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Phytochemical studies on stem part of methanolic extract of *Cissampelos pareira*

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Abstract
Objectives: The present study involves *in vitro* antioxidant studies on stem part of *Cissampelos pareira* and the identification of active phytoconstituents present in methanolic extract of stem part of *Cissampelos pareira* by HPTLC method.
Materials and methods: Phytochemical studies were carried out using three different solvents like hexane, ethyl acetate and methanol. The antioxidant activity of different extracts was studied by *in vitro* assay using DPPH and IC_{50} values were determined.Total phenolic and flavonoid contents and total antioxidant activity of methanolic extract of stem part of *Cissampelos pareira* were studied. By using HPTLC analysis the active phytoconstituents present in methanolic extract of stem part of *Cissampelos pareira* was also carried out.
Results: Preliminary phytochemical analysis revealed the presence of phenols, flavonoids, alkaloids, glycosides, saponins, steroids, carbohydrates, proteins and aminoacids. From the different extracts methanolic extract showed good antioxidant activity. HPTLC analysis of methanolic extract identified the presence of phenols and alkaloids.
Conclusion: The results suggest the antioxidant activity of methanolic extract of stem part of *Cissampelos pareira* is due the presence of phenols and alkaloids as evidenced by HPTLC analysis.

Keywords: Antioxidant, *Cissampelos pareira*, phytochemical analysis, HPTLC

Introduction

Medicinal plants have been playing an essential role in the development of human culture. Many of the modern medicines are produced from medicinal plants[1]. Plants always have been a great source of drugs, and most of the drugs that are presently available have been originally derived from plants, either directly or indirectly [2]. Plant and plant extracts have formed important position in modern medicine, due to their chemical and medicinal contents found in the natural form. The antioxidant compounds are mostly produced in plants in the form of secondary metabolites. There are over a thousand known phytochemicals classified as primary or secondary constituents based on their role in plant metabolism [3].

Phytochemicals classified as primary constituents includes the common sugars, amino acids, chlorophyll's, purines and pyrimidines of nucleic acids and proteins etc [4, 5]. Others classified as the secondary constituents are the chemicals consisting of alkaloids, flavonoids, terpenes, phenolics, lignans, plant steroids, curcumins, saponins, glucosides [5,6]. Of these secondary constituents, phenolics are seen to be the most numerous consisting of 45% of the secondary phytochemical constituents of plants, terpenoids and steroids 27%, alkaloids 18% and others 10% [7]. Phytochemicals possess nutraceutical importance [8].These secondary metabolites represent a large reservoir of structural moieties which work together exhibiting a wide range of biological activities [9, 10].

Cissampelos pareira (Family Menispermaceae), is a sub-erect or climbing herb, known as ambastha or malathangi in Indian traditional medicine [11]. The plant is common in orchards, hedges, parks and gardens on moist soils distributed throughout tropical and subtropical India, ascending up to an altitude of 2000 m, either creeping or twining around other plants. It is also common on the hilly tracts along watercourses.It has been extensively used in the traditional medicinal system since the ancient time for the treatment of numerous diseases such as ulcer, wound, rheumatism, fever, asthma, cholera, diarrhoea, inflammation, snakebite, malaria, rabies, and also recommended for blood purification [12].

Studies shown that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action [13, 14]. Phytochemical studies have shown that the major chemical components of this herb are alkaloids, flavonoids, tannins, volatile oils and glycosides. A detailed literature study revealed that alkaloids (mainly isoquinoline) are major constituents of *C. pareira* [15, 16].

Antioxidant potential of *C. pareira* has been demonstrated by various studies. Bafna and Mishra (2010) [17] evaluated the antioxidant property of the alkaloid enriched fraction from *C. pareira* roots based on the radical scavenging abilities against superoxide ion, DPPH, and lipid peroxidation in rat liver homogenate induced by iron/ADP/Ascorbate complex. Although some of the traditional uses have been well clarified and documented by modern pharmacological analysis, the correlation between its pharmacological activities and particular phytoconstituents still needs to be validated. Additionally, scientific validation of traditional knowledge of *C. pareira* is vital for ensuring safety, efficacy, and mechanism of action before clinical uses [12].

