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HPLC Assay, Phytochemical, FTIR Characterization and Studies on Antioxidant Activity of *Elephantopus scaber* (Linn) Using Six Different Soxhlet Leaf Extract

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ABSTRACT

In the present study, six solvents i.e., ethanol, methanol, aqueous, pet ether, ethyl acetate and chloroform were used independently for the extraction of bioactive phytoconstituents in Elephantopus scaber using successive soxhlet extraction method. There phytoconstituents were analyzed for their phytochemical properties, High Performance Liquid Chromatography (HPLC) assay, Fourier Transform Infra-Red (FTIR) profile and antioxidant status. The phytochemical analysis revealed the presence of alkaloids, flavonoid, saponin, steroid, tannin, terpenoids and cardiac glycoside. A reversed-phase HPLC analysis was performed using C18-150 × 4.6 mm column with 10 ul injection volume and Methanol: Water as a mobile phase in 60:40 ratios at 30°C. The detection was recorded at 370 nm (UV-detector). The highest content of Rutin (205 ± 0.05 µg/ml) and Quercetin (38.55 ± 0.02 µg/ml) was estimated in ethanolic extract. The FTIR spectroscopic studies revealed different characteristic stretching frequencies, peak values with various functional compounds in the studied extracts. We investigated the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) antioxidant activities of various E. scaber soxhlet leaf extracts. Results obtained by DPPH radical scavenging assay in the leaf extracts of E. scaber, indicated that the ethanolic leaf extracts exhibited significantly highest antioxidant radical scavenging activity (91.34 ± 0.03 with IC_{50} 139.6 µg/ml) as compare to methanolic and pet ether extracts. The chloroform extract indicated comparatively lowest scavenging activity (65.48 ± 0.03 µg/ml with IC_{50} 187.7 µg/ml). The aqueous and ethyl acetate extract does not revealed antioxidant activity. It can be concluded that E. scaber soxhlet leaf extracts can be used effectively in the production of potential natural antioxidant for commercial application.

Keywords: *Elephantopus scaber*, Phytochemical, HPLC, FTIR, DPPH, Antioxidant

INTRODUCTION

The phytochemicals presents in the medicinal plant extract are non-toxic, effective at low concentration, low cost and friendly with the environment. Phytochemical studies have attracted the attention of plant scientists due to the development of new and sophisticated techniques. These techniques played a significant role in the search for additional resources of raw material for pharmaceutical industry [1]. Plant leaf extraction is the main step for the recovery and isolation of bioactive phytoconstituents from plant materials [2]. High Performance Liquid Chromatography (HPLC) analysis is most widely used methodology and easily adapted for the flavonoids quantification during last 20 years [3]. Wosch et al., [4] reported that the quality of raw material and related products has been therapeutic use and it is possible by using analytical techniques like, Thin Layer Chromatography (TLC) and HPLC. Infrared spectroscopy is one of the most often used spectroscopic tools for the study of functional groups in plants. Identification of the chemical nature of phytochemical compounds present in the medicinal plants will provide some information on the different functional groups responsible for their medicinal properties [5].

Plants extracts consists in a complex mixture of several compounds as alcohols, phenols, carboxylic acid, esters, aldehydes, ketones, carbohydrates, terpenes, alkanes, alkenes, aromatics, alkyl halides, etc. in addition to crude extracts, purified fractions and pure compounds have already been used in an antioxidant approaches and several responses have been obtained. Conversely now a day it is necessary to search new sources and compound of specific antioxidant for determine objective. Phenolic compounds are commonly reported to have the antioxidant activities [6]. At present, there are many approaches available to search for new biologically active ingredients in the medicinal plants for the preparation of safe drugs. Many workers have extended their work to evaluate and discover new antioxidant, antimicrobial and antifungal ingredients from different kinds of natural sources like soil, microorganism, animals and plants [7]. The plants extract have been reported to exhibit functional properties like angiotensin I-converting enzyme inhibitors, antibiotics and cancer cells inhibitors. Several human diseases are known to be related to free radicals. The free radical scavenging medicines are antioxidants in nature. Consequently, a relationship exists between human health and minor nutrients exhibiting antioxidant activities, such as vitamin C, vitamin E, b-carotene, flavonoids, and other antioxidants [8].

The medicinal plant *E. scaber* is a member of the family Asteraceae known for its medicinal properties. In Ayurvedic system of medicine, the extract of *E. scaber* is described as antipyretic, alexipharmic and cough preventive medicine. It is also potentially effective on disorders of blood, heart and bronchitis. In Yunani system of medicine, this plant is described as an analgesic and its extract is used as a brain tonic. The leaves are used in pain and piles. The flowers are aphrodisiac. Plant is also used for liver troubles. The *E. scaber* has antitumor, hepatoprotective, antibacterial (Gram-positive and Gram-negative) and antiviral activities [9-11]. Chemical study started from 1960's and showed that constituents of *E. scaber* L. include flavonoids, triterpenoids and flavonoid esters [12].

The plant (root, leaf and bark) is used medicinally, because of the presence of epifriedelinol, lupeol and stigmasterol throughout the whole plant [13,14]. Deoxyelephantopin and isodeoxyelephantopin are found to be tumor inhibitors [15,16]. Previous phytochemical investigations on *Elephantopus* have also resulted in the isolation of flavonoids [17]. Triterpe-noids, diosgenin [18] and sterols. Among these compounds, sesquiterpene lactone is regarded as a chemotaxonomic marker for the genus *Elephantopus* [19]. *E. scaber* L. is known to contain a large number of bioactive compounds such as lipids, phytochemicals, pharmaceutics and pigments. For example, ethyl hexadecanoate, ethyl-9, 12-octadecadienoate, ethyl-(Z)-9-octadecenoate, ethyl octadecanoate, lupeol, stigmasterol, stigmasterol glucoside, deoxyelephantopin (1) and two new germacranolide sesquiterpene lactones named 17, 19-dihydrodeoxyelephantopin (2) and iso-17, 19-dihydrodeoxyelephantopin [20]. Previous bioactivity studies on *E. scaber* demonstrated that the extracts or compounds from this species have antibiosis, antivirus, and cytotoxicity activities. The cytotoxicity effect against HpeG2 cell line of the new compound was determined by MTT assay and the IC_{50} value as reported by Wang et al. [21].

