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ORIGINAL ARTICLE

PHENOLIC COMPOSITION, VOLATILE CONSTITUENTS AND ANTIOXIDANT POTENTIAL OF WILD EDIBLE FRUIT *ELAEOCARPUS TECTORIUS* (LOUR.) POIR. (*ELAEOCARPACEAE*)

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Abstract

Elaeocarpus tectorius (Lour.) Poir. is a wild edible fruit of Western Ghats in India, belonging to family *Elaeocarpaceae*. Ethnobotanically, the plant is known to possess multiple health benefits. In the present study, methanol extract of *E. tectorius* fruit was screened for total phenolic content, total flavonoid content and antioxidant activity. The *in vitro* antioxidant activity was evaluated using four different assays: total antioxidant capacity, reducing power assay, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Further, HPLC and GC-MS analysis was carried out to investigate the potential phenolic acids and volatile constituents present in the fruits. Results indicated that methanol extract of *E. tectorius* fruit successfully scavenged various free radicals evaluated in a dose dependent manner. HPLC and GC-MS profiling revealed the presence of six vital phenolic acids and twelve volatile constituents, respectively. Thus, observations of the present study offer a preliminary evidence of nutritive value of the *E. tectorius*, moreover this fruit can serve as a potential source of antioxidants and can be utilized in food and nutraceutical industries.

Rezumat

Elaeocarpus tectorius (Lour.) Poir. este un fruct sălbatic comestibil din India, aparținând familiei *Elaeocarpaceae*. Din punct de vedere etnobotanic, planta este cunoscută ca având beneficii multiple pentru sănătate. În studiul de față, extractul metanolic obținut din fructul *E. tectorius* a fost testat pentru determinarea conținutului fenolic și flavonoidic total, precum și a activității antioxidante. Activitatea antioxidantă *in vitro* a fost evaluată folosind: capacitatea antioxidantă totală, puterea de reducere a 2,2-difenil-1-picrilhidrazil (DPPH) și a acidului 2,2′-azino-bis(3-etilbenzotiazolin-6-sulfonic) (ABTS). De asemenea, a fost efectuată analiza HPLC și GC-MS pentru a determina posibilii acizi fenolici și compușii volatili prezenți în fruct. Rezultatele au indicat faptul că extractul metanolic din fructul *E. tectorius* a prezentat acțiune antioxidantă doză dependentă. Profilul HPLC a indicat prezența a șase acizi fenolici, iar GC-MS a indicat prezența a 15 compuși volatili. Astfel, observațiile studiului de față reprezintă o dovadă preliminară cu privire la valoarea nutritivă a *E. tectorius*.

Keywords: antioxidant activity, *Elaeocarpus tectorius*, GC-MS, HPLC, total flavonoid content, total phenolic content, wild fruit

Introduction

Plants are considered as primary and rich source of medicines. Plants and plant-based products are favourite choice among people across globe, as they are safe and have no side effects. Several medicinal plants are exploited in identification and elucidation of phytochemicals responsible for therapeutic nature. Different parts of plants possess wide array of phytochemicals that are known to have several health benefits such as antimicrobial and antioxidant activity [31]. Fruits are a rich and natural source of nutritional components and antioxidants. Epidemiological studies indicate that regular consumption and high dietary intake of fruits has beneficial effect against several chronic diseases including hypertension, diabetes and several forms of cancer [2, 4, 6, 34]. India is one of the richest tropical biodiversity countries consisting of several indigenous fruits. Western Ghats of Southern India is a biodiversity hotspot known for several indigenous and endemic fruits. Wild edible fruits are abundantly available during their glut season. Availability of nutritive and phytochemical information on these wild edible fruits is very scarce despite the several attempts that have been made in recent past. Fruits constitute good amount of phenolic acids that provide several health benefits other than nutritional fulfilment. The quantity of phenolic acids in fruits is dependent on multiple intrinsic and extrinsic factors including variety, geography, soil and plant health, climate and storage conditions [3, 9]. Phenolic acids are generally classified based on the number of phenol rings, such as stilbenes, flavonoids and tannins. These phenolic compounds consist of single or multiple

hydroxyl groups directly linked to an aromatic ring. Phenolic acids, namely, caffeic, chlorogenic, ferulic and *p*-coumaric acids are potential antioxidants in comparable to that of hydroxy derivatives of benzoic acid such as *p*-hydroxybenzoic, vanillic and syringic acids [17, 32].