The majority of phytochemical research on *C.pareira* has focused on its leaves and roots, leaving the stem part relatively understudied. This investigation seeks to elucidate the phytochemical profile of the stem using HPTLC, providing scientific evidence to support its traditional ethnobotanical applications and potentially revealing novel bioactive molecules.

Materials and methods

Plant preparation

Samples used in the study were stem part of *Cissampelos pareira* collected during the months of January to March.

Plant material was washed several times with water and allowed to shade dry at room temperature and authenticated.The voucher specimes (BC 10) were preserved in the Department.

Preparation of stem extract by different solvents

50g of dried powder of the *Cissampelos pareira* was subjected to successive cold extraction using three solvents based on their increasing polarity hexane, ethyl acetate and methanol for 72 h at a temperature not exceeding the boiling point of solvent. 100ml of each solvent used for extraction. Vigorous shaking is required for proper extraction. Finally filter the extract using whatman no 1 filter paper. Note down the weight of each bottle prior to the drying of the extract and after drying too. Calculate the weight of the extract from the difference. The extracts were concentrated to dryness The yield of methanolic extract of *Cissampelos pareira* was noteda nd the dried extracts were stored in a clean sample bottles unti further use.

$$\text{Percentage yield of extract} = \frac{(W1X100)}{W2}$$

W1= weight of extract residue obtained after solvent extraction, W2= weight of powder taken

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out for hexane, ethyl acetate methanol for the detection of phytoconstituents as per the standard conventional protocols [18].

Phytochemical tests

Phytochemical examinations were carried out for all the extracts as per the standard methods [19].

Phytochemical Screening

Phytochemical screening means to investigate the plant material in terms of its active constituents. The plant extract was subjected to qualitative tests for the identification of the phytoconstituents present in it *viz*, alkaloids, carbohydrates, glycosides, phytosterols, fixed oils & fats, phenolic compounds & tannins, proteins and free amino acids, flavonoids, lignins and saponins [20- 23].

Test for Phenolic compounds: Two to three drops of 1% ferric chloride ($FeCl_3$) solution was added into 2ml of extract. Phenolic compounds produce a deep violet colour with ferric ions.

Test for Flavonoids - Shinoda Test: To the alcoholic solution of the extract a few fragments of magnesium ribbon were added. To this concentrated hydrochloric acid (HCl) was added drop wise. Magenta colour was produced after few minutes which are the characteristic reaction of flavonoid.

Test for Tannins -Ferric Chloride test: Water extract was treated with 15 % ferric chloride test solution. The resultant colour was noted. A blue colour indicates condensed tannins; a green colour indicated hydrolysable tannins.

Test for Saponins: The extract was diluted with 20ml of distilled water and it was agitated on a graduated cylinder for 15 min. The presence of saponins was indicated by the formation of 1cm layer of foam.

Test for alkaloids

a. **Mayer's test:** To 1 ml of extract added 2ml of Mayer's reagent (Potassium mercuric iodide). Formation of a cream colored precipitate indicated the presence of alkaloids.
b. **Wagner's test:** To 1 ml of extract added 2ml of Wagner's reagent (Iodine in potassium iodide). Formation of brown/ reddish precipitate indicated the presence of alkaloids.
c. **Dragendroff's test:** To 1 ml of extract 1ml of Dragendroff's reagent (solution of potassium bismuth iodide) was added. Formation of orange – red precipitate indicated the presence of alkaloids.

Test for the carbohydrates

The aqueous extract 5 ml was treated with the reagent of the starch (iodine). Any shift to blue violet indicates the presence of starch.

Test for Glycosides

Fehling's Test for reducing sugars (In Glycosides): The extract was re-dissolved in water on the water bath. To 2 ml of the solution, in the test tube was added, 1ml each of Fehling's solutions A and B. The mixture was shaken and heated in a water bath for 10min. The colour obtained was recorded. A brick-red precipitate indicates reducing sugar.

Test for proteins

Xanthoproteic Test: Extract was treated with few drops of Concentrated HNO3.

Formation of yellow colour indicates the presence of proteins.

