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Pharmacognostical Standardization, Chromatograhic and Spectral Analysis of Methanolic Extract of *Echinops echinatus* Linn. Roots and Fractions

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MY, MMULH designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FZK supervised the study and author JHS, QAJ managed the analyses of the study. Authors ZK, GS managed the literature searches. Authors GS, MH, MAG revised manuscript. Authors MA and IN revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Echinops echinatus Linn. (Fam. Asteraceae) possesses medicinal value a good deal. The plant is a nerve tonic that stimulates liver and increases appetite, and is effective as anti-inflammatory and in jaundice. Objective of the current study was to standardize *Echinops echinatus (E. echinatus),* both macroscopically and microscopically. Pharmacognostic standardization with the help of different physicochemical parameters and fluorescence analysis was performed according to the WHO guidelines. Qualitative phytochemical analysis of crude methanolic extract (EME) and various fractions was done. TLC and column chromatographic techniques were employed for presence of various phytoconstituents. Five compounds were isolated from EME using column chromatography, which were characterized by techniques like FTIR and UV. The isolated purified compounds showed different hRf values ranging from 67 to 94. Results of this study may serve as biochemical markers for this medicinally important plant in the pharma industry and plant systematic studies. The current work will help in identification of the species pharmacognostically and anatomically; and phytochemical analysis may help in screening of active constituents responsible for the activity. The study will serve as a reference for correct identification and in checking any type of adulteration. This may also help in differentiating this species from closely related species of the same genus and family.

Keywords: Echinops echinatus; pharmacognostic; phytochemical; spectral analysis.

1. INTRODUCTION

The genus Echinops (Fam. Asteraceae) is thiste-like herb, consisting of various species found in Europe, Africa and Asia. Out of these species, *Echinops echinatus* and *Echinops niveus* are commonly found [1]. *Echinops echinatus* Linn. (Fam. Asteraceae), a xerophytic weed is widely distributed in deserted places and foothills of Potohar region; and is common in Lahore district [2]. Phytoconstituents belong to various classes viz. alkaloids, terpenoids, flavonoids, steroids, etc. [3,4]. Prabir identified apigenin 7-O-B-D- (4''-cis-p-coumaroyl) glucoside in *E. echinatus* (Chaudhri, 1987). A new alkaloid, echinozolinone was identified in *E. echinatus* as 3(2-hydroxylethyl- 4(3H)-qunazolinone. This was the first report of alkaloids from this plant and the first occurrence of 4-quinazolinone alkaloid in the Compositae [4]. The ethanolic extract yielded w–methylallophonic acid [5]. The plant is useful in diseases of liver, respiratory tract, intestines and inflammatory conditions [2,5]

2. MATERIALS AND METHODS

2.1 Identification and Authentication of Plant Material

Echinops echinatus Linn. plants were collected from different areas of Lahore, during February, March, 2018; and authenticated by taxonomist, Department of Botany, Government College University, Lahore. The voucher specimen (No.GC. herb. Bot. 526) was deposited in the Herbarium of pharmacognosy section, University College of pharmacy, University of the Punjab, Lahore.

2.2 Reagents and Equipments

All chemicals, solvents and reagents used were of analytical grade and were purchased from Merck and Sigma-Aldrich. Methanol , petroleum ether, chloroform, glacial acetic acid, anisadehyde, chloral hydrate, safranin, light green, iodine, HNO_3, H_2SO_4, HCl, glycerin, Mayer's Reagent solution, Wagner's reagent solution, Hager's reagent solution, copper sulphate, ferric chloride, TLC plates, TLC jars, glass column, separating funnel, silica gel 320 for column chromatography, silica gel G60, distillation apparatus (Quick fit, England), Heidholph Laborota 4000-efficient (Germany), Buchi Rotavapor R-20), oven (Memmert), Electric balance (Sartorius).

3. EXPERIMENTAL

3.1 Pharmacognostic Evaluation

3.1.1 Macroscopic evaluation

Macroscopic evaluation of the root was performed as per standard procedures [6,7].

3.2 Microscopic Evaluation

3.2.1 Powder microscopy

Binocular microscope was used to observe various cells. The so observed microscopic

structures were identified by comparing with the standard work [8,9].