The literature survey revealed that the systemic evaluation including pharmacognostic, HPLC analysis, FTIR and antioxidant study of this plant is poorly attended. The work is undertaken with objectives to investigated phytochemical constituents present in six different solvents leaf extracts extracted by soxhlet, to identify the main functional groups and to analyze the antioxidant activity of *E. scaber* using these soxhlet extracts.

MATERIAL AND METHODS

Plant material and chemicals

The leaf of *E. scaber* was used for present analysis. The plants were grown in the Botanical garden of The Institute of Science, Mumbai and taxonomically authenticated from Department of Botany, Blatter Herbarium, St. Xavier's College, Mumbai (Voucher specimen no.2943 of H. Santapau). The leaves were shade dried and grounded to fine powder. The 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), Quercetin and Rutin procured from Sigma Aldrich, L-Ascorbic acid (Himedia), Methanol (SD-Fine), HPLC grade water was used in the present analysis.

Preparation of plant extract for HPLC assay

For present study, ethanol, methanol, aqueous, pet ether, ethyl acetate and chloroform was used as solvents (HPLC-grade). The leaves of plant were collected and dried under shade. The dried material was mechanically powdered using 80 meshes and stored in an airtight container. 25 g of leaf powder was taken in 300 ml of ethanol, methanol, aqueous, pet ether, ethyl acetate and chloroform extracted by soxhlet apparatus (successive soxhlet extraction method) for 8 h. From this extract, 5 mg of sample was dissolved in each solvent and 5 µl of each stock was used for HPLC analysis.

Preparation of standard and sample solutions

Standard stock solution was prepared dissolving 5 mg of quercetin and rutin in 5 ml of methanol. It was then sonicated for 10 min. From the stocks, 10 µl each of the standard solutions and liquid leaf extract was injected in HPLC analysis.

Instrument and chromatography conditions (HPLC analysis)

The HPLC equipment of Agilent system was used including a 4-solvent delivery system, quaternary pump 0-20 ml/ min, a UV detector, and a 4-chamber in-line degasser. The analysis was performed on a Agilent ZORBAX RRHD Eclipse plus C18 column (150 mm × 4.6 mm i.d., 5 µm particle size) at ambient room temperature using with 10 µl injection volume and methanol (solvent A) and water (solvent B) mobile phase in 40:60 ratio at 30°C with a flow rate of 1.0 ml/min. Each run was followed by equilibration time of 15 min. Detection was carried out at 370 nm. Quercetin and Rutin was analyzed under same calibrated HPLC conditions. The calibration curves were used for the quantification of main bioactive compounds from *E. scaber*.

Method validation

Calibration curve and quantification of quercetin and rutin using HPLC assay

The HPLC method was validated in terms of specificity, accuracy, sensitivity (LOD and LOQ).

Specificity

The specificity of the method was determined by analyzing the bands of standard Rutin and Quercetin and unknown samples.

Accuracy

The accuracy of the method was determined by analyzing the percent recovery of the compound in samples. The analysis was done in three sets of different concentrations (10, 20 and 40 ng). The linearity of Rutin and Quercetin compound was validated by linear regression and correlation coefficient. The five point calibration curve was found to be linear in the range of 10-50 ppm. Regression equation and correlation coefficient for Rutin ($Y=6.382 \times (r^2=0.977)$) and for Quercetin ($Y=17.47 \times (r^2=0.977)$) revealed a good linearity response for developed method and it is represented in (Table 1 and Figure 1).

Precision

For the instrumental precision, the standard Rutin and Quercetin compounds (40 ppm) were analyzed in HPLC by repetitive method. The obtained peak area was expressed in terms of percent relative standard deviation (%RSD). The intermediate precision was carried out using six determinations of repeated concentrations of 20, 40 and 50 ppm of the Rutin and Quercetin compound for the period of 3 days and expressed as %RSD. The precision for proposed HPLC method was 0.16 and 0.45%.

Sensitivity

The sensitivity of the method was determined with values of Limit of Detection (LOD) and Limit of Quantification (LOQ). For the sensitivity of the method aliquots of standard solution of rutin, quercetin and *Elephantopus scaber* soxhlet leaf extract was analyzed. The lower limit of detection obtained for quercetin compound was 20 ppm and the limit of quantification was 50 ppm. The LOD for rutin metabolite was 10 ppm and the LOQ was 30 ppm.

Table 1: Method validation parameters for the quantification of studied standard compounds by using HPLC method

S. No.	Standard compound	Parameter	Results
1	Rutin	Rt	3.2
		r^2	0.977
		Linearity range (ppm)	Oct-50
		Precision	0.16
		Sensitivity	
		LOD (ng)	10
		LOQ (ng)	30
2	Quercetin	Rt	6.8
		r^2	0.977
		Linearity range (ppm)	Oct-50
		Precision	0.45
		Sensitivity	
		LOD (ng)	20
		LOQ (ng)	50

Preparation of plant extracts for FTIR analysis

The leaves of plants were collected and dried under shade. These dried materials were mechanically powdered and sheaved using 80 meshes. 25 g of leaf powder was taken in 300 ml of ethanol, methanol, aqueous, ethyl acetate, pet ether and chloroform (all the solvents were HPLC grade) and extracted by soxhlet apparatus (successive soxhlet extraction method) for 8 h. Then collected solutions were filtered through Whatman No.1 filer paper. The extracts were evaporated to dryness under reduced pressure by Rotary vacuum evaporator to obtain the respective extracts [22]. All the plant extract were analyzed independently. For the FTIR analysis 0.5 ml (10 mg/ml) of each plant extract were added drop wise and thoroughly mixed with KBr powder in a clean mortar and pestle. Then it was kept overnight in oven 150 to 200°C temperature to get totally moisture free. FTIR spectra were recorded using Perkin Elmer FTIR Spectrum-100 model, in The Institute of Science, Mumbai.

Preliminary phytochemical analysis

The Phytochemical studies are carried out using freshly prepared plant extracts of *E. scaber* leaves to detect the alkaloids, steroids, terpenoids, tannins, flavonoids, saponins and glycosides, etc., using standard procedures. The plant extract of 1 ml, independently prepared by soxhlet extraction method using ethanol, methanol, aqueous, pet ether, ethyl acetate and chloroform was used for the preliminary phytochemical analysis, the extract was taken in test tube and the chemical tests were following the method of Harborne B. 1998 [23].