The ability of a molecule to inhibit the oxidation of other molecule is termed as antioxidants. The imbalance between the free radical generation and antioxidant defence mechanism leads to oxidative stress which is considered to be the main cause for many disorders and chronic diseases [1]. Thus, the only prerequisite to avoid oxidative stress is the dietary intake of food rich in antioxidants. Various natural antioxidants present in the plant and plant-based products successfully inhibit oxidation reaction by donating an electron to free radicals generated through oxidative stress [33]. Phenolic acids, coloured pigments and vitamins are natural source of antioxidants, while, synthetic antioxidants such as butylated hydroxy-anisole, propyl gallate, tert-butylhydroquinone and butylated hydroxytoluene are all reported to have adverse side effects [5, 28]. Hence the quest for exploring natural antioxidants is increasing day by day in this context, several attempts are being made by the scientific community.

Elaeocarpus tectorius (Lour.) Poir. (*Elaeocarpaceae* family) is a tall tree that grows up to 40 meters, found in the higher altitude of the Nilgiris, a part of Western Ghats and UNESCO World Network of Biosphere Reserves. Fruits are green in colour, sweet to taste and commonly available during the months of July to August. Locally, the fruit is called by different names such as *bikkihannu* and *bikkipalzam* in *Tamil* language. Previous studies show that the fruit is known to comprise following nutritive composition: moisture (%): 59.30, proteins (mg/100 g): 1.4, fibres (mg/100 g): 1.6, phosphorous (mg/100 g): 26.00, iron (mg/100 g): 3.10, calcium (mg/100 g): 37.00, carotenes (mg/100 g): 190.00, thiamine (mg/100 g): 0.02, niacin (mg/100 g): 0.30 and riboflavin (mg/100 g): 0.06, [20]. Traditionally, fruits are consumed by *badagas*, a tribal community of Nilgiris forests and it is commonly used against microbial infections and several health issues including rheumatism and piles [20, 25-27]. The aim of this study was to evaluate the total phenolics, total flavonoids, individual phenolic acids, volatile constituents present in the methanolic extract of *E. tectorius* fruit and further assessment of the anti-oxidant potential by using multiple *in vitro* assays.

Materials and Methods

Reagents

All chemicals used in the present study were of analytical grade. Ascorbic acid, aluminium chloride, potassium ferricyanide, ferric chloride, Folin-Ciocâlteu reagent, sodium carbonate, trichloroacetic acid, glacial acetic acid, sodium phosphate (monobasic and dibasic) and methanol (HPLC grade) were purchased from Merck Chemicals, Mumbai, India. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-Azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) and phenolic acids such as gallic, protocatechuic, chlorogenic, vanillic, caffeic, syringic, *p*-coumaric, ferulic and trans-cinnamic acids were obtained from Sigma-Aldrich Company Ltd. (Bangalore, India).

Plant material and sample preparation

Mature and healthy fruits of *E. tectorius* were collected from Nilgiri hills of Ooty in Tamil Nadu, India. An identified plant specimen was deposited at the Herbarium of Department of Botany, Karnatak University, Dharwad, India (KU/AM/DT-012). Fruits were washed with deionized water, cleaned, chopped in to small pieces, oven dried at 45 ± 2°C and dried samples was crushed in to fine powder. Extraction was then carried out using methanol as a solvent in a sonicator at keeping a ratio of 1:10, sample and solvent, respectively. Further it was centrifuged at 10,000 rpm at 4°C for 15 min. The homogenate was filtered through polytetrafluoroethylene (PTFE) membrane filter (0.45 µm). Finally, the sample was completely dried in a rotary evaporator.