Test for Phytosterols

Liebermann-Burchard's Test: One gram of the extract was treated with chloroform and filtered. The filtrate was treated with few drops of acetic anhydride boiled and cooled, concentrated sulphuric acid was added through the sides of the test tube. The formation of brown coloured ring at the junction of two liquids confirmed the presence of steroids.

Test for fixed oil and fats

Press the extract in between the two filter papers, a permanent stain indicates the presence of fixed oil. Extract was treated with few drops of 0.5N potassium hydroxide and few drops of phenolphthalein and heat, formation of soap indicates the presence of fixed oil and fats.

***In vitro* antioxidant studies**

The plant extract were subjected to free radical scavenging activities such as DPPH assay. The plant extracts were subjected to quantification of phytoconstituents such as flavanoids, total phenolic content and total antioxidant capacity.

DPPH radical scavenging activity [24]

The determination of the free radical scavenging activity of each of the crude extract was carried using the DPPH (1-diphenyl-2-picrylhydrazil) assay with a slight modification.

Various concentrations of 50, 100, 200 and 400 µg/ml of sample extracts in methanol were prepared. 1.0 ml of a 0.1 Mm DPPH in methanol was added to 2.5 ml solution of the extract or standard, and allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to yellow was then measured at 518 nm on a spectrophotometer. The percentage of inhibition was calculated.

$$\text{Percentage inhibition} = \left(\frac{\text{control}-\text{test}}{\text{control}}\right) \times 100$$

The total phenolic content in the methanol extract was determined by Folin-Ciocalteu method [25] Total flavonoid content was measured with the aluminium chloride colorimetric assay [26]. The antioxidant activity of the extract was evaluated by the phosphomolybdenum method [27].

Determination of total phenolic content

The total phenolic content in the water and methanol extract was determined by Folin-Ciocalteu method. For the preparation of calibration curve 1 ml aliquots of 50, 100,150 and 200µg/ml gallic acid solutions were mixed with 5ml Folin-Ciocalteu reagent (diluted tenfold) and 4ml (75g/l) sodium carbonate. The final volume of the tubes were made up to 10ml with distilled water and allowed to stand for 90min at room temperature. Absorbance of sample was measured against the blank at 750nm using a spectrophotometer. A calibration curve of gallic acid was prepared and the results were expressed as GAE (Gallic acid equivalents)/g dry extract.

Determination of total flavonoid

Total flavonoid content was measured with the aluminium chloride colorimetric assay. Methanolic extracts at a concentration of 400µg/ml and different dilution of standard solution of quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. Add 0.3 ml of 5 % Sodium nitrite, 0.3 ml of 10% Aluminium chloride was added after 5 minutes. After 6 minutes incubation at room temperature, 1ml of 1 M Sodium hydroxide was added to the reaction mixture. Immediately the final volume was make up to 10 ml with distilled water. Absorbance of sample was measured against the blank at 510 nm using a spectrophotometer. Total flavonoid content of the extract was expressed as percentage of quercetin equivalent per gram dry weight of sample.

Determination of total antioxidant activity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method. The assay is based on the reduction of Mo(VI) –Mo(V) by the extract and subsequent formation of a green phosphate /Mo(V) complex at acid pH. A 0.3ml extract was combined with 3ml of reagent solution (0.6M sulphuric acid, 0.28mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95ºC for 95min.Then the absorbance of the solution was measured at 695nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3ml) in place of extract was used as the blank. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

High performance thin layer chromatography (HPTLC)

Sample applied on the HPTLC plate as a spot. Ethyl acetate: Methanol: Water at the ratio of 10:1.35:1 used as mobile phase. Mobile phase drawn through the stationary phase capillary action. Samples are separated in to their components which remain in their position on the layer after the mobile phase has been evaporated and visualized the bands.

Rf value=(Distance moved by solute front)/(Distance moved by solvent front)

Statistical analysis

Results were expressed as mean ± S.E and all statistical comparisons were made by means of one way ANOVA test

Results

Phytochemical screening

Preliminary phytochemical analysis of *Cissampelos pareira* revealed the presence of different types of secondary metabolites in hexane, ethyl acetate and methanol fractions as shown in Table 1.