Preparation of plant extracts for DPPH analysis

The plant extracts was prepared using 10 mg/mL soxhlet leaf extracts of all the six samples. The plant leaf extracts was taken in Eppendorf tube and each extract was sonicated for 5 min. From these stock solution 5 different aliquots of each extracts were make (50, 100, 150, 200 and 250 µg/ml) and directly used for DPPH assay.

Antioxidant activity with DPPH radical scavenging assay

DPPH scavenging activity was measured using the method described by Brand-Williams et al., with slight modifications. DPPH methanolic solution (0.002%) were used. For the present analysis DPPH concentration is reduced by the existence of an antioxidant at 515 nm and the absorption gradually disappears with time. Aliquot of *E. scaber* leaf extract was prepared (50, 100, 150, 200, 250 µg/ml each extract). From the stock solution 180 µl DPPH and 20 µl each plant extract was mixed together. After a 30 min incubation period at room temperature in the dark, the absorbance was measured at 515 nm, using a Synergy H1-Multi plate reader (Biotek) instrument. Ascorbic acid used as standard and methanol used as blank [24]. Ascorbic acid was used as the positive control. The IC_{50} values (concentration at which 50% of decolourization was obtained). The experiments were performed in triplicate and percentage scavenging activity was calculated using following equation:

$$[(Absorbance_{control}-Absorbance_{sample}/Absorbance_{control}] \times 100.$$

Statistical analysis

The analysis performed in triplicate the data represented as mean ± standard deviation. The statistical analysis was performed by non-linear regression (curve fit) using graph pad prism software and Microsoft excel 2007. The p value was < 0.05 regarded as significant.

RESULTS

Phytochemical tests, HPLC assay, FTIR analysis and DPPH antioxidant activity was performed for present studies. The results obtained summarized below.

Preliminary phytochemical analysis

The results of phytochemical analysis of 6 different soxhlet extracts were given in Table 2. In the present phytochemical analysis ethanolic *E. scaber* leaf extract was accounted for flavonoid, tannin, terpenoids, cardiac glycoside. The methanol extract was tested positively for Flavonoid, tannin, saponin, steroid, terpenoids and cardiac glycoside. Aqueous extract has flavonoids, tannins, terpenoids and cardiac glycoside. Ethyl acetate showed moderate amount of flavonoids, saponins, steroids, tannins, terpenoids and cardiac glycoside. While pet ether extracts showed presence of all these compounds. Chloroform extract showed alkaloids which are not accounted by other 5 leaf extract. In the present phytochemical analysis we observe that, all the extract showing moderate amount of flavonoids, minimum amount of tannins and rich amount of terpenoids.

Table 2: Preliminary phytochemical screening of *Elephantopus scaber* leaf extracts indifferent solvent system

S. No.	Metabolites	Chemical test	Ethanol	Methanol	Water	Ethyl acetate	Pet ether	Chloroform
1	Alkaloid	Dragendroffs	-	-	-	-	-	+
2	Flavonoid	Conc.H_2SO_4	++	++	+	++	++	++
3	Saponin	Foam test	-	++	-	+	+	+
4	Steroid	Acetic acid+H_2SO_4	-	++	-	++	++	++
5	Tannin	10% Lead acetate	+	++	+	+	+	+
6	Terpenoids	Chloroform+H_2SO_4	+++	+++	++	++	++	++
7	Cardiac glycoside	Acetic acid+$FeCl_3$ +H_2SO_4	++	++	+	+	++	+

+++=Rich amount; ++=Moderate amount; +=Minimum amount; -=absent

HPLC assay

HPLC analysis detected the standard Rutin compound with retention time of 3.252 min and quercetin with 6.837 min. Both the constituents were resolved at 100% area (Figure 2 and Table 3). The Retention Time (Rt) of rutin and quercetin in *E. scaber* ethanolic soxhlet leaf extract, was noted to be 3.347 min and 6.589 min respectively (Figure 3 and Table 3), which are coinciding with the standards Rt values for both the compounds, respectively. The amount of rutin and quercetin in the ethanolic leaf extract was observed to be 205.89 ± 0.05 µg/ml and 38.55 ± 0.02 µg/ml, respectively. The methanolic soxhlet leaf sample extract Rt was indicated to be 3.340 min and 6.600 min, respectively (Figure 4 and Tables 3 and 4), corresponding to the standards Rt values. The amount of Rutin and Quercetin in the soxhlet leaf sample extract was indicated to be 60.59 ± 0.09 µg /ml and 2.60 ± 0.04 µg/ml respectively. In the aqueous soxhlet leaf sample extract only Rutin was resolved. The Rt was 3.310 min in aqueous soxhlet leaf sample extract (Figure 5 and Tables 3 and 4), identical to the standard Rt value respectively. The amount of rutin in the soxhlet leaf sample extract was found to be 196.96 ± 0.05 µg/ml. The other three soxhlet leaf sample extracts (Pet ether, ethyl acetate and chloroform) does not yield content for rutin and quercetin.

Table 3: Retention time, height and %area of standards rutin, quercetin and studied soxhlet leaf extract of *Elephantopus scaber* using HPLC assay

S. No.	Standards/soxhlet leaf extract		Retention time (min)	Area [mAU]	Height [mAU]	Area (%)
1	Rutin		3.184	684.009	37.218	100
2	Quercetin		6.823	1737.37	59.997	100
3	Ethanolic	Rutin	3.347	1314.32	536.84	58.14
		Quercetin	6.589	673.51	36.07	6.1
4	Methanolic	Rutin	3.34	386.696	27.164	89.464
		Quercetin	6.6	45.54	2.171	10.536
5	Aqueous	Rutin	3.31	1257.04	113.47	100
		Quercetin	-----	-----	-----	-----

Table 4: Content of rutin & quercetin in studied soxhlet leaf extract of *Elephantopus scaber* using HPLC assay

S. No.	Soxhlet leaf extract	Quantified Standard compound	HPLC Content (in µg/ml)
1	Ethanolic	Rutin	205.89 ± 0.05
		Quercetin	38.55 ± 0.02
2	Methanolic	Rutin	60.59 ± 0.09
		Quercetin	2.60 ± 0.04
3	Aqueous	Rutin	196.96 ± 0.05
		Quercetin	-----