3.3 Fluorescence Analysis

E.echinatus dried powder of the roots was studied after treating with water, NaOH, HCl, HNO_3, H_2SO_4, picric acid, acetic acid, methanol and ethanol, using ordinary and UV.

3.4 Physico-chemical Analysis

Percentage of moisture content or loss on drying, total ash value, acid insoluble and water soluble ash value, extractive values and swelling and foaming index of *E. echinatus* (powder) were examined [6].

3.5 Extraction and Fractionation

Plants were well washed to remove all the external dirt and unwanted material. 1 kg of powdered material was macerated in 2 L of methanol for 72 h at room temperature. The soaked material was filtered three times for coarse filtration. The filtrate was filtered through Whatman Grade-1filter paper. The filtrate was evaporated under controlled pressure and temperature (-760mm Hg at 45-50^{o}C) on the rotary evaporator. The filtered extract was made free from solvent. A dark brownish green gummy extract was placed in oven, and percentage yield was calculated. Moreover, successive solvent extraction was used as previously described by Tiwari et al. [10]. Extracts were dried, weighed, labelled and placed at 4°C. Methanolic crude extract and two fractions so obtained were named as follows:

EME = Methanolic crude extract;
EPE= Petroleum ether fraction;
ECE= Chloroform fraction.

3.6 Preliminary Phytochemical Sreening

Crude as well as its fractions, i.e; EME, EPE, ECE were screened to identify the phytoconstituents like alkaloids, glycosides, flavonoids, tannins, saponins and phenols, etc., was carried out by using standard conventional procedures [11,6,7].

3.7 Column Chromatography

A glass column of 55x 4.5 cm. was used. Chloroform was used for packing the column. 12 g of EME was adsorbed on 10 g of silica gel. The column was first run with chloroform then the polarity of the system was changed [12].

3.8 Thin Layer Chromatography

20 x 5 cm glass plates by applying 30 g silica gel were used for this purpose [13,24]

3.9 Ultraviolet Visible Absorption

EME was analyzed in UV-Visible range between 200-800 nm.

3.10 Infra-Red Spectroscopy

IR spectra of EME were scanned over the range from 4000-400 cm-1.

4. RESULTS

4.1 Pharmacognostic Evaluation

4.1.1 Macroscopic evaluation

E. echinatus is an erect, 1-3 ft. high low growing much branched herb with white cottony stems. The fresh leaves are simple and sessile, 3-5 inch long, pinnatifid with lobes ending in spines up to 20 mm long; undersurface white tomentose. Flower heads are 1 flowered, numerous, aggregated into a white ball, 2.2 -2.4 cm in diameter, subtented by stout spines; and flowers are tubular with narrow lobes. Achens are 1/6 inch long, densely silky, surrounded by the connate hardened inner involucral bracts [1].

4.2 Microscopic Evaluation

4.2.1 Powder microscopy (root)

Fine powder revealed that root contains annular vessels showing pits and spiral vessels with parenchyma cells. Reddish brown Cork cells are also present.

4.2.2 Physico-chemical analysis

Percentage of moisture content or loss on drying, total ash value, acid insoluble and water soluble ash value, extractive values and swelling and foaming index are shown in Table 6.

4.2.3 Fluorescence analysis

The results of fluorescence analysis are shown in Table 7.

4.3 Preliminary Phytochemical Sreening

E. echinatus as well as its fractions, i.e; EME, EPE, ECE confirmed the presence of phytoconstituents like alkaloids, glycosides, flavonoids, tannins, saponins and phenols (Table 1).

Table 1. Phytochemical evaluation (various fractions of *E.echinatus*)

Test	*Ee* M	*Ee* Pe	*Ee* Pe
Alkaloids Hager's tes	+	–	+
Mayer's test	–	–	–
Wagner's test	–	–	–
Glycosides Borntrager's test	–	–	–
Tannins Fe Cl_3 test	+	–	+
Flavonoids Shinoda test	+	–	+
Saponins Froth test	+	–	+
Phenolic contents $FeCl_3$ test	+	–	–

+ = present *- = absent*

4.3.1 Column chromatography

Results of pooled fraction of EME by column chromatography are shown in Table 5.

