such as plant extract [6].

Bioautography screening methods are simple and reliable methods based on the biological activities. This method can be easily and successfully combined with different chromatographic techniques but TLC-B is the simplest and widely used technique among all combinations. It is the technique where TLC is linked to biological assay or the chemical detection indicating the biological properties [6,17]. A good number of such TLC-B techniques have been developed and successfully being applied for the similar activities where all the steps involved in the process such as separation of compounds, determination of the bio activity and visualization of the results are performed in situ directly on a single TLC plate [17]. In the TLC-B technique, a widely used derivatization reagent is DPPH (2,2-diphenyl-1-picrylhydrazyl) which is a stable free radical and shows the development of yellow or cream color on the pink or purple background on TLC plate as an indicator of the presence of antioxidant substances. This method is very popular as in enjoys some advantages such as flexibility, simplicity, and high throughput [6,14,18,19].

***In vitro* assays**
All the experiments were performed in triplicate, and the values are expressed as mean ± SD.

Statistical analysis
The data were represented as mean ± SD (n=3) and analyzed by one-way ANOVA followed by *t*-test. Statistical significance was considered based on $p<0.05$ or $p<0.01$.

DPPH radical scavenging activity
The test extracts of leaves and stem were found to be good scavengers bearing the IC_{50} values of 111.652 μg/ml, 111.685 μg/ml, respectively, which are comparable with that of standard (IC_{50} value of 95.334 μg/ml). Whereas the seeds extract had shown very negligible value (IC_{50} value of 1961.369 μg/ml).The scavenging activity of leaves, stems were found to be significant ($p<0.01$) at concentrations

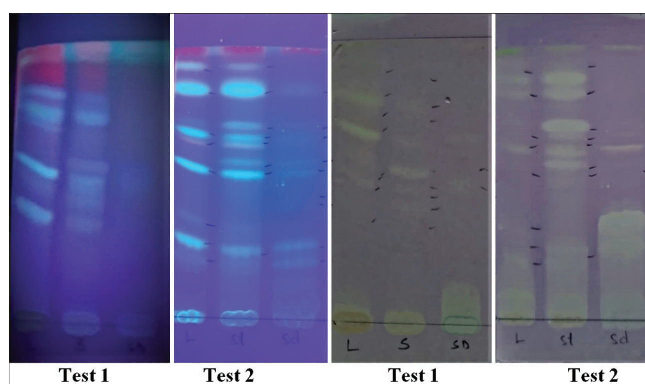

Fig. 1: TLC chromatograms at 366 nm and after spraying with DPPH. Test 1: Toluene: Ethyle acetate: Formic acid (5:4:0.2). Test 2: Toluene: Ethyle acetate: Methanol (5:3:2)

Table 2: Thin layer chromatography bioautography study of antioxidant activity (solvent system: Toluene: Ethyl Acetate: Formic acid [5:4:0.2])

Leaves		Stems		Seeds	
R_f (UV at 366 nm)	R_f (after spraying DPPH)	R_f (UV at 366 nm)	R_f (after spraying DPPH)	R_f (UV at 366 nm)	R_f (after spraying DPPH)
0.88	-	0.86	-	-	0.67
0.79	0.79	0.76	0.76	0.54	-
-	0.76	0.70	-	0.46	0.46
-	-	-	0.61	-	-
0.73	-	0.54	0.54	-	-
0.67	0.67	0.46	-	-	-
0.54	0.54	0.40	0.40	-	-
0.46	-	0.32	-	-	-
0.39	-	-	-	-	-

UV: Ultra Violet-Visible Spectroscopy

Table 3 : Thin-layer chromatography bioautography study of antioxidant activity (Solvent system: Toluene: Ethyl acetate: Methanol [5:3:2])

Leaves		Stems		Seeds	
R_f (UV at 366 nm)	R_f (after spraying DPPH)	R_f (UV at 366 nm)	R_f (after spraying DPPH)	R_f (UV at 366 nm)	R_f (after spraying DPPH)
0.93	0.93	-	0.99	-	0.99
-	-	0.93	0.93	0.84	0.84
-	0.91	0.84	0.84	0.72	-
0.84	0.84	0.72	0.72	0.69	0.69
0.69	-	0.69	0.69	0.61	-
-	-	-	0.67	0.57	0.57
0.64	0.64	0.64	-	0.27	-
0.54	-	0.57	-	0.22	-
0.34	-	0.54	-	-	-
-	0.31	0.24	0.24	-	-
0.28	-	-	-	-	-
0.27	-				
0.21	0.21				

UV: Ultra Violet-Visible Spectroscopy

of 100 μg/ml, 150 μg/ml and 200 μg/ml. The results are shown in Fig. 2.

The molecule DPPH is a stable free radical which is a widely used reagent for investigation of the free radical scavenging activity of various compounds. Unlike other free radicals, DPPH molecule is not dimerized as the spare electron is delocalized over the whole molecule. The deep violet color of the DPPH solution in ethanol/methanol which shows absorption maxima at 517 nm is also due to the delocalization of the electron. When any compound is added to it which can donate hydrogen atom, the DPPH is reduced with loss of the color. This is measured at 517 nm to assess the free radical scavenging ability of the test compound [6,20-22].

Inhibition of lipid peroxidation

The test extracts of leaves and stems were found to possess good activity in inhibition of lipid peroxidation having IC_{50} values of 106.683 μg/ml and 106. 26 μg/ml, respectively, and for ascorbic acid 94.866 μg/ml, but seeds extract has shown very negligible result (IC_{50} value of 1831.72). The activity of the test extracts of leaves and stems was found significant at all the doses (p<0.01 or p<0.01). The comparative study for test extracts of leaves, stems, seeds, and standard results are shown graphically in Fig. 3.