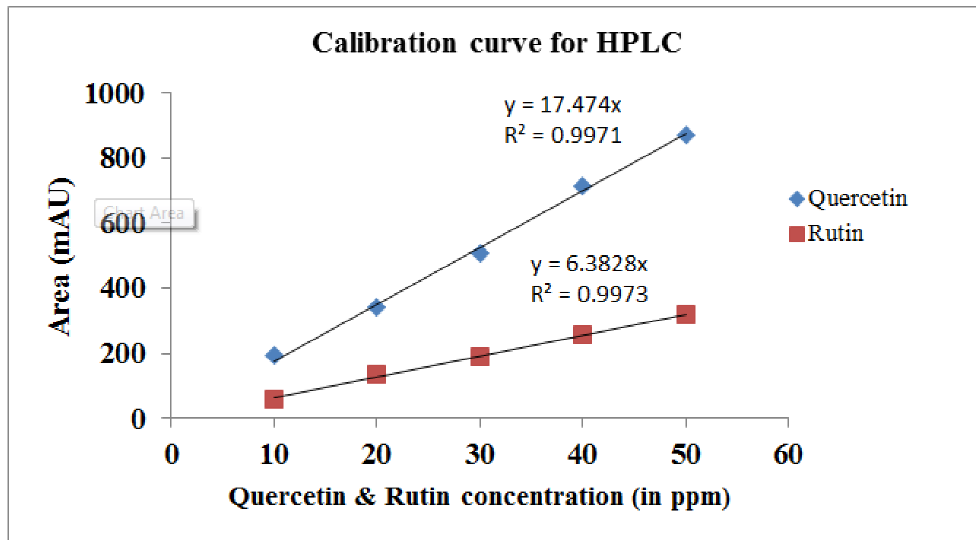

Figure 1: Calibration curve of standard rutin and quercetin for HPLC analysis

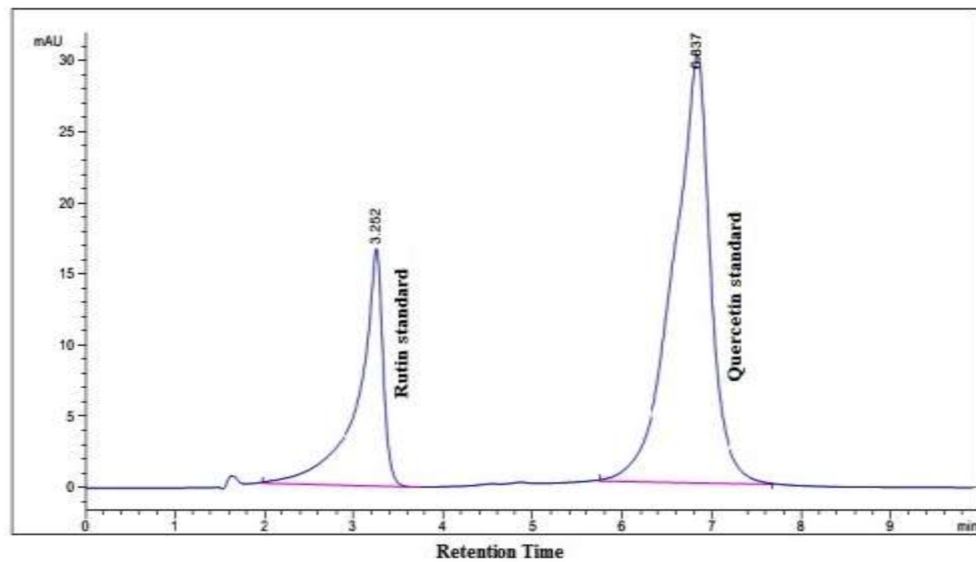

Figure 2: HPLC Chromatogram for standard rutin and quercetin

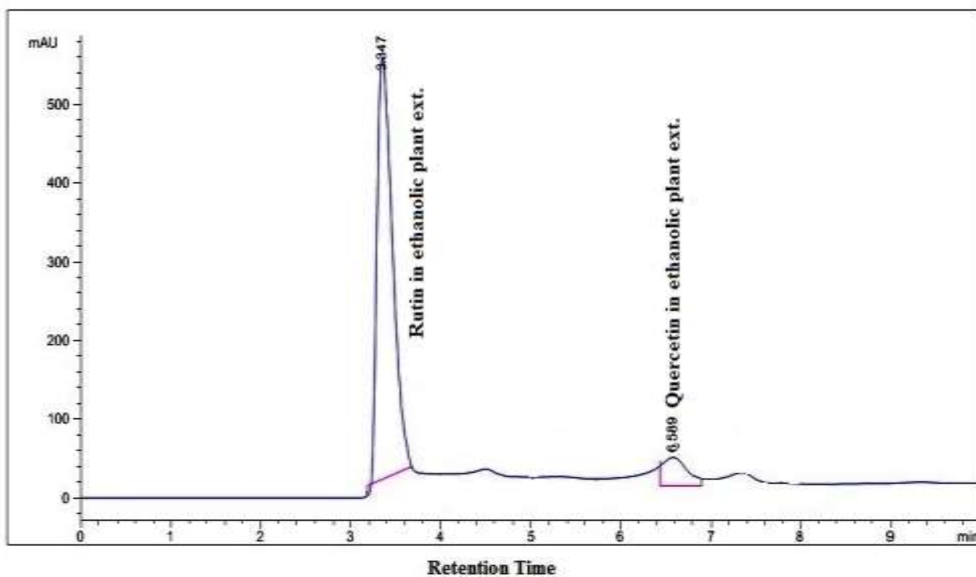

Figure 3: HPLC Chromatogram of ethanolic leaf extract of *Elephantopus scaber*

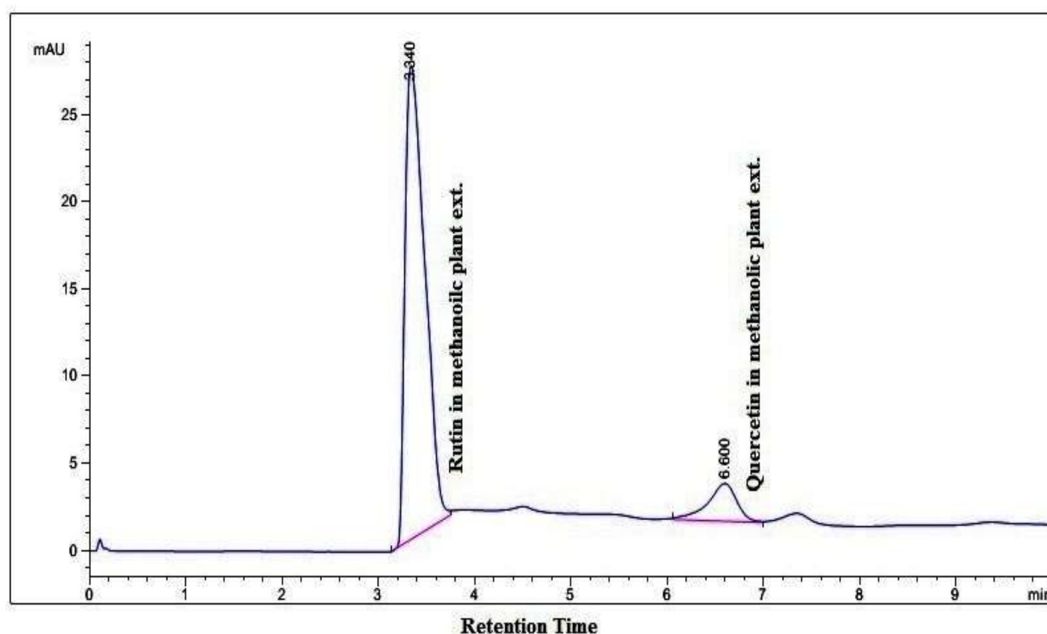

Figure 4: HPLC Chromatogram of methanolic leaf extract of *Elephantopus scaber*

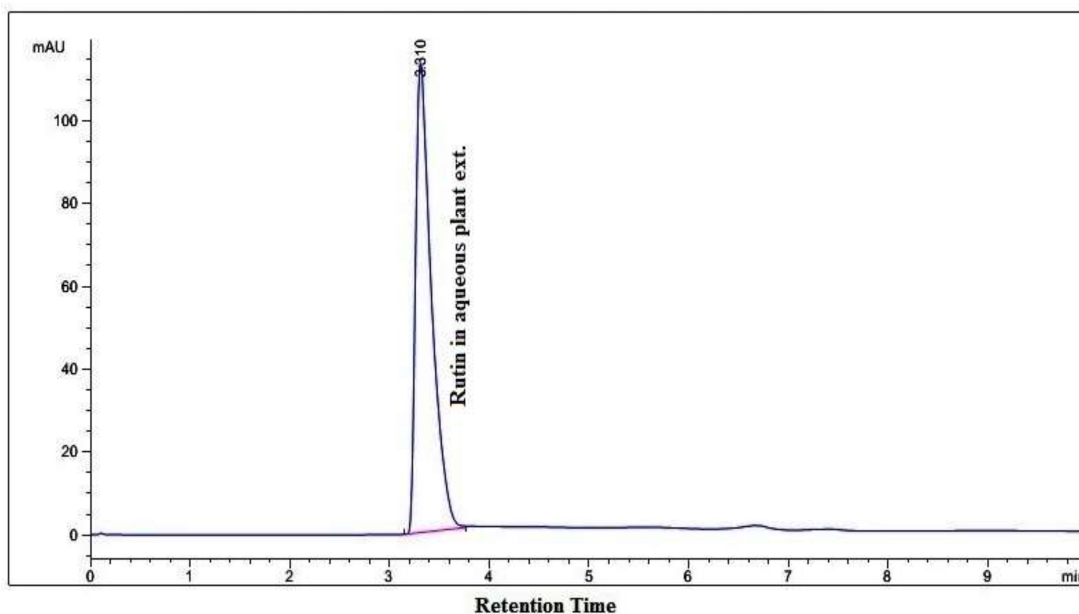

Figure 5: HPLC Chromatogram of aqueous leaf extract of *Elephantopus scaber*

FTIR analysis

The FTIR spectrum was used to identify the functional group of the active components based on the peak value in the region of infrared radiation. The soxhlet leaf extract of *E. scaber* was subjected to FTIR and the functional groups of the components were separated based on its peak ratio. The results of FTIR peak values and functional groups were represented in Table 5. The IR absorption spectra of the six *E. scaber* soxhlet extracts was studied and recorded in the 400-4000 cm^{-1} region (Figures 5-11). These spectra show that there were clear differences between all the functional groups. The obtained results is summarized as, FTIR analysis confirmed the presence of alcohols, phenols, alkanes, alkenes, aromatics, carboxylic acids, esters, ethers, alkyl halides compounds which shows major peaks at, 3416-3410, 2930-2925, 2861-2852, 1638-1605, 