4.3.2 Thin layer chromatography

Results of TLC of EME, EPE, ECE are shown in Table 2,3,4.

4.3.3 Ultraviolet visible absorption

Results of UV-Visible spectra of the five compounds (*Ee*1 to *Ee* 5) isolated from fractions of *E.echanitus* are shown in Fig. 1,3,5,7 and 9.

4.3.4 Infra-Red spectroscopy (IR)

Results of IR spectra of of the five compounds (*Ee*1 to *Ee* 5) isolated from fractions of *E. echanitus* are depicted in Fig. 2,4,6,8 and 10.

5. DISCUSSION

Despite different sophisticated modern research techniques and tools, macroscopic and microscopic methods are still the simplest, reliable, precise and economical methods for correct identity of the plant source. As per WHO [6], the macroscopic and microscopic description is first criterion for identity and purity of material. Organoleptic standardization is a qualitative test based on the study of macroscopical characters. In current study, research was conducted on roots of a medicinal plant *E. echinatus.* The microscopic studies of the powder showed different histological structures. Different stains differentiate different cells on the basis of their chemical nature [14,15,16]. Fluorescence is an important phenomenon for purity and quality of the sample and their chemical constituents [17, 18]. Powder, qualitative and fluorescence standards provide valuable information for authentication. Preliminary phytochemical screening showed the presence of various phytoconstituents in the plant which may have diversified therapeutic value for curing ailments; for example, saponins, flavonoids, tannins, alkaloids and phenols have anti-inflammatory activities whereas flavonoids, alkaloids, tannins and phenols have hypoglycemic and liver protective potential [19]. Water soluble extractive value indicates sugars, inorganic compounds and acids; and alcohol soluble extractive value shows polar components like flavonoids, steroids and phenols etc. To prevent chemical decomposition and microbial contamination low moisture content is needed. Due to presence of mucilage swelling index was in range of 5 ml; while foaming index was less than 100, i.e., insignificant. By estimating ash value quality and also the purity of powdered sample can be determined. Total ash determines that how much care is required in preparation of a crude drug [20]. Ash value is also signifies adulterant added for adulteration [21]. Ash value usually represents inorganic salts which are present in the drug sample [6]. Total ash value indicates the inorganic composition or earthy materials presence [19].

Ten pooled fractions were obtained from EME based on TLC analysis. Five major compounds (*Ee*1 to *Ee* 5) were isolated and purified from EME by silica gel column and TLC (Tables 1 and 2). Compound Ee-1 isolated from the first column fraction, showed (Fig. 1) absorption maximum in UV as: λmax=213 nm and λmax=269 nm (Fig. 1). The strong absorption at λmax=213 nm was probably due to presence of open chain diene; while at λmax=269 nm was due to substituted ring [22]. IR spectrum of Ee-1 compound (Fig. 2) showed abroad intermolecular hydrogen bonding around 3437 cm-1 due to –OH showing presence of some alcoholic/phenolioc hydroxyl group. The band at 2078 cm-1 showed stretching vibration

present in alkane. The presence and the number of –CH3=CH2 and ≡CH groups in the molecule were further indicated by the peaks in the finger print region at 1500, 1200 and 1000 cm-1. A strong peak at 1637 cm-1 indicated a coupled C=C-C=C conjugated diene (alkene) with aromatic ring [23,22,19].

Compound Ee-2, a light yellow oily compound, isolated from the second column fraction. The strong absorption at λmax=217 nm (Fig. 3) was probably due to ה to ה* transition [22,19]. IR spectrum of the compound (Fig. 4) showed absorption maximum at 3370 (medium) and 1459 (sharp) cm-1. A band at 3370 cm-1 is absorption frequency of triple bond showed alkyne, i.e., ≡C-H or –C≡C-H. Presence of a medium band at 2937 cm-1 showed C-H aliphatic asymmetric stretch.