Estimation of total phenolic content

The total phenolic content in the methanolic fruit extract of *E. tectorius* was determined following the protocol described by Kumar *et al*. [14]. Known aliquots of methanol extract and standards (1 mg/mL) were taken in separate test tubes and volume was made up to 3 mL by adding deionized water. Folin-Ciocâlteu's reagent (0.5 mL) was mixed to each tube and incubated for 3 min at room temperature, a solution of sodium carbonate (20%, 2 mL) was added, thoroughly mixed and further these tubes were kept in boiling water bath for 1 min. Absorbance was recorded at 650 nm using a double beam UV-Visible Spectro-photometer (UV-1800, Shimadzu, Japan) against a blank reagent and calibration curve was plotted using various concentrations of gallic acid (20 - 100 µg/mL) and expressed as milligrams of gallic acid equivalents *per* gram dry weight (mg GAE/g dw).

Estimation of total flavonoid content

Estimation of flavonoid content in the sample was done following the protocol described by Helmja *et al*. [10], with slight variation in reaction volumes. Briefly, methanolic fruit extract (500 µg/mL) and standards were pipetted out in a separate test tubes and volume was made up to 0.5 mL with deionized water. Sodium nitrite (5%; 0.03 mL) was added to each tube, after 5 min incubation at room temperature, aluminium chloride solution (10%; 0.06 mL) was added, incubated for 5 min at room temperature and further sodium hydroxide solution (1 M, 0.2 mL) was mixed and total volume was made up to 1 mL with deionized water by adding 0.210 mL. Absorbance

was measured at 510 nm against a blank reagent. A calibration curve was prepared using different concentrations of catechin (20 - 100 µg/mL). From the standard curve, concentration of flavonoids in the methanolic fruit extract was determined and expressed as milligrams of catechin equivalents *per* gram dry weight (mg CE/g dw).

Total antioxidant capacity

Determination of total antioxidant activity was done following a method of Prieto *et al*. [24] with a slight modification in the reaction mixture. Briefly, 0.2 mL of methanolic fruit extract was taken in a test tube. To this, 1.8 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added. The tubes were kept at 95°C for 90 min in a water bath and allowed to cool. The absorbance was measured at 695 nm against a blank reagent using double beam UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan). The values are represented as µg ascorbic acid equivalents (AAE) *per* gram dry weight.

Reducing power activity

Determination of reducing power activity of methanol extract was done following the method of Oyaizu [21]. Different concentrations of sample (200 - 1000 µg/mL, made up to 1.0 mL with methanol) was mixed with freshly prepared 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%), and this was kept at 50°C for 20 min in a water bath allowing the reaction to complete. After incubation period, 2.5 mL of 10% trichloroacetic acid was added, further this was centrifuged at 650 rpm for 10 min. Supernatant (2.5 mL) was mixed with deionized water (2.5 mL) and further 0.5 mL of $FeCl_3$ (0.1%) was added. Finally, absorbance was read at 700 nm using a double beam UV-Visible Spectrophotometer (UV-1800, Shimadzu, Japan).

DPPH radical scavenging activity

DPPH radical scavenging activity of methanolic fruit extract of *E. tectorius* was determined spectrophotometrically following the protocol of Lee *et al*. [15] and Jagtap *et al*. [11]. DPPH solution (24 mg dissolved in 100 mL of methanol) was further diluted with methanol until the absorbance was achieved to 1.1 ± 0.01 units at 517 nm using a double beam UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan). 3 mL of DPPH solution was mixed to various concentrations of sample (200 - 1000 µg/mL), vortexed and incubated for 30 min at 37 ± 2°C in dark. Methanol with no added samples was taken as control. Percentage radical scavenging activity (RSA) was calculated using the formula:

$$\% \text{ RSA} = [(A_{control}-A_{sample})/A_{control}] \times 100,$$

where, A = absorbance at 517 nm.