1549-1541, 1405-1400, 1107-1101, 831-820 and 523-502 respectively (Figures 5-11 and Table 5). The FTIR analysis results of six soxhlet leaf extract proved the presence of alcohols, phenols, alkanes, alkenes, aromatics, carboxylic acids, esters, ethers, alkyl halides. The FTIR analysis revealed the similarity and variation between various solvent system soxhlet leaf extracts of *E. scaber* based on the functional group presence and absorption spectrum [25]. The peaks at 3416-3410 cm^{-1} are corresponding to Hydrogen-bonded O-H Stretching frequency having phenols, alcohols functional groups. The peaks at 2930-2925 cm^{-1} are assigning to H-C-H asymmetric and symmetric stretching and having alkanes functional groups. The band at 2861-2852 cm^{-1} represents as alkanes groups. The peaks at 1638-1605 cm^{-1} assigned to C-C=C symmetric stretching frequency and correspond to alkenes groups. The peaks at 1549-1541 cm^{-1} are assign C-C=C asymmetric Stretching and represents as aromatic rings. The band at 1405-1400 cm^{-1} assigned to H-C-H bend and having alkanes groups. The peaks at 1107-1101 cm^{-1} are assigning to C-O stretching and correspond to carboxylic acids, esters, ethers functional groups. The peak at 831 to 820 cm-1 is assigned to C–Cl stretching and represents alkyl halides groups. The peaks at 523 to 502 is assign to C–Br stretching correspond to the alkyl halides functional groups respectively (Table 5 and Figures 5-11) [26-28].