Table 1: Phytochemical screening of stem of *Cissampelos pareira*

Constituents	Hexane	Ethyl Acetate	Methanol
Alkaloids	+	+	+
Flavonoids	+	+	+
Phenolics	+	+	+
Tannins	-	-	+
Glycosides	-	+	+
Fats and oils	+	+	-
Steroids	+	-	-
Saponins	-	+	+
Carbohydrates	+	+	+
Proteins & amino acids	+	+	+

(+) sign indicates the presence of constituents; (-) sign indicates the absence of constituents

***In vitro* antioxidant studies**

The plant extract were subjected to free radical scavenging activities such as DPPH assay. The plant extracts were subjected to quantification of phytoconstituents such as flavanoids, total phenolic content and total antioxidant capacity.

DPPH radical scavenging activity

The hexane, ethyl acetate and methanol extracts of *Cissampelos pareira* exhibited a significant dose dependent inhibition of DPPH activity. Methanol extract showed maximum scavenging activity. Ascorbic acid was used as standard. Percentage (%) scavenging activity was plotted against log concentration and from the graph IC_{50} value was calculated. Results given in Table 2.

Table 2: DPPH radical scavenging activity of different extracts of stem part of *Cissampelos pareira*

Concentration	Percentage of DPPH radical scavenging activity			
	Hexane	Ethyl acetate	Methanol	Ascorbic acid
50	44.69±0.13	43.18±0.12	37.87±0.12	39.39±0.14
100	48.48±0.26	49.24±0.16	46.96±0.23	45.45±0.08
200	60.61±0.21	63.63±0.21	50.75±0.14	56.81±0.13
400	65.59±0.32	71.96±0.13	69.69±0.21	65.15±0.21
IC_{50} (µg/ml)	154.19	153.40	141.28	139.40

Estimation of phytoconstituents

The methanolic extract of *Cissampelos pareira (*MECP) showed highest radical scavenging ability when compared to other extracts. Also the methanol extract of plant contained almost all phytochemicals. Hence MECP was used for further *in vitro* studies.

Determination of total phenolic, flavonoid and antioxidant activity of *MECP*

MECP showed a high total antioxidant activity, phenolic contents and total flavonoid contents. The results are given in Table 3.

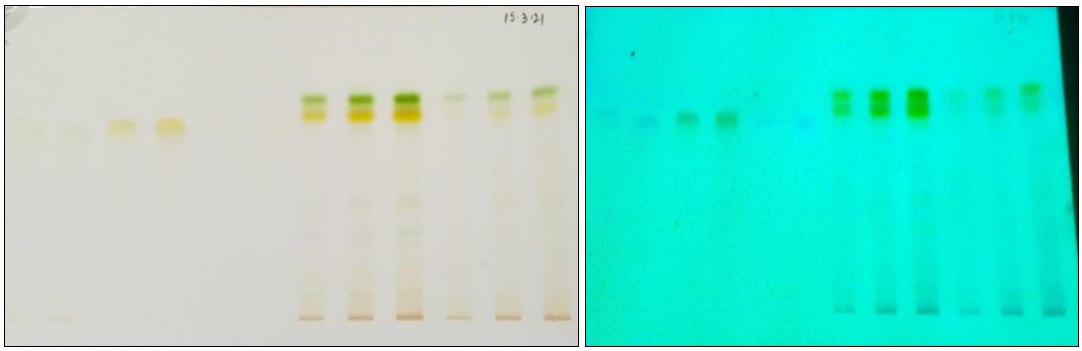
Table 3: Total phenolic, flavonoids and antioxidant activity of *Cissampelos pareira*

Extract	Total Phenolic content (mgGAE/g dry extract)	Flavonoids(mg QE/g dry extract)	Total antioxidant activity (mg ascorbic acid/g dry extract)
Methanol	20.85 ± 1.74	2.30 ±0.29	147± 2.05

Values are mean ± SD (n=3)

High performance thin layer chromatography (HPTLC)

Qualitative analysis of HPTLC of MECP showed presence of phenol and alkaloid in the sample. For HTPLC Catechin, quercetin, reserpine were used as the standards. Ethyl acetate: Methanol: Water (10: 1.35: 1) was used as mobile phase. When MECP was analysed by HPTLC revealed the presence of phenol and alkaloids when compared with the standards catechin, quercetin and reserpine Figure 1, 2 and 3 & Table 4 and 5. Due to the presence of these compounds methanolic extract of *Cissampelos pareira* showed higher antioxidant activity.



