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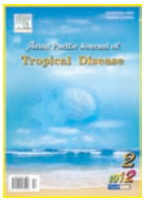

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In vitro antioxidant activities and HPTLC analysis of ethanolic extract of *Cayratia trifolia* (L.)

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ABSTRACT

Objective: To assess the preliminary phytochemical constituents, free radical scavenging activities and HPTLC analysis of the whole plant of the ethanolic extract of *Cayratia trifolia*. **Methods:**2, 2- diphenyl-1-picryl-hydrazyl, reducing power assay, superoxide radical scavenging, hydroxyl scavenging and nitric oxide scavenging assays were used in this study. The HPTLC analysis was also performed to confirm the presence of alkaloids and flavonoids in the ethanolic extract of *Cayratia trifolia*.**Results:** The preliminary phytochemical screening has shown the presence of alkaloid, flavonoids, tannins, saponins and phenolic compounds. The ethanolic extract of *Cayratia trifolia* possesses the free radical scavenging activities. The HPTLC analysis assessed that, the ethanolic extract of *Cayratia trifolia* has five alkaloid compounds and one flavonoid compounds. **Conclusions :**It is concluded that, the *Cayratia trifolia* contains natural source of antioxidants and the ethanolic extract of this plant possess a good free radical scavenging activity. In future, it can be used as a bioactive source of natural antioxidants and are potential natural resources for pharmacology of functional foods.

1. Introduction

Free radicals occurring from metabolism or environmental sources interrelate continuously in biological systems. These play a major role in the formation of chronic and degenerative diseases including cancer, autoimmune, inflammatory, cardiovascular, neurodegenerative diseases and aging [1,2,3]. Recent studies have confirmed that free radicals would injure nearby structures including DNA, proteins or lipids. Radical scavenging antioxidants are mainly significant in protecting cells from the injury of free radical [4]. Thus, antioxidants with free radical scavenging activities may have enormous significance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated [5]

Variety of bioactive substances present in medicinal plants is widely used against various diseases. The demand for natural food constituents has resulted in broad research on naturally occurring antioxidants which are able to deactivate highly reactive free radicals [6]. In this respect, flavonoids and alkaloids which are usually found in medicinal plants have been accounted to have high antioxidant activity as well as multiple biological effects [7]. Currently, the synthetic antioxidants might be unsafe and its toxicity has been criticized. It is generally assumed that frequent use of plant- derived phytochemicals may contribute to shift the stability in the direction of a sufficient antioxidant status. As a result, attention in natural antioxidants, in particular plant origin, has deeply amplified in recent years [8].

Cayratia trifolia Linn. Domin Syn. (Vitaceae) commonly known as Fox grape in English is native to India, Asia and Australia. It is a perennial climber having trifoliated leaves with (2-3 cm), long petioles and ovate to oblong-ovate leaflets. Flowers are small greenish white brown in color [9]. Fruits are fleshy, juicy, dark purple or black, nearly spherical, about 1 cm in diameter [10]. The whole plant is used as anti diuretic, in tumors, neuralgia and splenopathy [11]. This plant also contains kaempferol, myricetin,

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quercetin, triterpenes and epifriedelanol. The bark extract has been reported to have antiviral, antibacterial, antiprotozoal, hypoglycemic, anticancer and diuretic activities in animal models [12]

The aim of the present study is to evaluate phytochemical constituents and the free radical scavenging properties of the ethanolic extract of *Cayratia trifolia*. HPTLC analysis was also performed to confirm the presence of alkaloids and flavonoids in the ethanolic extract of *Cayratia trifolia*.

2. Materials and methods

2.1. Plant collection

Cayratia trifolia was collected from in and around Thanjavur district, Tamil nadu, India. The plant was authenticated by Dr. P. Sathyanarayanan, Botanical survey of India, TNAU Campus, Coimbatore. The voucher number is BSI/SRC/5/23/2010-2011/Tech.1527. Fresh whole plant material was washed under running tap water, tipped on slain overnight, air dried and powdered.

2.2. Preparation of extract

The powder was continuously soaked with petroleum ether, chloroform, ethyl acetate, ethanol and aqueous were kept in the shaker for 48 hour at room temperature. The extracts were collected and concentrated at 40 ℃ under reduced pressure using rotary evaporator. The dried extracts were stored at 4 ℃ until further use.