ABTS assay

ABTS assay was done following the procedure described by Thaipong *et al*. [30] with slight variation in the reaction volume. Preparation of stock solutions was done by taking 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution. Working solution was prepared by mixing these two stock solutions in equal volume and reaction mixture was kept for incubation in dark at room temperature. Finally, this solution was diluted by taking 1 mL reacted ABTS solution with 60 mL methanol to obtain an absorbance of 1.170 ± 0.02 units at 734 nm using a double beam UV-Visible Spectrophotometer (UV-1800, Shimadzu, Japan). For each assay, ABTS solution was prepared freshly. A 300 µL of various concentrations of fruit extract was mixed with 2700 µL of the ABTS working solution, which was allowed to react in dark. Finally, absorbance was read at 734 nm.

HPLC analysis of phenolic acids

HPLC analysis was performed with the aid of liquid chromatograph multi gradient system equipped with manual injector and a diode-array detector (LC-10A, Shimadzu, Japan). The analytical column was a C18 column (250 × 4.6 mm) packed with 5 µm particle size. Solvent A consisted of acetonitrile whereas solvent B consisted of 2% acetic acid in water. Gradient profile was followed according to the method of Kim *et al*. [13]: 100% B to 85% B in 30 min, 85% B to 50% B in 20 min, 50% B to 0% B in 5 min and 0% B to 100% B in 5 min, flow rate of 1.0 mL/min was maintained and sample volume of 10 µL was injected with a total programme run time of 70 min and simultaneous observation at 280 and 320 nm. Phenolic acids in sample were identified and quantified by comparing peaks with that of the congruent retention time of reference standards; that were spiked instantly after sample analysis.

GC-MS analysis

GC-MS analysis of the methanol extract of *E. tectorius* fruit was done following the procedure described by Geetha *et al*. [7]. Briefly, GC-MS (GCMS-QP2010, Shimadzu, Japan) equipped with fused silica capillary column (Rtx-5MS, crossbond diphenyl dimethyl polysiloxane) was used for GC-MS detection with an electron ionization system at ionization energy of -70 eV. Helium (99.999%) was used as carrier gas with a flow rate of 1 mL/min and sample volume injected was 1 µL. Injector temperature and ion source temperature was set to 250°C and 200°C, respectively. The oven temperature was programmed at 110°C to 200°C with a rate of 10°C/min and with a final temperature of 260°C at 5°C/min. Interface temperature was kept at 250°C. Compounds were identified based on the retention time, retention index, mass spectra of each putative compound with those of the NIST library (2005). The area percentage of the identified compounds was expressed in terms of percentage with peak area normalization.

Statistical analysis

All assays were carried out in triplicate and results were represented as mean ± standard deviation (SD) on a dry weight basis. Statistical analysis was carried out with XLSTAT software (2014.5.03, Addinsoft, NY) using ANOVA and differences at $p < 0.05$ were considered significant.

Results and Discussion

Phenolic acids as secondary metabolites are found abundantly and naturally in various parts of plants. Fruits constitute several vital phenolic acids and are responsible for taste, colour, aroma and flavour. Several epidemiological studies have proved that dietary intake of fruits rich in phenolic acids is known to be beneficial against several chronic degenerative diseases. Therefore, till to date more than 8000 polyphenols have been identified and their biological significance is being investigated [22]. In the present study, the total phenolic and flavonoid content observed in the methanolic extract of *E. tectorius* fruit was 10.96 ± 0.14 mg GAE/g dw and 1.25 ± 0.04 mg CE/g dw, respectively.