Compound Ee-3 was a light yellow compound and chromatographically pure. The strong absorption at λmax=215 nm (Fig. 5) was probably due to ה to ה* transition, the compound may be α,β conjugated six-ring or acyclic ketone; while at λmax=275 nm is the positive identification of a ketone or aldehyde carbonyl group [22,13]. IR spectrum around 3419 nm (Fig. 6) emphasizes the stretching vibration of –OH with intermolecular H-bonded at OH. Two bands at 2924 and 2853 cm-1showed the presence of single bonds due to C-H stretching; or these may be saturated C-H (-CH3) and C-C in the form of 2 or 3 bonds [23,22,19].

Compound Ee-4 was a dark yellow compound and chromatographically pure. The strong absorption at λmax=207 nm (Fig. 7) was probably due to ה to ה* transition, which suggested that the compound may be α, β unsatured ketone or aldehyde [22,19]. IR spectrum of Ee-4(Fig. 8) showed a band at 3387 cm-1 due to alkyne, i.e., ≡C-H or –C≡C-H. Presence of two bands at 2943 and 2881 cm-1 showed single bonds due to C-H stretching; or these may be saturated C-H (-CH3) and C-C in the form of two or three bands.

Table 2. Comparative thin layer chromatographic analysis of methanol extract of *E. echinatus.*

Solvent system		No. of compounds	Detection		hRf value
Solvents	Ratio		UV Light	Iodine	
MeOH:CHCL3	90:10	2	Blue, Blue	Yellow, Yellow	31,6
MeOH:CHCL3	90:20	2	Blue, Purple	Yellow, Brown	16,34
MeOH:CHCL3	80:20	2	Light blue, Blue	Yellow, Yellow	34,33
MeOH:CHCL3	80:30	2	Sky blue, Purple	Light Yellow, Yellow	30,32
MeOH:CHCL3	70:20	2	Bluish green, Blue	Yellow, Dark Yellow	18,28
MeOH:CHCL3	70:30	2	Blue, Pink	Yellow, Dark Yellow	30,38
MeOH:CHCL3	60:40	3	Sky blue, Pink, Blue	Yellow, Brown, Light green	34,46,40
MeOH:CHCL3	60:50	3	Blue, Sky blue, Blue	Yellow, Dark yellow, Light yellow	18,34,32
MeOH:CHCL3	40:60	2	Off white, Sky blue	Yellow, Yellow	14,48
MeOH:CHCL3	40:70	2	Blue, Bluish green	Yellow, Dark yellow	31,43
MeOH:CHCL3	40:80	2	Light green, Grey	Light yellow, Brown	12,43
MeOH:CHCL3	20:80	1	Grey	Yellow	15
MeOH:CHCL3	20:90	2	Sky blue, Blue	Light yellow, Dark yellow	16,41
MeOH	100%	2	Light green, Blue	Brown, Yellow	15,38

Where : MeOH= methanol; $CHCL_3$= chloroform

Table 3. Comparative thin layer chromatographic analysis of pet.ether extract of *E. echinatus.*

Solvent system		No. of Compounds	Detection		hRf value
Solvents	Ratio		UV Light	Iodine	
Pet.ether:CHCL3	95:5	1	blue	Dark yellow	42
Pet.ether:CHCL3	90:10	2	light blue, blue,	Yellow, dark yellow	35,18
Pet.ether:CHCL3	90:15	2	light blue, dark blue	Yellow, Yellow	16,48
Pet.ether:CHCL3	90:20	2	blue, blue	Light Yellow, dark Yellow	30,75
Pet.ether:CHCL3	80:10	2	grey, light blue	Yellow, light brown	21,49
Pet.ether:CHCL3	80:15	2	grey, Purple	Light Yellow, light brown	28,68
Pet.ether:CHCL3	80:20	2	blue, Purple	Yellow, Dark yellow	35,68
Pet.ether:CHCL3	70:10	2	Pink, blue,	Yellow, Yellow	15,38
Pet.ether:CHCL3	70:20	1	dark blue	light brown	24
Pet.ether:CHCL3	70:30	2	Blue, light Blue	Yellow, Yellow	45, 82
Pet.ether:CHCL3	70:40	2	Light yellow, blue	Light yellow, Yellow	70,45
Pet.ether:CHCL3	65:35	2	Blue, red	Light yellow, Light yellow	78,93
Pet.ether:CHCL3	65:40	2	light blue, dark blue	Brown, Yellow	60,47
Pet.ether:CHCL3	65:50	1	Blue	Yellow	80
Pet.ether:CHCL3	60:20	2	light blue, dark blue	Yellow, Yellow	35,73
Pet.ether:CHCL3	60:30	2	light blue, grey	Yellow, light yellow	45,80
Pet.ether:CHCL3	60:40	2	light yellow, blue	Light yellow, dark yellow	60,74
Pet.ether:CHCL3	60:50	2	pink, blue	Yellow, Yellow	57,95
Pet.ether:CHCL3	50:50	2	blue, blue	Yellow, Yellow	59,78
Pet.ether:CHCL3	50:60	2	blue, light blue	Light yellow, yellow	66,98

