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EXTRACTION, CHARACTERIZATION AND PREPARATION OF STRUCTURAL ANALOGUE OF BIOACTIVE CHEMICALS FROM LEAVES OF *ECHINOPS ECINATUS* PLANT.

1. Introduction-

Echinops echinatus Family: Asteraceae that is native to More or less throughout India and Afghanistan. It is an erect branched herb about a meter high. It has short, stout stems, branching from the base, covered with white cottony hair. It is used as medicinal plant used in urinary disorder, liver disorder, heart diseases, etc. The root is abortifacient aphrodisiac. The seeds are sweet and aphrodisiac (Aurveda).

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent. The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent.

2. Material and Method

Collection of plant material

The fresh leaves of *Echinops echinatus* climber were collected from Botanical garden of A.C.S College campus Dist-Amravati (Maharashtra) The experimental site is located between coordinates 20.91° N, 77.75°E and an altitude of 342 m in foothills of Central India experiencing the subtropical climate during winter season in the month Feb 2014 and the Authentication of plant was confirmed by botanist (Prof.S.K Tippat, Department of Environment Science , Art, Commerce & Science College Amravati).

Chemicals and microbial cultures

All the chemicals and standard antibiotics used in this work were purches from Sigma Aldrich, Merck and Hi-media, Mumbai India. The reference bacterial strains used in this study were obtained from American Type Culture Collection (ATCC) and Microbial Type Culture Collection (MTCC) Institute of Microbial Technology, Chandigarh, India. They were selected from gram positive and gram negative bacteria to represents a broad spectrum of potential pathogens that pose significant threats in the medical field

Preparation of plant extract

The plant were dried over ambient temperature and the dried sample were grind properly and dried powder sample was extracted in Methanol at 65ºC, by using soxhlet apparatus and extracts were concentrated by gradually evaporating the respective solvent on rotary evaporator . The concentrated extract was collected in sterile bottles and kept in a cool and dark place prior to analysis.(U.S.Khandekar et al)

2.1 Phytochemical analysis (Qualitative analysis)

Test for Alkaloids: - 0.4 g extract of each plant was mixed with 8 ml of 1% HCl, warmed and filtered. 2 ml of each filtrate was titrated separately with (a) Mayer's reagent and (b) Dragendorff's reagent (c) Wagner Test, Yellow precipitation for Mayer's reagent, Red precipitation for Dragendorff's reagent and formation of brown / Reddish precipitate for Wagner reagent was observed to indicate the presence of alkaloids. (Harborne 1973)

Determination of flavonoids: - Two methods were used to determine the presence of flavonoids in the plant sample. (Sofowara, 1993)

Cyanide test:-Put small pieces of magnesium ribbon into extract of sample and few drop of con HCl .The presence of bubble clour ranging from orange to red with indicate flavonoids .Red to crimson indicate presence of flavonoids. Crimson to magenta indicate presence of flavonoids Green or blue was presence reaction either aglycone. (Shah et al 2011)

Test for Phenolic(Tannins) compound

Ferric chloride test*:* - The extract (50 mg) is dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution is added. A dark green color indicates the presence of phenolic compounds.

Lead acetate test*:* - The extract (50 mg) is dissolved in distilled water and to this, 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

Gelatin test*:* - To the extract 1% gelatin solution containing sodium chloride was added. Formation of white precipitation indicates the presence of tannins. (W.C Evans et al 1989)

Test for steroids: - 0.5 ml of the each extract was dissolved in 3 ml of chloroform and was filtered. To The filtrate, concentrated sulphuric acid was added by the sides of the test tube, which formed a lower layer. A reddish brown colour ring with a slight greenish fluorescence was taken as the indication for the presence of steroids. (Sazada S et al 2009)

Test for terpenoids *(*Salkowski test):- 5 ml (1 mg/ml) of each extract was mixed in 2 ml of chloroform, and then 3 ml concentrated H_2SO_4 was carefully added to form a layer. A reddish brown colouration of the inter face was formed which showed positive results for the presence of terpenoids. (Harborne, 1973)

Test for Saponins:-

Foam Test*:* - 0.5 gm of extract was shaken with 2 ml distilled water if foam produce persist for ten minute it, indicated the presence of saponins. (Trease, GE and Evans WC, 1989)

Test for glycosides:-

Legal's Test: - Extracts were treated with sodium Nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red color indicates the presence of cardiac glycosides. (Trease, GE and Evans WC, 1989, N. Raaman. Phytochemical Techniques 2006)