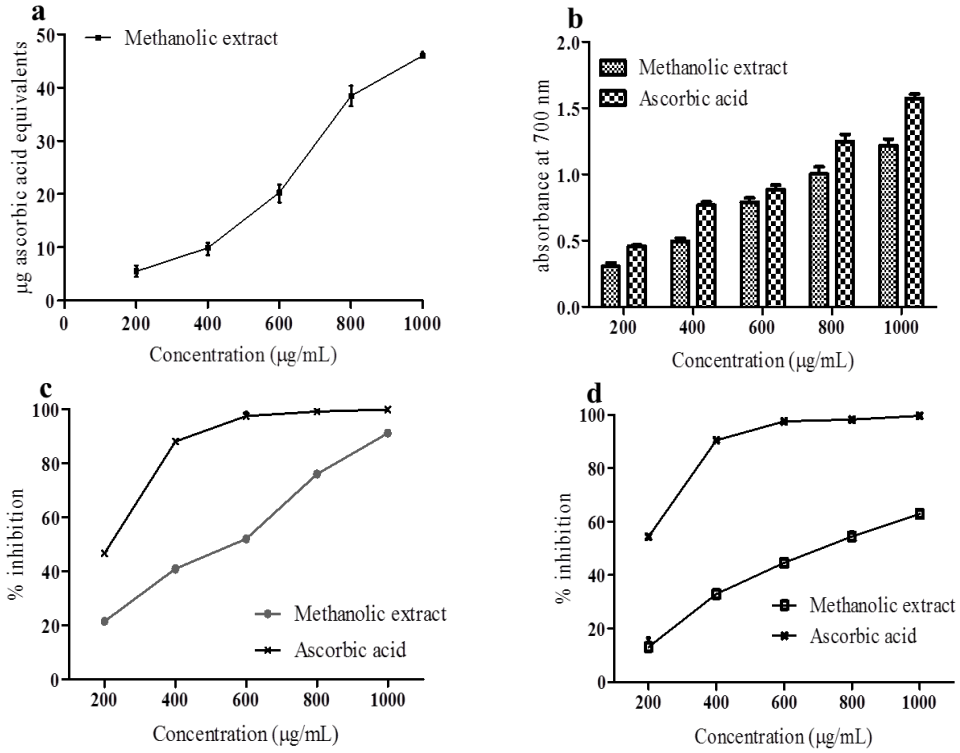

Figure 1.

Antioxidant activity of methanolic fruit extract of *E. tectorius* against various radicals: (a) Total antioxidant activity, (b) Reducing power effect, (c) DPPH radical scavenging activity and (d) ABTS radical scavenging activity

Total antioxidant activity was determined by phospho-molybdenum method and expressed as mg ascorbic acid equivalents (AAE)/g dw. The methanolic fruit extract comprised a good proportion of antioxidant capacity (Figure 1a). However, the methanol fruit extract had 10.626 mg ascorbic acid equivalents/g dw. The reducing power ability of the extract is dependent upon the reducing agents present in it. The methanol extract exhibited a significant reducing power effect signifying that it comprises fair amount of reducing agents (Figure 1b). Higher absorbance values indicate higher reducing power ability. Thus, the methanolic fruit extract of *E. tectorius* showed higher reducing power with increased concentration. The DPPH assay is a simple, fast and one of the oldest techniques, which is widely accepted and employed for assessing the antioxidant activity of particular test sample. Generally, 2,2-diphenyl-1-picrylhydrazyl reacts with hydrogen donors such as phenolic acids and vitamins present in the test sample. The intensity of the change in colour of DPPH reaction mixture increases with the increasing concentrations of the test sample. The disappearance of colour is predominantly dependent on the presence of the antioxidants such as phenolic acids and vitamins in the sample. The radical scavenging activity of methanol fruit extract exhibited strongest inhibition at the concentration of 1000 µg/mL with a 92.91 ± 0.18 % of RSA (Figure 1c). The IC_{50} value for the DPPH radical was found to be 571.7 ± 2.04 µg/mL. The ABTS assay is considered as a best tool to define the antioxidant ability of bioactive compounds. Many recent reports prove that plant extracts comprise various phenolic acids that are responsible for the antioxidant activity against ABTS free radicals. In the present study, methanolic fruit extract of *E. tectorius* exhibited a potential scavenging activity against ABTS

radicals (Figure 1d) with an IC_{50} value of 469.0 ± 1.82 µg/mL. This may be attributed to the presence of vital phenolic acids and bioactive constituents in the fruit.