Table 5: FTIR functional groups of *Elephantopus scaber* soxhlet leaf extract in studied solvents

S. No.	Solvent used (soxhlet leaf extract)	Functional group
1	Ethanol	Alkyl halides, phenols, alcohols, carboxylic acids, esters, ethers, aromatic rings, alkanes, alkenes
2	Methanol	Phenols, alcohols, alkanes, alkenes, aromatic rings, carboxylic acids, esters, ethers, alkyl halides
3	Aqueous	Alkyl halides, phenols, alcohols, carboxylic acids, esters, ethers, aromatic rings, alkanes, alkenes
4	Ethyl acetate	Phenols, alcohols, alkanes, alkenes, aromatic rings, carboxylic acids, esters, ethers, alkyl halides
5	Pet ether	Alkyl halides, phenols, alcohols, carboxylic acids, esters, ethers, aromatic rings, alkanes, alkenes
6	Chloroform	Phenols, alcohols, alkanes, alkenes, aromatic rings, carboxylic acids, esters, ethers, alkyl halides

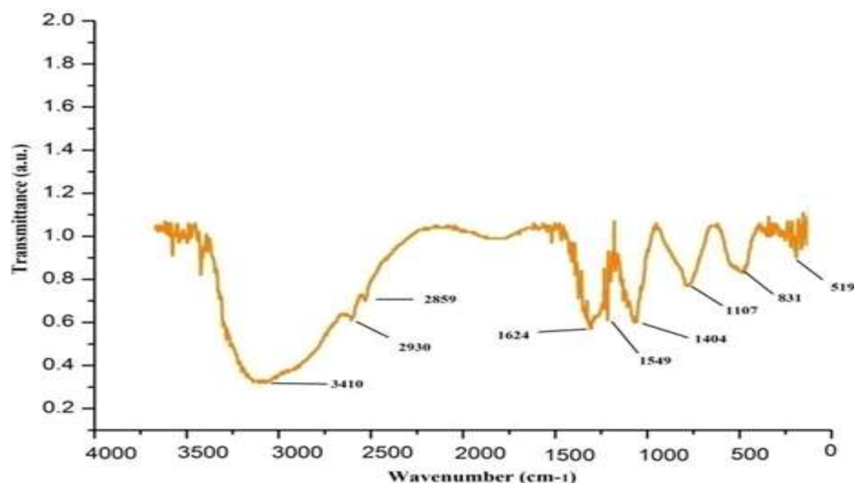

Figure 6: FTIR spectra of ethanol soxhlet leaf extract of *Elephantopus scaber*

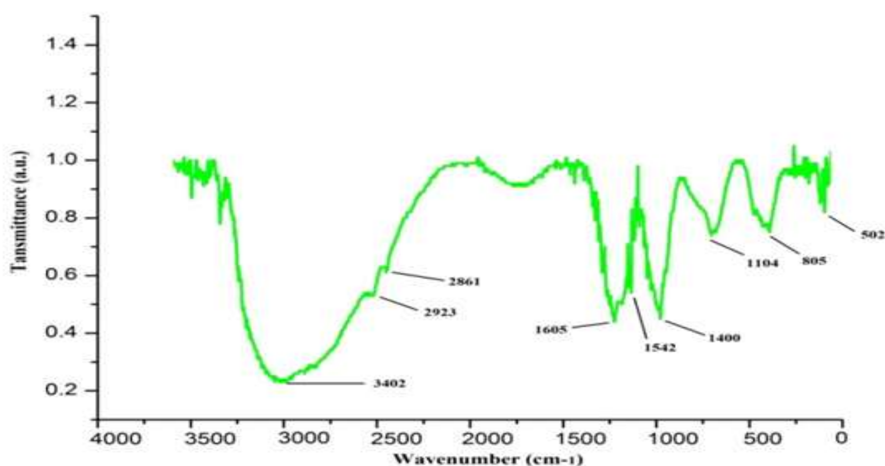

Figure 7: FTIR Spectra of Methanol soxhlet leaf extract of *Elephantopus scaber*

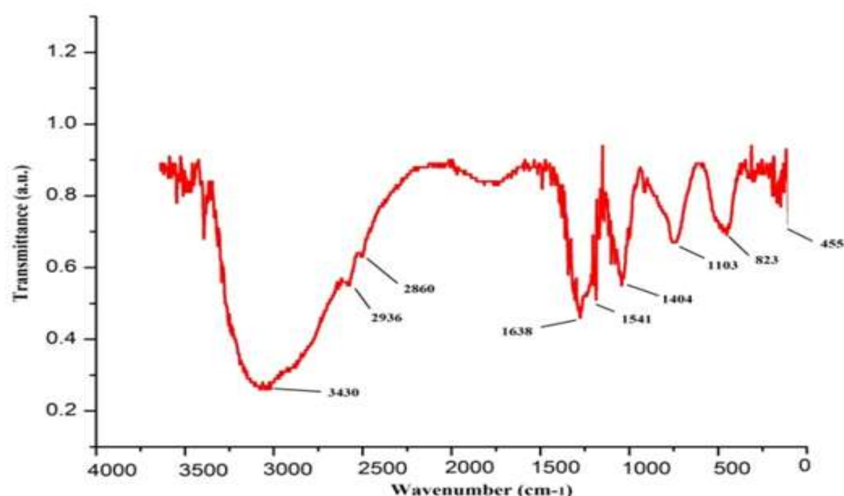

Figure 8: FTIR Spectra of aqueous soxhlet leaf extract of *Elephantopus scaber*

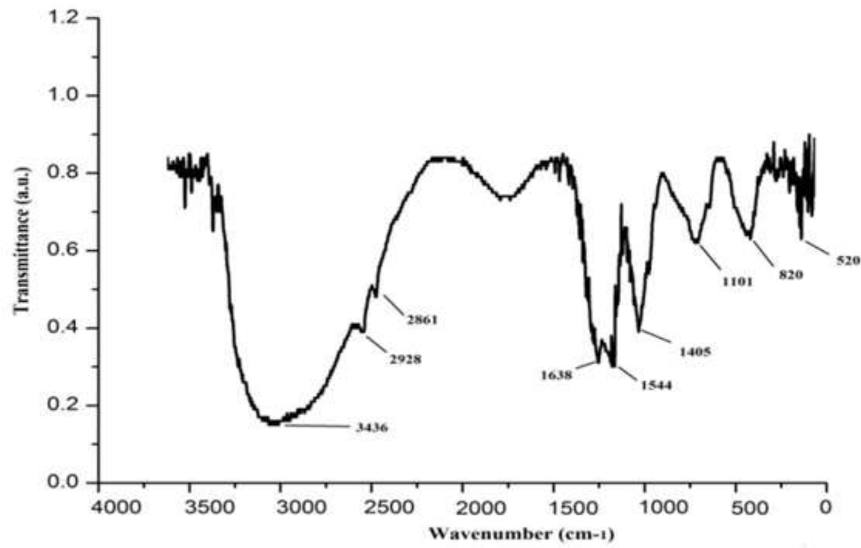

Figure 9: FTIR Spectra of ethyl acetate soxhlet leaf extract of *Elephantopus scaber*