Where : $CHCL_3$= chloroform; Pet.ether= petroleum ether

Compound Ee-5 was a dark yellow oily compound and chromatographically pure. The strong absorption at λmax=237 nm (Fig. 9) was probably due to ה to ה* transition, the compound may be an acyclic diene with 2-alkyl group, one each on α and β position; while at λmax=275 nm was due to disubstituted, benzene rings and is the positive identification of a ketone or aldehyde carbonyl group, it gives rise to yellow colour of the compound [23,22,19]. IR spectrum of the compound Ee-5 (Fig. 10) showed absorption maximum at 3409 (medium) 2925 (sharp) and 1636 (sharp) cm-1. IR spectrum of Ee-5 showed a broad intermolecular hydrogen bonding around 3409cm-1 due to –OH showing some alcoholic/phenolioc hydroxyl group. A strong peak at 1636 cm-1 indicated a coupled C=C-C=C conjugated diene (alkene) with aromatic ring; it may be α,β unsaturated carbonyl compounds, usually much weaker than C=O band [23,22,19]. A band at 1378 cm-1 showed C-H bend for –CH3 symmetrical deformation; while another band at 1272 cm-1 was for –CH3 group stretch. All the five compounds Ee 1- Ee 5 contain –OH, -COOH, or ketonic group and a double bond with conjugated diene system in their molecules.

Table 4. Comparative thin layer chromatographic analysis of chloroform extract of *E. echinatus*

Solvent system		No. of	Detection		hRf
Solvents	Ratio	compounds	UV Light	Iodine	value
CHCL3:MeOH	90:7	1	light blue	Yellow	98
CHCL3:MeOH	90:10	1	Off white	Yellow	98
CHCL3:MeOH	90:15	1	blue	Light Yellow	98
CHCL3:MeOH	85:15	1	blue	dark Yellow	92
CHCL3:MeOH	85:20	1	bluish green	dark Yellow	92
CHCL3:MeOH	80:20	1	Light grey	Brown	78
CHCL3:MeOH	80:25	1	bluish green	light brown	79
CHCL3:MeOH	70:30	1	Light pink	Light Yellow	88
Pet.ether: CHCL3:MeOH	95:5:1	1	Sky blue	Yellow	80
Pet.ether: CHCL3:MeOH	95:10:2	1	Dark blue	Yellow	80
Pet.ether: CHCL3:MeOH	95:16:3	1	blue	Light yellow	45
Pet.ether: CHCL3:MeOH	95:20:5	2	Yellow, light brown	Light pink, yellow	80,45
Pet.ether: CHCL3:MeOH	90:10:3	2	Blue , Yellow	Yellow, Yellow	56,55
Pet.ether: CHCL3:MeOH	90:10:5	1	blue	dark Yellow	77
Pet.ether: CHCL3:MeOH	90:10:7	2	dark blue, blue	Yellow,yellow	73,85
Pet.ether: CHCL3:MeOH	90:15:10	2	dark blue, light blue	yellow, yellow	74,90
Pet.ether: CHCL3:MeOH	85:15:10	2	pink, grey	Yellow, Yellow	94,55
Pet.ether: CHCL3:MeOH	80:20:5	1	Blue	Yellow	73
Pet.ether: CHCL3:MeOH	80:20:10	2	Pink, purple	Light yellow, yellow	75,78
Pet.ether: CHCL3:MeOH	70:25:5	1	light blue	Yellow	88