Individual phenolic acids in methanolic fruit extract of *E. tectorius* were detected by HPLC (Figure 2). The major peaks were identified by comparison with nine standard phenolic acids spiked (Figure 2a), in which phenolic acids such as gallic (29.53%), vanillic (3.423%), syringic (4.461%), *p*-coumaric (7.241%), ferulic (5.680%) and *trans*-cinnamic acids (10.93%) were found (Figure 2b). However, phenolic acids, *viz*., caffeic acid, chlorogenic acid and protocatechuic acid were not detected among the standards analysed. Although, unknown major peaks at 13th and 50th min with an area % of 23.266 and 10.495, respectively, were also detected. Prakash *et al*. [23] reported the presence of three phenolic acids in wild edible fruit, *E. sikkimese*, *viz*., caffeic, ferulic and gallic acids. However, in the current study, *E. tectorius* showed wide array of phenolic acids.

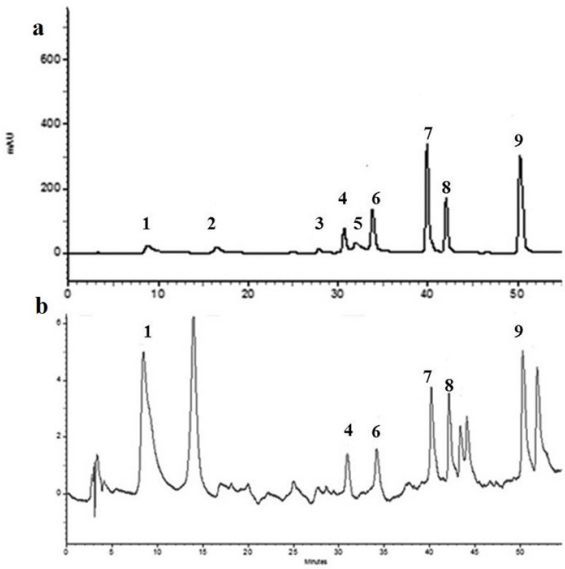

Figure 2.

HPLC chromatogram depicting standard phenolic acids (a) and methanolic fruit extract *E. tectorius* (b) at 280 nm: (a) highlights chromatogram of standard phenolics, viz. (1) gallic, (2) protocatechuic, (3) chlorogenic, (4) vanillic, (5) caffeic, (6) syringic, (7) *p*-coumaric, (8) ferulic, and (9) *trans*-cinnamic acids; (b) corresponds to phenolic acids detected in methanolic fruit extract of *E. tectorius*.

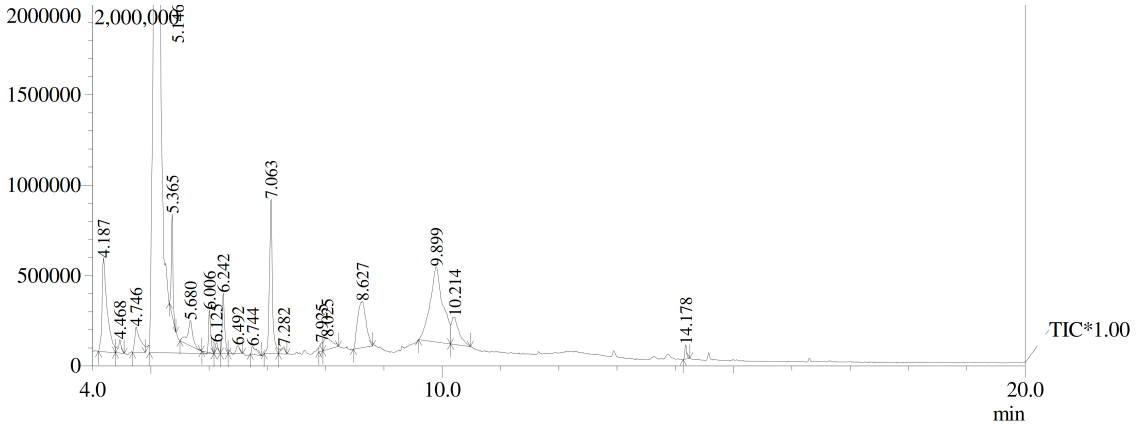

Figure 3.