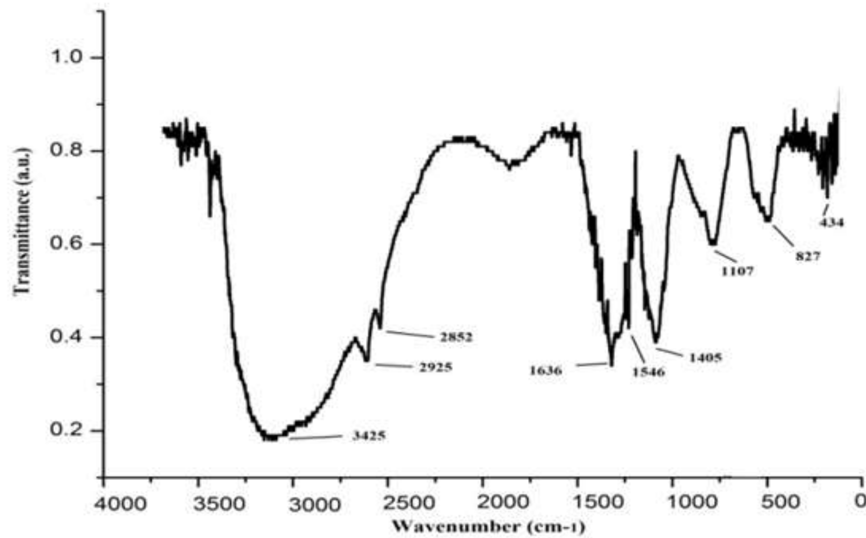

Figure 10: FTIR Spectra of pet ether soxhlet leaf extract of *Elephantopus scaber*

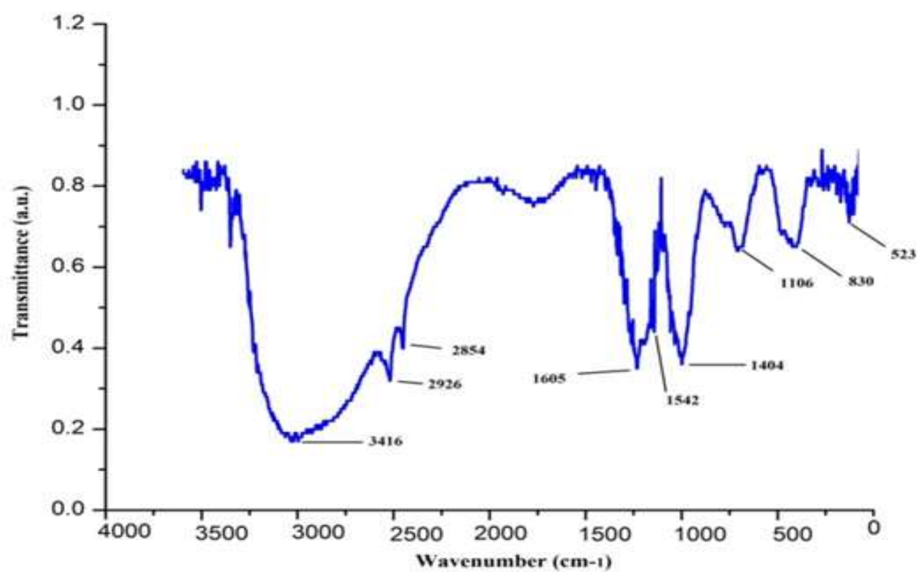

Figure 11: FTIR Spectra of chloroform soxhlet leaf extract of *Elephantopus scaber*

Antioxidant activity of six soxhlet leaf extract of *E. scaber* studied

In present analysis six different types of soxhlet leaf extracts were evaluated using DPPH radical scavenging ability. DPPH is widely used for screening of medicinal plants to investigate their antioxidant potential. This is a useful parameter to evaluate the antioxidant activity and compare the antioxidant capacity of different solvent soxhlet leaf extract samples prepared in different solvents. The results obtained are summarized in Table 2. The present results revealed that the lowest area values correspond to the most antioxidant substances in the extracts. The DPPH free radical scavenging activity of analyzed sample extracts was concentration dependent. The analysis was carried out taking absorbance at 515 nm; this can be useful for quantification of DPPH assay.

Different soxhlet extract showed considerable variations in scavenging activities (Table 2). The plant leaves extracts DPPH radical scavenging activities ranging from 20.50 ± 0.01 to 91.34 ± 0.03 at concentrations of 50, 100, 150, 200 and 250 µg/ml respectively (Table 2). Besides, most of the samples exhibited marked DPPH scavenging activity in a concentration dependent manner. The highest scavenging activity of ethanolic extracts was 91.34 ± 0.03 and lowest 11.84 ± 0.05 µg/ml with IC_{50}=139.7. While the highest scavenging activity of methanolic extract was revealed as 83.37±0.01 and lowest was 24.82 ± 0.09 µg/ml with IC_{50} 195.0. The pet ether highest scavenging activity was 53.07 ± 0.05 and lowest was 15.48 ± 0.09 µg/ml with IC_{50}=144.3. In chloroform extract the highest scavenging activity was 20.50 ± 0.01 and lowest, 1.10 ± 0.03 µg/ml with IC_{50}=187.7. The ethyl acetate and aqueous extract did not show DPPH scavenging activity. Ascorbic acid used as reference standard and the standard calibration curve plotted in the range of 20 to 140 µg/ml, showed scavenging activity 62.41 ± 0.40 µg/ml with IC_{50}=52.82. From the results obtained (Table 2), we may deduce that the ethanolic extract have stronger scavenging activity than all other extracts. According to the IC_{50} value of the samples the order of their activities was ethanol>methanol>pet ether>chloroform. The results were found to be statistically significant ($P<0.05$) at IC_{50} (Table 6).