Visible light UV radiation

Fig 1: Chromatogram of HPTLC

Table 4: Alkaloid peaks of sample *Cissampelos pareira*

Peak	Start position	Start Height	Max position	End position	Area %	Assigned substance
1	0.08 Rf	0.7 AU	0.09 Rf	0.12 Rf	0.98 %	Unknown*
2	0.14 Rf	0.1 AU	0.17 Rf	0.19 Rf	3.30 %	Unknown*
3	0.19 Rf	40.6 AU	0.22 Rf	0.24 Rf	3.74 %	Unknown*
4	0.24 Rf	35.5 AU	0.25 Rf	0.27 Rf	1.59 %	Unknown*
5	0.27 Rf	21.0 AU	0.33 Rf	0.36 Rf	9.28 %	Unknown*
6	0.36 Rf	75.7 AU	0.37 Rf	0.41 Rf	4.74 %	Unknown*
7	0.41 Rf	18.2 AU	0.46 Rf	0.47 Rf	4.84 %	Unknown*
8	0.48 Rf	63.1 AU	0.49 Rf	0.56 Rf	7.22 %	Unknown*
9	0.60 Rf	22.8 AU	0.64 Rf	0.68 Rf	4.68 %	Unknown*
10	0.69 Rf	1.2 AU	0.79 Rf	0.81 Rf	9.01 %	ALKALOID
11	0.81 Rf	93.0 AU	0.86 Rf	0.90 Rf	2.79 %	Unknown*
12	0.90 Rf	30.7 AU	0.92 Rf	1.02 Rf	7.83 %	Unknown*

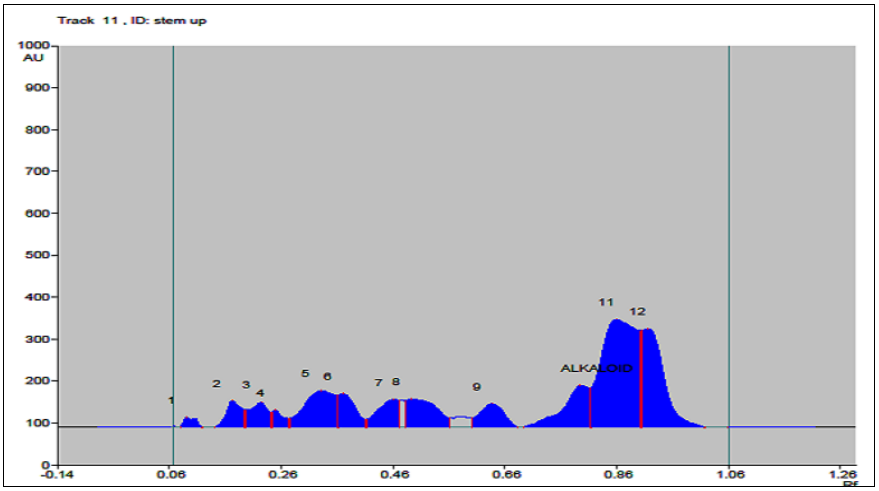


Fig 2: Chromatogram of HPTLC

Table 5: Phenol peaks of sample *Cissampelos pareira*

Peak	Start positior	Start Height	Max position	End position	Area %	Assigned substance
1	0.08 Rf	0.7 AU	0.09 Rf	0.12 Rf	0.98 %	Unknown*
2	0.14 Rf	0.1 AU	0.17 Rf	0.19 Rf	3.30 %	Unknown*
3	0.19 Rf	40.6 AU	0.22 Rf	0.24 Rf	3.74 %	Unknown*
4	0.24 Rf	35.5 AU	0.25 Rf	0.27 Rf	1.59 %	Unknown*
5	0.27 Rf	21.0 AU	0.33 Rf	0.36 Rf	9.28 %	Unknown*
6	0.36 Rf	75.7 AU	0.37 Rf	0.41 Rf	4.74 %	Unknown*
7	0.41 Rf	18.2 AU	0.46 Rf	0.47 Rf	4.84 %	Unknown*
8	0.48 Rf	63.1 AU	0.49 Rf	0.56 Rf	7.22 %	Unknown*
9	0.60 Rf	22.8 AU	0.64 Rf	0.68 Rf	4.68 %	Unknown*
10	0.69 Rf	1.2 AU	0.79 Rf	0.81 Rf	9.01 %	PHENOL
11	0.81 Rf	93.0 AU	0.86 Rf	0.90 Rf	2.79 %	Unknown*
12	0.90 Rf	30.7 AU	0.92 Rf	1.02 Rf	7.83 %	Unknown*