GC-MS chromatogram of methanolic fruit extract of *E. tectorius*

The volatile constituents present in the methanolic fruit extract of *E. tectorius* were detected by GC-MS analysis (Figure 3) with a total run time of 35 minutes. 12 compounds were identified with the aid of mass spectra and comparison with NIST library. The predominant compounds retention time, retention index, area (%), molecular formula, molecular weight and nature of compound are presented in Table I. Among the identified volatile constituents, 5-hydroxymethylfurfural, 1,6-anhydro-β-D-glucopyranose and 1,5-anhydro-D-mannitol were predominant. Several studies indicate that dry fruits are rich source of 5-hydroxymethylfurfural, whereas some studies indicate the adverse effects of 5-HMF as well [8, 18, 25]. Recent studies suggest that 5-HMF may possess several favourable bioactivities including *in vitro* antioxidant, antimutagenic and antihypoxic activities [12, 16, 19, 29, 35].

Table I

GC-MS profiling of methanolic fruit extract of *E. tectorius*

Retention time (min)	Compound name	Retention index	Area %	Molecular formula	Molecular weight	Nature of compound
4.746	1,5-Anhydro-D-mannitol	672	8.30	$C_6H_{12}O_5$	164.15	Sugar
5.146	2-Methylcyclopentanone	729	1.28	$C_6H_{10}O$	98.14	Ketone
5.365	3-Heptanol	884	0.26	$C_7H_{16}O$	116.20	Alcohol
5.680	5-Hydroxymethylfurfural	956	39.94	$C_6H_6O_3$	126.11	Aldehyde
6.006	Hexanoic acid	967	1.77	$C_6H_{12}O_2$	116.16	Fatty acid
6.125	6-Oxoheptanoic acid	1022	1.17	$C_7H_{12}O_3$	144.16	Keto acid
6.242	3-Hydroxy-2-methyl-4-pyrone	1061	0.75	$C_6H_6O_3$	126.11	Flavone
6.492	4-Methylhexyl acetate	1267	0.22	$C_9H_{18}O_2$	158.24	Alcohol
7.925	1H-Azonine, octahydro-1-nitroso-	1526	1.42	$C_8H_{16}N_{20}$	119.16	Nitro compound
9.899	Hexadecanoic acid	1959	0.21	$C_{16}H_{32}O_2$	256.42	Fatty acid
10.214	5-(2-Propynyloxy)-2-pentanol	2273	0.28	$C_8H_{14}O_2$	142.19	Alcohol
14.178	4-(1-Hydroxyethyl)-γ-butanolactone	2348	1.20	$C_6H_{10}O_3$	130.14	Furanone

Conclusions

The quantitative estimation of the methanolic fruit extract of *E. tectorius* showed the presence of total phenolic content and total flavonoid content of 10.96 ± 0.14 mg GAE/g dw and 1.25 ± 0.04 mg CE/g dw, respectively. Moreover, HPLC analysis revealed it to be a good source of various vital phenolic acids, *viz*., gallic, vanillic, syringic, ferulic, *p*-coumaric and *trans*-cinnamic acids. Further, GC-MS profiling indicated the presence of various volatile constituents wherein 5-hydroxymethylfurfural was found to be the major compound. Methanolic fruit extract of *E. tectorius* exhibited potential antioxidant activity against various free radicals assayed, which may be attributed to the phenolics and flavonoids present in the fruit. In conclusion, the observed results offer nutraceutical significance of the fruit that can be utilized in the food industry. Nevertheless, extensive study is essential in order to exploit the potential bioactivities of *E. tectorius* fruit against certain diseases as traditionally it is acclaimed.

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