Table 6: Antioxidant activity of soxhlet leaf extracts of *E. scaber* indifferent solvent system

S. No.	Soxhlet extracts	Concentration (µg/ml)	DPPH radical scavenging ability	
			Scavenging ability in % (µg/ml)	IC_{50} value in µg/ml
1	Ethanol	50	11.84 ± 0.05	139.6
		100	25.74 ± 0.03	
		150	34.49 ± 0.06	
		200	69.24 ± 0.08	
		250	91.34 ± 0.03	
2	Methanol	50	11.38 ± 0.09	195
		100	21.41 ± 0.02	
		150	32.11 ± 0.05	
		200	50.14 ± 0.04	
		250	83.37 ± 0.01	
3	Aqueous	50	N.A.	------
		100		
		150		
		200		
		250		
4	Ethyl acetate	50	N.A.	------
		100		
		150		
		200		
		250		
5	Pet ether	50	15.48 ± 0.09	144.3
		100	22.77 ± 0.07	
		150	34.16 ± 0.03	
		200	41.68 ± 0.08	
		250	53.07 ± 0.05	
6	Chloroform	50	15.48 ± 0.09	187.7
		100	20.50 ± 0.02	
		150	31.12 ± 0.07	
		200	58.76 ± 0.05	
		250	65.48 ± 0.03	
7	Ascorbic acid (Standard)	20-140 µg/ml	62.41 ± 0.40	52.82

N.A.–Not active; Data are presented as mean ± SD ($P<0.05$). IC_{50}: Concentration of the analyzed extracts causing 50% DPPH radical scavenging ability

DISCUSSION

The phytochemical analysis was carried out in six types of extracts in *E. scaber*. The variations detected in the existence of these metabolites in different solvent extract might be due to the dissolving ability of phytoconstituents with respect to the type of solvent used in the experiment. Flavonoid, saponin, steroid, tannin, terpenoids and cardiac glycoside were evident in the system but Mohan et al., [12] and Kamalakannan et al., [29] also have reported the existence of these constituents but not alkaloid. The reports indicated that the alkaloid compounds are hardly detected in *E. scaber*. The analysis performed in our lab also support this view since we were unable to trace alkaloid content in ethanol, methanol, aqueous, pet ether and ethyl acetate mediated extract. However in our observation we have observed the presence of alkaloids only in the extract sample was chloroform was used as a solvent.

Analysis of the six soxhlet leaves extract of *E. scaber* samples was processed by HPLC assay three solvent extracts (ethanol, methanol and aqueous) indicated the presence of rutin and quercetin compounds only. In this study the primary peaks in each sample have been detected at 370 nm by comparison of retention time with individual time of 3.184 min for rutin and 6.823 for Quercetin under uniform condition.

Comparatively out of six soxhlet leaf extracts, ethanolic leaf extract gives high yield (Rutin 205 ± 0.05 and Quercetin 38.55 ± 0.02 µg/ml), followed by aqueous leaf extract (Rutin 196.96 ± 0.05 µg/ml and Quercetin - nil) followed by methanolic leaf extract (Rutin 60.59 ± 0.09; Quercetin 2.60 ± 0.04 µg/ml). The proposed method was conveniently used for the analysis of rutin and quercetin flavonoids and it is highly sensitive at 370 nm. This method has many advantages like, rapid extraction, easy sample preparation, short time between injections and targeted flavonoids easily detected in common solvents (ethanol, methanol & aqueous). Thus the proposed method was quick, specific, precise, linear and easy to perform.

FTIR spectroscopic technique showed the presence of alcohols, phenols, alkanes, alkenes, aromatics, carboxylic acids, esters, ethers, and alkyl halides compounds. These constituents can be isolated and further screened for different biological activities depending upon their beneficial use. Further study is needed to find out the structural analysis of these compounds by use of different analytical methods such as NMR and GC-MS. The FTIR analysis is very helpful to find the functional groups in the medicinal plants. The presences of various functional groups have been noticed in the present analysis. FTIR spectroscopy is proved to be a reliable and sensitive method for detection of bimolecular composition. Ashok Kumar and Ramaswamy [5] reported the FTIR analysis and functional groups of various Indian medicinal plants using different solvents systems.

The soxhlet leaf extract was investigated for the natural antioxidant activity in *E. scaber*. The result revealed that the phenolic compounds are present in the plants and therefore hence, plant shows health promoting activities. The flavonoid shows the significant antioxidant activity. In the present analysis ethanolic soxhlet leaf extract showed highest antioxidant property compared to other five solvent extracts. The antioxidant DPPH radical scavenging activity (IC_{50}=158.207) of pet ether extract of *E. scaber* was reported by Subramanian et al., [30] and it was approaching our finding (IC_{50}=144.3). The number of reports available on other antioxidant property reported by Tagne et al., [31] in Cameroonian medicinal plants, Liao et al., [8] in *Talinum triangulare* and Wong-Paz et al., [6] in plants from semiarid Mexican region. Zorzetto et al., [32] analyzed antioxidant activity of three plants of *Hypericum* species from the Canary Islands. All these workers have reported the significant free radical scavenging ability of plant extract prepared in various solvent systems. Our observation revealed the existence of this property in the *E. scaber* extract prepared in all the six solvent.

CONCLUSION

The results obtained from present study revealed that *E. scaber* indicates rich amount of phytoconstituents. The flavonoid compounds were successfully quantified by reverse-phase high performance liquid chromatography in the ethanolic, methanolic and aqueous soxhlet leaf extract. The content of rutin was highest estimated in ethanolic extract followed by aqueous and methanolic extract. The content of quercetin was highest in the ethanolic leaf extract followed by methanolic extract. The result obtained revealed that ethanol is the best solvent for the extraction of *E. scaber* leaf and flavonoid estimation using soxhlet extraction. This method is used for both qualitative and quantitative analysis of raw leaves and their commercial products. The various functional groups were observed in the different soxhlet extracts with symmetrical and asymmetrical stretching frequency in the groups of alcohols, phenols, alkanes, alkenes, aromatics, carboxylic acids, esters, ethers, alkyl halides compounds. In the studied six soxhlet extract, the ethanolic soxhlet leaf extract indicates highest radical scavenging property. *E. scaber* is the potential sources to obtain bioactive phenolic compounds with high antioxidant properties which can be used in the pharmaceutical companies as antioxidant agents. The soxhlet leaf extract of *E. scaber,* an important property as anticancer, antianalgesic and antidiabetic plant was a analyze using six different solvent system by successive solvent extraction method. Based on these findings it is concluded that this plant is rich in important metabolites which are extractable in any organic solvents. The flavonoids (Rutin and Quercetin) were easily quantified using ethanol, methanol and aqueous solvents.

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