Table 2.Phytochemical analysis of *Echinops echinatus*

S.N	Phytochemical	Tests performed	Aqueous extract	Methanolic extract
1	**Steroid**	Ring Test	-	-
3	**Tannins and Phenolic Compound**	Ferric Chloride Test	-	++
		Lead Acetate Test	+	+++
		Gelatin Test	-	-
4	**Terpenoids**	Salkowski Test	+	+
5	**Alkaloids**	Mayer Test	-	+
		Dragendroff's Test	-	+++
		Wagner Test	-	++
6	**Flavonoids**	Cynadine Test	+	++
7	**Glycosides**	Legal test	+	+++
8	**Saponins**	Foam Test	-	-

+++ indicates: strong presence, + indicates: weak presence, - indicates: strong absence

2.2 Antimicrobial Activity of *Echinops echinatus* leaves

Antimicrobial activity organic extracts methanolic of *Echinops echinatus* leaves were determined by Agar disc diffusion assay according to the Manual of antimicrobial susceptibility testing (Cavalieri 2005)was used to assay the various antibiotics for bactericidal activity against test strains of E. coli, K. pneumonia, S.epidermidis , S. aureus, P. acnes, S.typhi. the steps of this method are carried out by following steps.

Sterilization

All the glass wares were thoroughly washed and cleaned with double distilled water and wrap each glass ware with brown paper and then autoclaved at 120°C at 15 lb pressure for 20 minutes and then dried in hot air oven at 160°C.

Nutrient Agar Medium

13 gram of the powdered medium (HIMEDIA) was dissolved in 1000 ml sterile distilled water in a conical flask. The weighed amount was mixed properly and allowed to dissolve by heating over a water bath. The conical flask was then plugged with cotton wool and wrapped with aluminum foil. The flask was then Autoclaved at 120 degree for 20 minutes. The sterilized medium was then poured over sterilized glass petriplates (Borosil) and allowed to cooled and solidified.

Test Bacteria

Escherichia coli (ATCC-14948), Staphylococcus aureus (ATCC-33591), Klebsiella pneumonia (MTCC-4030), Staphylococcus Epidermidis (MTCC-3086), propioni bacterium acnes(ATCC-1951),, salmonella typhi (ATCC-25812),were purchased from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and used for assessment of antibacterial activity.

Nutrient Broth Medium

0.5 of the powdered Nutrient Broth medium (HIMEDIA) was dissolved in 25 ml sterile distilled water in a conical flask and transferred this solution in small test tubes and these test tubes was then plugged with cotton wool and wrapped with aluminum foil. Then Autoclaved at 120 degree for 20 minutes. After autoclaving cool this solution inoculating the bacteria each of the test tube by using inoculating needle and transferred these test tubes in incubator at 37^0c for 18 to 24 hours for growing inoculating bacteria.

Swabbing and preparation of impregnated disc

Deep the cotton swab into broth culture of the organisms. Gently squeeze the swab against the inside of the tube to remove excess fluid. Use the swab to streak a nutrient agar plate for a lawn of growth. Allow to plates dry for about 1 hrs. Sterile disc and antibiotic disc such as tetracycline, streptomycin can be place on the surface of the nutrient agar plates. Each test plate comprises of four discs. One positive control, which is a standard commercial antibiotic disc, one negative control (sterile disc), and one experimental sterile disc (for plant extract)

Pour the plant extract about Twenty micro liter on experimental sterile disc and ethyl acetate used as negative control poured on second sterile disc, then transferred the all Petri plates in incubator at 37^0c for 24 hours. After incubation at 37 °C for 24 hrs, the zones of inhibition were measured. The experiment was done three times and the mean values were presented. Positive controls were used in experiments antibiotics (Tetracycline -10 mcg) as a standard.