Where : $CHCL_3$= chloroform; MeOH= methanol; Pet.ether= petroleum ether

Table 5. Comparative thin layer chromatographic analysis of pooled column fractions of methanol extract of *E. echinatus*

Pooled fraction	Eluting Solvent	No. of compounds	h R_f value	UV light	Iodine	Leiberman
1	CHCl3 (100%)	1	94	Light blue	Yellow	Light grey
2	CHCl3 (100%)	1	91	Light blue	Yellow	Light grey
3	CHCl3 (100%)	1	90	Light blue	Yellow	No colour
4	CHCl3:Meoh (95:5)	1	86	Light blue	Yellow	No colour
5	CHCl3:Meoh (95:5)	1	80	Light blue	Dark yellow	No colour
6	CHCl3:Meoh (95:10)	2	80,83	Light blue, Blue	Dark yellow	Light grey, Grey
7	CHCl3:MeoH (90:20)	1	45	Off white	Dark yellow	No colour
8	CHCl3:Meho (80:20)	2	53,88	Sky blue, Blue	Yellow, Light	Yellow

Pooled fraction	Eluting Solvent	No. of compounds	h R_f value	UV light	Iodine	Leiberman
9	CHCl3:Meoh (8020)	1	67	Bluish green	Yellow	Light grey
10	CHCl3:Meoh (80:20)	1	68	Bluish green	Yellow	No Color

Table 6. Physico-chemical analysis (*Echinops echinatus* powder)

Sr.#	Physico-chemical character	Value
1	Moisture content	10 %
2	Toatal ash	18 %
3	Acid insoluble ash	2 %
4	Water soluble ash	15 %
5	Swelling index	10 ml
6	Foaming index	≤ 100 cm
7	Extractive value in water	14%
8	Extractive value in ethanol	13%