2.3. Phytochemical screening

The preliminary phytochemical screening was carried out for petroleum ether, Chloroform, ethyl acetate, ethanol and aqueous extract of *Cayratia trifolia* [13]

2.4. Free radical scavenging activities

The free radical scavenging activities of the ethanolic extract of *Cayratia trifolia* was determined by using various *in vitro* assays like 2, 2- diphenyl-1-picryl-hydrazyl (DPPH), reducing power antioxidant, superoxide radical scavenging, hydroxyl scavenging and nitric oxide scavenging.

2.4.1. DPPH radical scavenging assay

The DPPH radical scavenging activity of ethanolic extract of *Cayratia trifolia* was tested by standard method of Blois [14]. Briefly, the reaction mixture contained 100 μM DPPH in methanol, various concentrations (40-200 μg/mL) of the extracts and incubated for 30 minutes at room temperature. The decrease in absorbance was measured at 517 nm. The scavenging activity was calculated as a percentage of the radical reduction. All tests were performed six times. Butylated Hydroxyl Toluene (BHT) was used as a reference compound.

2.4.2. Reducing power assay

The reducing power capacity of the plant was assessed by the modified method of Oyaizu [15]. Various concentrations (40-200 ug/mL) of the extract (0.5 mL) were mixed with 0.5 mL phosphate buffer (0.2 M, pH 6.6) and 0.5 ml potassium hexacyanoferrate (0.1%), following 50 ℃ incubation in a water bath for 20 minutes. After incubation, 0.5 mL of TCA (10%) was added to end the reaction. The upper portion of the solution (1 ml) was mixed with 1 ml distilled water, and 0.1 mL FeCl3 solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against a suitable blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Vitamin C was used as a positive control.

2.4.3. Superoxide radical scavenging

The superoxide scavenging activity of the ethanolic extract of *Cayratia trifolia* was measured by reduction of nitroblue tetrazolium (NBT) [16]. Briefly, Tris HCl buffer (3 mL, 16 mM, pH 8.0) was mixed with 1 ml NBT (50 μM) solution, 1 ml NADH (78 μM) solution and the plant extract (40-200 μg/mL). The reaction was initiated by the addition of 1 mL of phenazine methosulfate (PMS) solution (10 μM) to the mixture. The reaction mixture was incubated at 25 ℃ for 5 min and the absorbance was read at 560 nm against the subsequent blank sample. All tests were performed six times. Vitamin C was used as a reference drug.

2.4.4. Hydroxyl radical scavenging

The hydroxyl radical scavenging activity of the ethanolic extract of *Cayratia trifolia* was measured with a slight modification of Elizabeth and Rao [17]. All the solutions were freshly prepared. The 1ml reaction mixture contained, 2-deoxy-2-ribose (2.8 mM); KH2PO4-KOH buffer (20 mM, pH 7.4); FeCl3 (100 μM); EDTA (100 μM); H2O2 (1.0 mM); ascorbic acid (100 μM) and various concentrations (40-200 μg/mL) of the test sample. After incubation for 1 h at 37 ℃, 0.5 mL of the reaction mixture was added to 1 Ml of 2.8% TCA, then 1 mL of 1% aqueous TBA was added and the mixture was incubated at 90 ℃ for 15 minutes to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Vitamin C was used as a positive control. Percentage of inhibition was evaluated by comparing the test and blank solutions.

2.4.5. Nitric oxide radical scavenging

The Nitric oxide was generated by sodium nitroprusside and measured by the Griess Illosvoy reaction [18]. The reaction mixture contained 10 mM SNP, phosphate buffered saline (pH 7.4) and various doses (40-200 μg/mL) of the

test solution in a final volume of 3 ml. After incubation for 150 min at 25 ℃, 1 mL sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 mL of the incubated solution and allowed to stand for 5 min. Then 1 ml of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25 ℃. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with NED was measured spectrophotometrically at 540 nm against a blank sample. All tests were performed six times. Vitamin C was used as a standard reference.