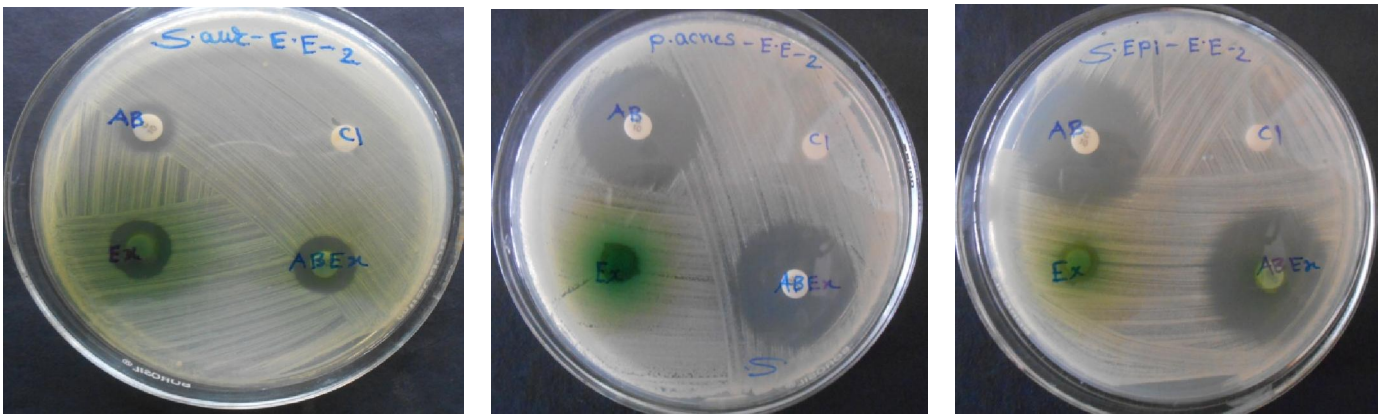
Table 3 Antimicrobial activity of *Echinops echinatus* leaves

Organisms	Test Samples (Growth inhibition [a]) mm			
	AB	**Ex**	**AB-Ex**	**Cl**

S.aureus	14±0.3	15±0.2	16±0.3	00
P.acne	26±0.1	10±0.3	26±0.1	00
S.epidermidis	34±0.3	09±0.4	34±0.3	00
E.coli	28±0.2	00	28±0.2	00
K. pneumonia	24±0.3	00	24±0.3	00
S.typhi	29±0.3	00	29±0.3	00

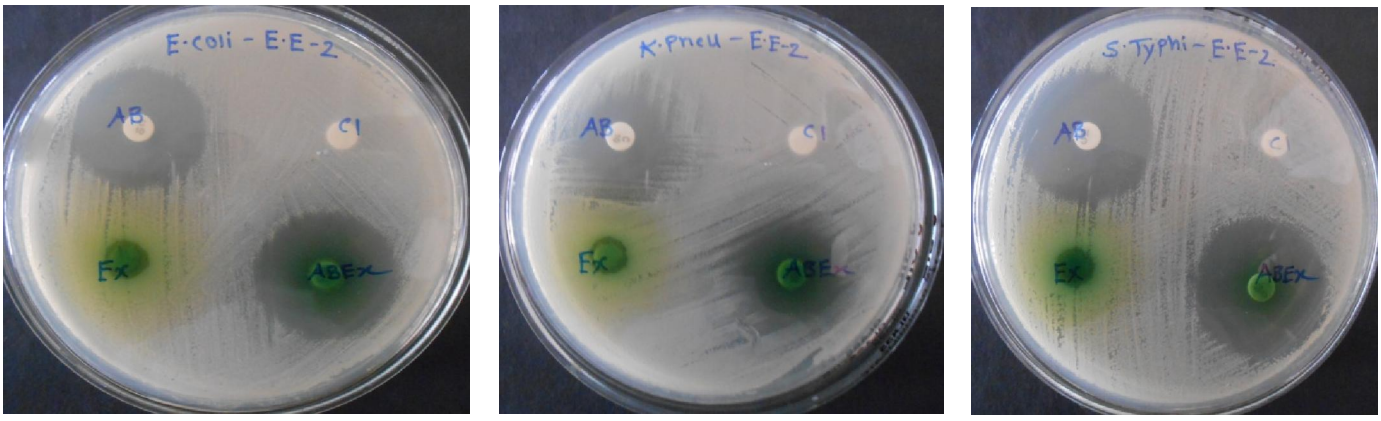
[a]- Values are represented as the mean ±S.D. of experiments.

A) Gram positive bacteria -



S.aureus **P.acne** **S.epidermidis**

B) Gram negative bacteria -



E.coli **K. pneumonia** **S.typhi**

Figure - 2: Antibacterial activity of *Echinops echinatus* Leaf against six bacteria, in each image: AB- Antibiotic disk, Cl - Sterile disk (control), Ex- Extract disk, AB+ Ex- Antibiotic + Extract disk.