The process of lipid peroxidation is usually proceeds involving free radical chain reaction leading to the cellular damage and thereby associated with various diseases such as cardiovascular diseases, diabetes, and even cancer [6]. Many of studies indicate that dietary antioxidant supplements mainly which are rich in phenols and polyphenols play a major role in controlling or preventing these diseases by slowing or reducing the level of oxidative stress, principally

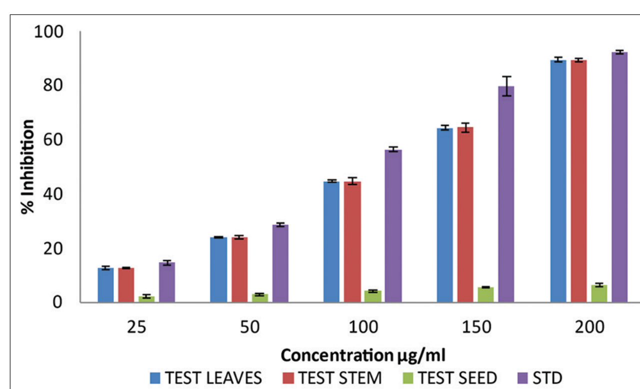

Fig. 2: DPPH Radical scavenging activity. TEST = Test extracts for leaves, stem and seed, STD = Standard ascorbic acid

by protecting lipoproteins from lipid peroxidation [23,24]. In this assay, egg yolk homogenate was used as the lipid substrate. Two polyunsaturated fatty acids, namely, linoleic acid and linolenic acid are present in this. In the presence of $FeSO_4$, lipid peroxidation occurs which produces malonaldehyde. This malonaldehyde reacts with TBA to produce pink color. Addition of test extract to this system reduces or inhibits the process of lipid peroxidation which is indicated by reduced intensity of the pink color which is measured by spectrophotometer at 532 nm [25].

The comparative study between all the IC_{50} values of test extracts of leaves, stem, and seed which were obtained from the DPPH method performed in this study are given in the Fig. 2 which explains that

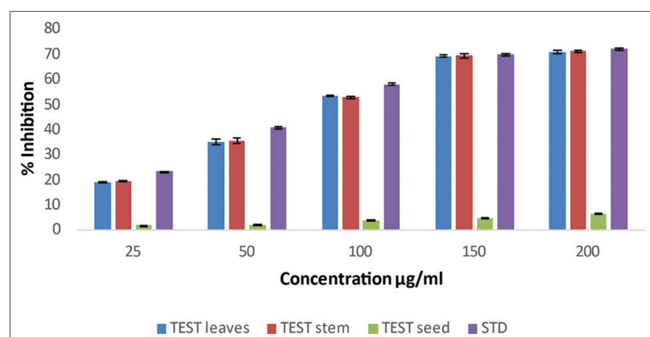

Fig. 3: Inhibition of lipid peroxidation. TEST = Test extract, STD = Standard ascorbic acid

between all the extracts performed, leaves extract shows the best activity with the lowest IC_{50} values and stem extract also shows the similar activity, but seed extracts show very negligible activity among all.

Moreover, for lipid peroxidation method, the comparative study of test extracts of leaves, seed, and stem show that between all the extracts performed, leaves extract shows the best activity with the lowest IC_{50} values and stem extract also shows the similar activity but seed extracts show very negligible activity among all and that are given in the Fig. 3.

Very often the terms “antiradical” and “antioxidant” are used synonymously and misunderstood to be same. However, there is a great difference between them in terms of where action and mechanism of action are concerned. Antiradicals are react and scavenge with free radicals, but antioxidants inhibit or stop the process of oxidation [26]. The *in vitro* experimental models for testing the scavenging abilities of free radical used to show in the present study for the antiradical activity and inhibition of lipid peroxidation at the same time which proved the test extract to have classical antioxidant property also. The antioxidant and free radical scavenging properties may follow either of two methods that are hydrogen atom transfer (HAT) or single electron transfer/electron transfer (SET/ET). In this investigation, study models like inhibition of lipid peroxidation assay falls in the HAT category and the methods such as DPPH free radical scavenging assay find its place in the SET/ET category [6,27]. A huge number of studies on alkaloids, phenols, and flavonoids show the evidences in favor of them that these secondary plant metabolites do possess antioxidant ability, and there is a strong positive correlation between these secondary plant metabolites with that of the antioxidant activity of different extracts [6,28-30]. The present study reveals the strong presence of alkaloids, phenols, and flavonoids in the leaves, stem of *C. ternatea* when extracted with methanol. The positive results among the different extracts and 2 methods performed in this study indicate that the methanol leaves and stem extracts possess fairly good amount of antioxidant and antiradical properties, but seed extract shows very poor activity. Both of these findings may be correlated together to state that the antiradical and antioxidant activities of methanol extracts of the leaves and stem of *C. ternatea* are may be due to the presence of alkaloids, phenols and flavonoids. Based on the findings of the present study, further investigations on these alkaloids, phenols and flavonoid compounds may be done to establish these statements.

CONCLUSION

C. ternatea has long been considered as well-known perennial herb. The findings of different *in vitro* models used in this study state that leaves, stem of this plant can act as potential antioxidant through both the mechanisms such as HAT or electron transfer indicating its possible efficiency in combating the huge number of diseases caused by oxidative stress. However, seed extract possesses no significant result on antioxidant and antiradical activity. Further study of these findings on *C. ternatea* may be encouraging to establish its journey from a beautiful ornamental plant to a potential natural antioxidant.

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AUTHORS’ CONTRIBUTIONS

For the preparation of the article, each author has contributed equally.

CONFLICT OF INTEREST

The authors declare no conflict of interest associated with this study.

AUTHORS’ FUNDING

Self.

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