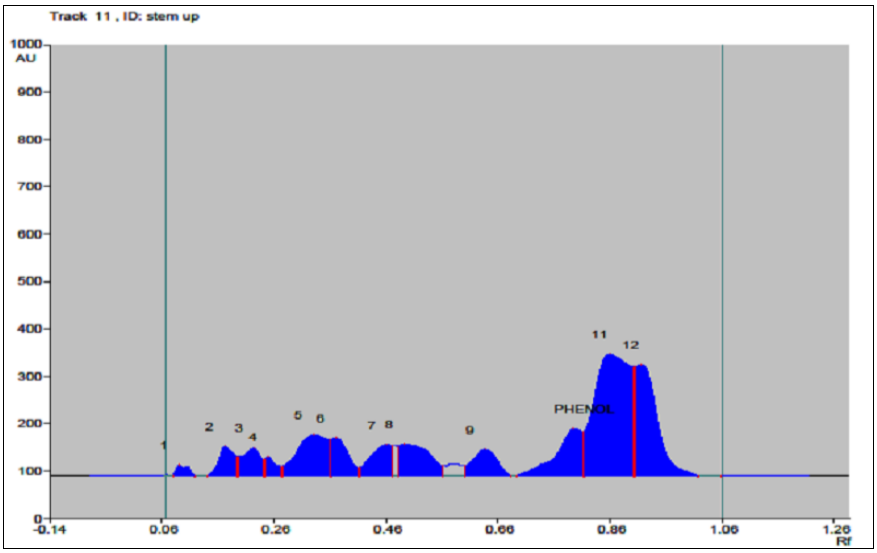


Fig 3: Chromatogram of HPTLC

Discussion

The phytochemical screening in the present study revealed the presence of carbohydrates, glycosides, proteins, amino acids, steroids, phenolics, tannins, flavonoids, saponins, terpenes and alkaloids. Most of the phytochemical constituents were present in the extracts of methanol, ethyl acetate, chloroform and hexane. Steroids were found only in the hexane extract. Several studies have described the antioxidant properties of medicinal plants which are rich in phenolic compounds [28, 29]. Alkaloids have been associated with medicinal properties is their cytotoxicity.

Antioxidant that scavenges free radicals played an important role in various disorders. Studies showed that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action. The alkaloids, tannins, flavonoids and phenol compounds play a major role in preventing a number of chronic diseases by a definite physiological action on the human body like anti-inflammatory, anti-thrombotic, anti-oxidant, hepatoprotective and anticarcinogenic activities [30].

In the present study MECP showed higher activity. The radical scavenging assays were decreased in the following order: methanol > ethyl acetate > hexane. The hexane, ethyl acetate and methanol extracts of *Cissampelos pareira* exhibited a significant dose dependent inhibition of DPPH activity. The MECP showed highest radical scavenging ability when compared to other extracts. MECP showed a high total antioxidant activity, phenolic contents and total flavonoid contents.

HPTLC method were used for the separation of phytoconstituents in MECP. Qualitative analysis of HPTLC showed the presence of phenols and alkaloids in the sample when compared with the standards catechin, quercetin and reserpine.

Conclusion

The present study showed the antioxidant activity of stem part of C. pareira in various extracts such as hexane, chloroform, ethyl acetate and methanol. From the investigation it is understood that methanolic extract showed greatest free radical scavenging activity, so it is used for further *in vitro* studies. Qualitative analysis of HPTLC showed the presence of phenols and alkaloids in the sample when compared with the standards catechin, quercetin and reserpine validating its traditional uses.

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