2.3 Antioxidant activity of *Echinops echinatus*

The radical scavenging activity of the plant extracts against 2,2 Diphenyl -1-Picryl hydroxyl radical were determined by UV spectrophotometer carry 60 (Agilent). Antioxidant present in plant usually quantified employing folins reagent i.e. DPPH is used as a quantify antioxidant .DPPH assay is often used to evaluate the ability of antioxidant to scavenge free radical which are known to be a major factor in biological damage to caused by oxidative strace (Sochor J, 2010)

Reaction:- Reduction of DPPH from stable free radical (purple). Antioxidant react with DPPH is often used to evaluate the ability of antioxidant to scavenge free radical which is known to be major factor which stable free radical become paired in the presence of H donor and reduce to DPPH-H to yellow colour.

$$DPPH + H\text{-}A \longrightarrow DPPH\text{-}H + A$$

Extract was examined by comparing it to activity of known antioxidant such as ascorbic acid by scavenging activity.

Sample Preparation:-

The plant of *Echinops echinatus* firstly dry at room temperature after drying the sample get grind with the help of mixer. Then to prepare plant extract of methanol with the help of soxhlet apparatus at 62^0C. After extract preparation the extract were filter with the help of Whatman filter paper no.1.and reduce the sample to dry and stored in refrigerator.

Antioxidant activity (DPPH free radical scavenging activity) of methanolic extract:-

The free radical scavenging activity of the extracts, based on the scavenging activity of the stable 2, 2-diphenyl-1- picrylhydrazyl (DPPH) free radical was determined by the method described by (Shen et al, 2010). The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid was used as standard in 1000-5000 µg/ml solution. 3.94 mg of DPPH was prepared in 100 ml methanol and 2.96 ml of this solution was mixed with 40 µl of sample solution and standard solution separately. These solution mixtures were kept in dark for 20 min and optical density was measured at 517 nm using UV-Vis Spectrophotometer (UV-1700 Shimadzu). DPPH solution was used as blank. The optical density was recorded and % inhibition was calculated using the formula given below

$$\textbf{\% of DPPH Radical Scavenging activity \% RSA} = \frac{\textbf{Abs}_{\textbf{control}} - \textbf{Abs}_{\textbf{sample}}}{\textbf{Abs}_{\textbf{control}}} \times 100$$

Abs control is the absorbance of DPPH radical and methanol. Abs sample is the absorbance of DPPH radical + sample extract was the measure. Absorbance values were corrected for free radical decay using blank solution. And IC_{50} value can calculate by using calibration curves verses percentage of inhibitions.

Simple Reads Report

Collection Time:	5/12/2016 4:02:32 PM
Method:	
Version	5.0.0.999
Instrument:	Cary 60
Ave Time (sec)	1.0000

Read	Abs	nm
DPPH (Control)	0.7753	517.0
E.E-1	0.6261	517.0
E.E-2	0.4967	517.0
E.E-3	0.2757	517.0
E.E-4	0.1745	517.0
E.E-5	0.1341	517.0

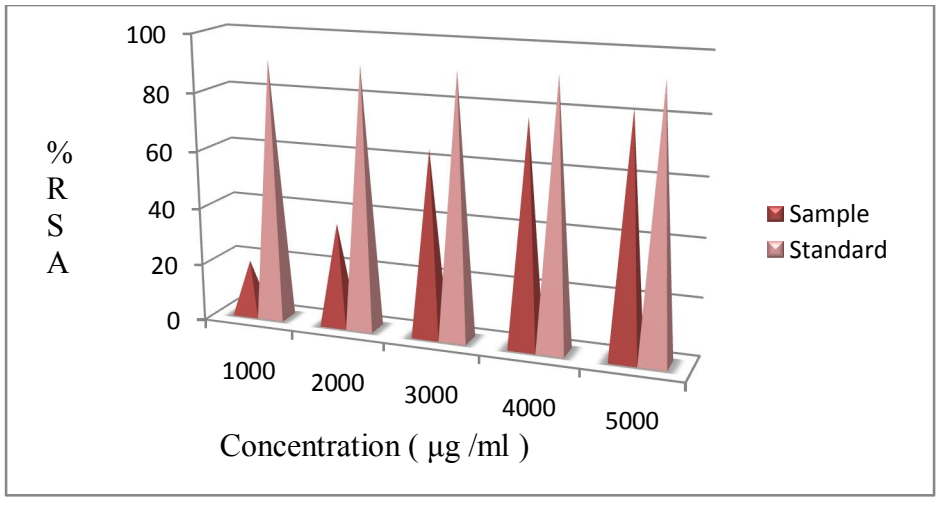
Simple Reads Report

Collection Time:	12/20/2015 4:38:17 PM
Method:	
Version	5.0.0.999
Instrument:	Cary 60
Ave Time (sec)	1.0000