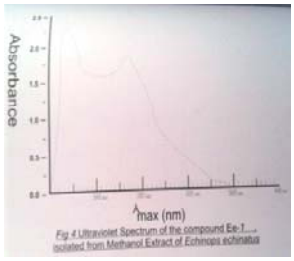

Fig. 1. UV peak of compund Ee-1

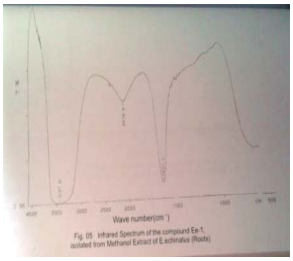

Fig. 2. IR peak of compund Ee-1

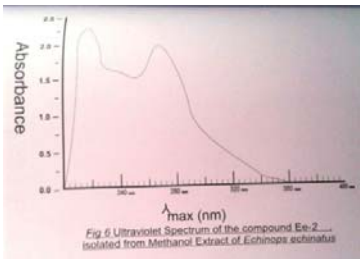

Fig. 3. UV peak of compundEe-2

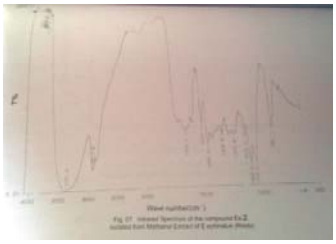

Fig. 4. IR peak of compund Ee-2

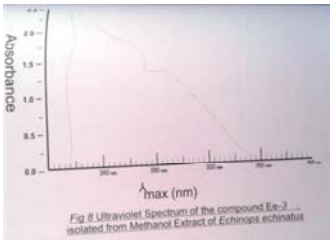

Fig. 5. UV peak of compund Ee-3

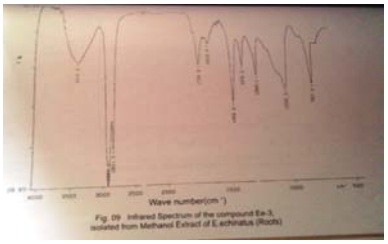

Fig. 6. IR peak of compund Ee-3

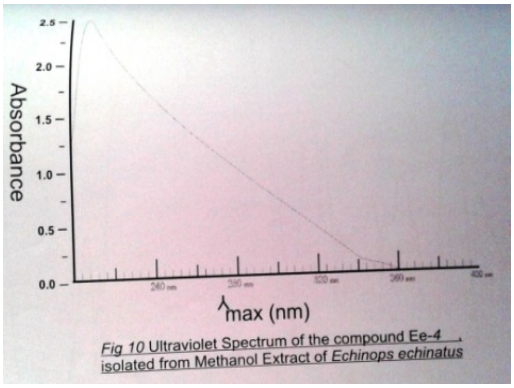

Fig. 7. UV Peak of Compound Ee-4

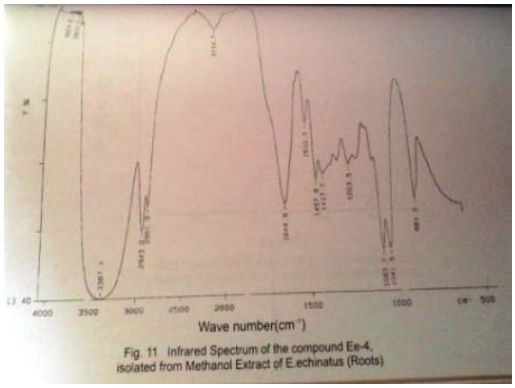

Fig 8. IR peak of compundEe-4

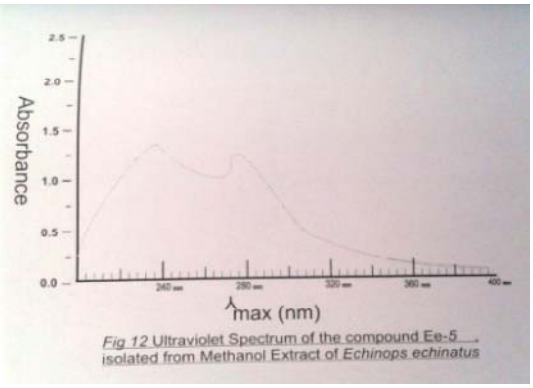

Fig. 9. UV Peak of Compound Ee-5

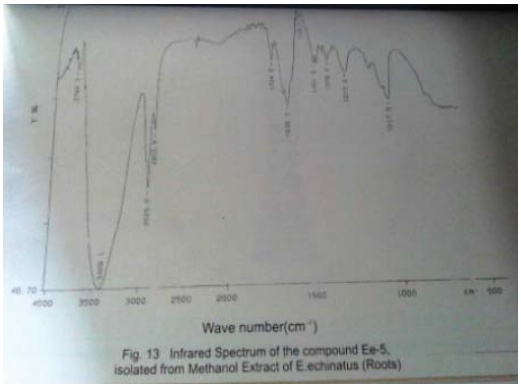

Fig. 10. IR peak of compund Ee-5

Table 7. Fluorescence analysis (*Echinops echinatus* powder)

Sr.#	Powdered crude drug+ reagent	Ordinary light	UV light	
			254 nm	365 nm
1	Powder as such	Light yellow	Brownish grey	Fluorescent yellow
2	Powder +H_2O	Brownish yellow	Yellowish green	Yellow
3	Powder +1N NaOH	Yellowish brown	Grey	Light green
4	Powder +HCl	Brownish yellow	Blackish brown	Dark brown
5	Powder +H_2SO_4	Brownish black	Black	Black
6	Powder +Picric acid	Bright yellow	Green	Brownish green
7	Powder + Acetic acid	Buff yellow	Light brown	Yellow with bright particles
8	Powder+ HNO_3	Reddish brown	Dark brown	Greyish black
9	Powder+ Methanol	Light green	Greyish green	Light green
10	Powder+ Ethanol	Light green	Light green	Fluorescent green

6. CONCLUSION

Standardized pharmacognostic evaluation for this plant has yet not been much reported in literature. The roots powder subjected for macroscopic, microscopic pharmacognostic analysis provides important information which may be helpful in the authentication of the sample and also to check adulteration for quality control of raw material. The pharmacognostic parameters observed in present study, being reported for the first time may be helpful for standardization and preparation of the crude drug's formulation and inclusion in various pharmacopoeias to be utilized as a potential therapeutic agent for treating various diseases.

The current observation may be helpful to differentiate this species from other species of family Asteraceae. UV and IR spectra may be useful for spectral analysis of the plant.

CONSENT

It's not applicable.

ETHICAL APPROVAL

It's not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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