2.5. HPTLC Analysis

2.5.1. HPTLC analysis for alkaloids

2 μL of the ethanolic extract was loaded as 5mm band length in the 3 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin through developing chamber (after saturated with Solvent vapor) with respective mobile phase (alkaloid) and the plate was developed in the respective mobile phase Ethyl acetate-Methanol-Water (10 : 1.35 : 1) up to 90 mm. The developed plate was dried in hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at White light, UV 254 nm and UV366 nm. The developed plate was sprayed with respective spray reagent (Dragendorff's reagent followed by 10% ethanolic sulphuric acid reagent) and dried at 100 ℃ in hot air oven. The plate was photo-documented in day light mode using photo-documentation (CAMAG REPROSTAR 3) chamber. Scanning: After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at daylight 500 nm. The peak table, peak display and peak densitogram were noted.

2.5.2. HPTLC analysis for flavonoids

2 μL of the ethanolic extract was loaded as 5 mm band length in the 3 x 10 Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with respective mobile phase (flavonoid) and the plate was developed in the respective mobile Ethyl acetate-Formic acid-Glacial acetic acid-Water (10 : 1.1 : 1.1 : 2.4) phase up to 90 mm. The developed plate was dried by hot air to evaporate solvents from the plate. The plate was kept in photo-documentation chamber (CAMAG REPROSTAR 3) and captured the images at white light, UV 254 nm and UV366 nm. The developed plate was sprayed with respective spray reagent 1% ethanolic Aluminium chloride and dried at 100 ℃ in hot air oven. The plate was photo-documented in day light and UV 366 nm mode using photo-documentation (CAMAG REPROSTAR 3) chamber. After derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 500 nm. The peak table, peak display and peak densitogram were noted.

3. Results

3.1. Phytochemical screening

The preliminary phytochemical screening results are illustrated in Table1. These results indicate that, the ethanolic extract of *Cayratia trifolia* has variety of secondary metabolites (Alkaloid, flavonoids, terpinoids, tannins, saponins, proteins and carbohydrates) than other extracts. Based on these results ethanolic extract of *cayratia trifolia* was selected for free radical scavenging activity and HPTLC analysis.

3.2. Free radical scavenging activities

3.2.1. DPPH radical scavenging assay

DPPH Radical Scavenging assay is the standard method to measure the antioxidant potential of compounds [19]. The DPPH approach revealed scavenging capacity of ethanolic extract of *Cayratia trifolia* and the IC_{50} value was 74 ± 0.83 μg/mL which was compared with standard BHT (87 ± 1.12 μg/mL) (Figure 1). The percentage inhibition of plant extract and BHT are more are less similar at 200 μg/mL (88% and 85% respectively). In this assay, these results confirmed that, the ethanolic extract has high inhibition at low concentration compared with standard reference.

3.2.2. Reducing power assay

The reducing power capability was measured by transformation of Fe^{3+} to Fe^{2+} in the presence of ethanolic extract of *Cayratia trifolia* and the reference of Vitamin C. These results were illustrated in Figure 2. At 200 μg/mL, the absorbance of plant extract and Vitamin C were 0.82 and 0.76 respectively. These results indicate that the plant extract has high activity than Vitamin C.

3.2.3. Superoxide radical scavenging

The superoxide radicals produced from dissolved oxygen by PMS-NADH coupling can be measured by their ability to reduce NBT [20]. The decrease in absorbance at 560 nm with ethanolic extract of *Cayratia trifolia* and the reference of Vitamin C indicates their abilities to reduce superoxide radicals in the reaction mixture and their IC_{50} values were 133±2.34 μg/mL and 169±0.92 μg/mL respectively. The percentage of inhibition at 200 μg/mL of ethanolic extract and Vitamin C were 78% and 63% respectively (Figure 3). Compared with reference of Vitamin C, plant extract has high inhibitory activity at low concentration.

3.2.4. Hydroxyl radical scavenging

In the Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction

mixture, the plant extract and standard reference shows their inhibition power of hydroxyl radical-mediated deoxyribose as illustrated in Figure 4. The IC_{50} values of Ethanolic extract of *Cayratia trifolia* and standard Vitamin C were 102±1.72 μg/ml and 93±0.48 μg/mL respectively. The IC_{50} value of extract is less than the standard. The percentage of inhibition at 200 μg/mL of ethanolic extract and Vitamin C were 85% and 84% respectively.

3.2.5. Nitric oxide radical scavenging

This assay revealed the abilities to inhibit the free radicals of ethanolic extract of *Cayratia trifolia* and standard Vitamin C as illustrated in figure 5 and their IC_{50} values were 77 ± 1.34 μg/mL and 122 ± 0.58 μg/mL respectively (Figure 5). The IC_{50} value of plant extract is less than the standard. The percentage of inhibition at 200 μg/ml of ethanolic extract (high) and Vitamin C (less) were 89% and 80% respectively. All the experimental results were expressed as mean ± SD of six replicates.