Read	Abs	nm
DPPH(Control)	0.8784	517.0
STD-1	0.0806	517.0
STD-2	0.0792	517.0
STD-3	0.0771	517.0
STD-4	0.0705	517.0
STD-5	0.0672	517.0

Fig- Simple read report of sample *Echinops echinatus* and Ascorbic Acid (Standard) at different concentration

Types of Sample	Concentration ug/ml	% Radical Scavenging activity
Echinops echinatus Plant Extract (Sample)	1000	19.24416
	2000	35.93448
	3000	64.43957
	4000	77.49258
	5000	82.70347
Ascorbic Acid (Standard)	1000	90.82423
	2000	90.98361
	3000	91.22268
	4000	91.97404
	5000	92.34973



Graph- DPPH radical scavenging activity of *Echinops echinatus*

3.2 GC-MS Analysis of *Echinops echinatus*

Gas Chromatography:-

Gas Chromatography of the plant extract was carried out on a 6890 Gas Chromatography model 5765 equipped with direct injector and split ratio set to 10:1. (DB-5) (5% phenyl polysioxane, 30m length 250u internal diameter; 0.25um film coating) fused capillary column. Helium was the carrier gas at 1.0 ml min. The oven temperature program was programmed to start at 35 ^{o} hold for 2 min then temp at 20 ^{0}c per min to 300 ^{0} c and hold for 5 min. Injector and detector temperature were 220 ^{o} c and 230^{o} c respectively .Injection size was 0.02 ul neat.

Gas Chromatography and Mass Spectroscopy:-

A JEOL GCmate II benchtop double-focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-2000^{1} software was used for all analyses. Low-resolution mass spectra were acquired at a resolving power of 1000 (20% height definition) and scanning from m/z 25 to m/z 700 at 0.3 seconds per scan with a 0.2 second inter-scan delay. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 65 to m/z 750 at 1 second per scan.

Identification of chemical constituents:-

Identification of the chemical constituents was done on the basis of retention index (RI) using a mass spectra library search NIST and by com-paring the mass spectral and retention data with literature (P. Abirami, 2012). The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