3.3. HPTLC Analysis

3.3.1. HPTLC analysis for alkaloids

Orange-yellow brown colored zone at daylight mode was observed from the chromatogram after derivatization (Figure 6), which confirmed the presence of alkaloid in the extract (compared with the standard). The Rf value in the extract was found to be 0.11, 0.16, 0.20, 0.27, 0.37, 0.54, 0.65, 0.75, 0.82, 0.91 (Table 2) of peaks 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and that of alkaloid standard was 0.43. Among them the peaks 4, 5, 6, 7, 10 showed the presence of alkaloid (Figure 7).

3.3.2. HPTLC analysis for flavonoids

Yellow coloured fluorescent zone at UV 366 nm mode was observed from the chromatogram after derivatization (Figure 8), which confirmed the presence of flavonoid in the given standard and in the extract. The Rf value for the ethanolic extract was found to be 0.07, 0.32, 0.37 and 0.97 of peaks 1, 2, 3 and 4 (Table 3). Among them, the peak 4 showed the presence of flavonoid (Figure 9).

4. Discussion

Free radicals are the major cause of various chronic and degenerative diseases, in the living systems. The vast amounts of synthetic molecules are available for free radical scavenging antioxidants but adverse side effects are associated with these compounds. An alternative solution for this problem is to consume the naturally available antioxidants from the medicinal plants because they are having lower side effects and comparatively safe [21]. Many of the naturally occurring antioxidant compounds like alkaloids, flavonoids were isolated from various medicinal plants [22]. Previous studies revealed that the *Cayratia trifolia* is medicinally important and used in the treatment of various diseases [23].

Isolation of pharmacologically active components from medicinal plant is the long and tedious process. Therefore, the phytochemical screening is necessary to eliminate unnecessary separation procedures. This method is performed to allow localization and targeted isolation of new or useful constituents with potential activities and also this procedure enables recognition of known metabolites in extracts or at the earliest stages of separation and is significant for inexpensive [24]. In this study, the preliminary phytochemical screening ascertained the presence of alkaloids, flavonoids, phenolic components, tannins, steroils and steroids, saponins, amino acids and carbohydrates which confirmed that, *Cayratia trifolia* have pharmacologically active components.

The *in vitro* antioxidant activities of the ethanolic extract of *Cayratia trifolia* was investigated by various assays. The free radical scavenging test using the stable DPPH radical is a widely employed method to evaluate antioxidant activities, since it needs relatively short time compared with other methods. The effect of antioxidants on DPPH radical was thought to be due to their hydrogen donating ability. The DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule [25]. The reducing power is highly related to the amount of phenolic compounds especially flavonoids that is present in the extracts, which can serve as electron donor to terminate the radical chain reaction [26]. Various *in vitro* models like DPPH, superoxide radical scavenging assay, hydroxyl radical scavenging assay and nitric oxide scavenging assay that were evaluated also supported the free radical scavenging activities of ethanolic extract of *Cayratia trifolia*.

The phytochemical components like alkaloids, flavonoids, terpenoids, tannins, glycosides are known to be responsible for the antioxidant activities of the plants. The alkaloids which are nitrogen-containing naturally occurring compound, commonly found to have antimicrobial properties due to their ability to intercalate with DNA of the microorganisms [27]. Flavonoids in the plants have strong free radical scavenging properties. The multiple pharmacological properties of flavonoids, such as anti-inflammatory, antibiotic and cardiovascular activities are to a large extent, linked to their polyphenolic and hence radical scavenging nature and act as primary antioxidants or free radical scavengers [28,29]. It is interesting to note that the phytochemicals alkaloids and flavonoids are commonly associated with various pharmacological activities of natural products [30]. The presence of alkaloids and flavonoids in the ethanolic extract of *Cayratia trifolia* was confirmed by Preliminary phytochemical studies and HPTLC analysis. The present study was concluded that the ethanolic extract of *Cayratia trifolia* possess a good free radical scavenging activity which may due to the presence of alkaloids and

flavonoids.

Conflict of interest statement

We declare that we have no conflict of interest.

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