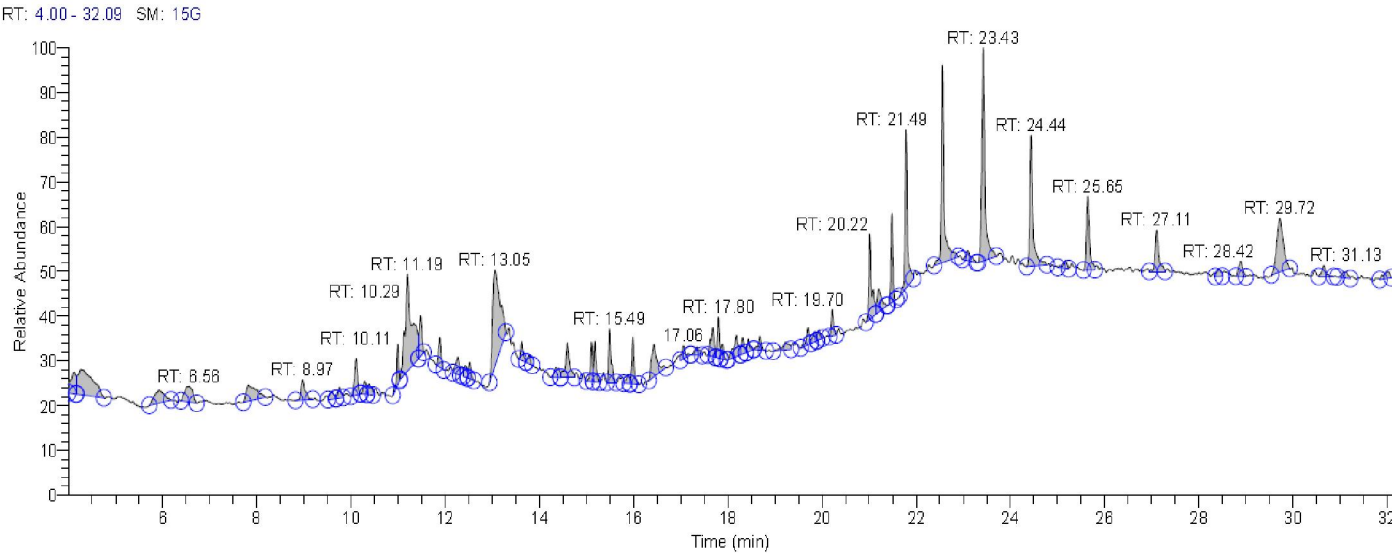


Fig- 2. Gas chromatogram of leaves extract of *Echinops echinatus*

Table No 2 :- Chemical Composition of *Echinops echinatus* leaves

Sr. No	Rention Time	Name of chemical constituent	Molecular Formula	Peak Area %
1	5.92	1HAzonine, octahydro1nitroso	$C_8H_{16}N_2O$	18.70
2	10.11	Ethanone,1(3butyl2hydroxy5methylphenyl)	$C_{13}H_{18}O_2$	1.37
3	11.19	Phenol, 2(1,1dimethyl2propenyl) 3,6dimethyl	$C_{13}H_{18}O$	7.98
4	13.05	Quinic acid	$C_7H_{12}O_6$	4.38
5	16.43	Phthalic acid, butyl 2ethylbutyl ester	$C_{18}H_{26}O_4$	2.10
6	17.80	Phytol	$C_{20}H_{40}O$	1.08
7	21.01	Eicosane	$C_{20}H_{42}$	2.94
8	21.49	Diisooctyl phthalate	$C_{24}H_{38}O_4$	2.07
9	21.78	Tetracosane	$C_{24}H_{50}$	29.03
		Tetratriacontane	$C_{34}H_{70}$	
		Triacontane	$C_{30}H_{62}$	
10	29.72	Propanoic acid, 3,3'thiobis, didodecyl ester	$C_{30}H_{58}O_4S$	4.28

3.3. Isolation of major chemical component by Column and thin layer chromatography

3.3.1 Column Chromatography

Slurry was prepared by mixing 60gm of silica gel with ethanol and then the column was packed with the slurry. Then the 10ml plant extract was applied on top of column and six fractions were collected by eluting the column with Benzene & ethyl acetate at different proportions. Out of these six fractions, fraction three which was contained the major component was subjected to further purification by using preparative thin layer chromatography.(Laurence Dinan et al 2001, Satyajit D. Sarkar, Lutfan Nahar, 2012)

3.3.2 Thin layer Chromatography

The second fraction (200mg) obtained from column chromatography was applied in the form of aband, on preparative silica gel plates The solvent system, Benzene: Ethyl acetate (1:1) was used as a developing solvent system. The required band (F_3Y) was scraped off and collected in a beaker to which methanol was added and filtered. The filtrate was then concentrated under reduced pressure and the residue was kept in a refrigerator for further investigation for GC-MS analysis, ^{1}HNMR and IR Spectroscopy.

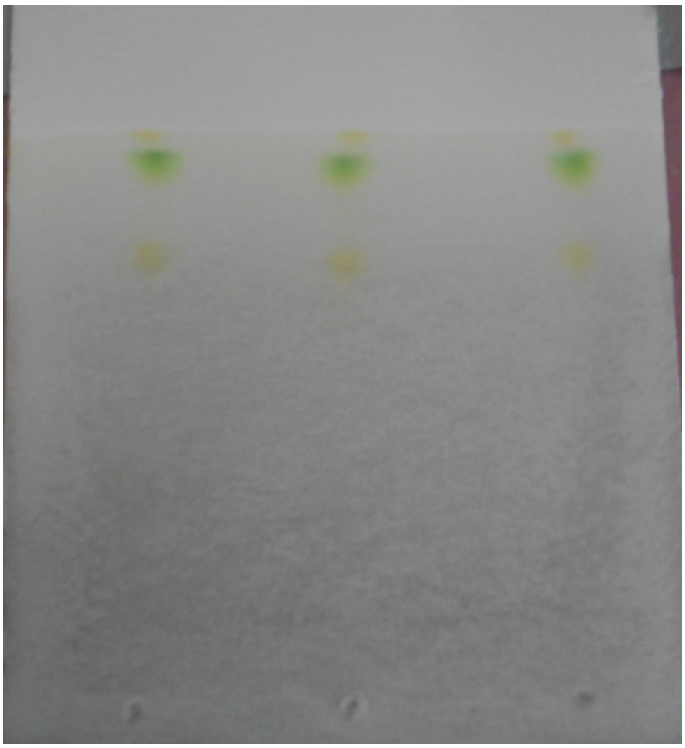
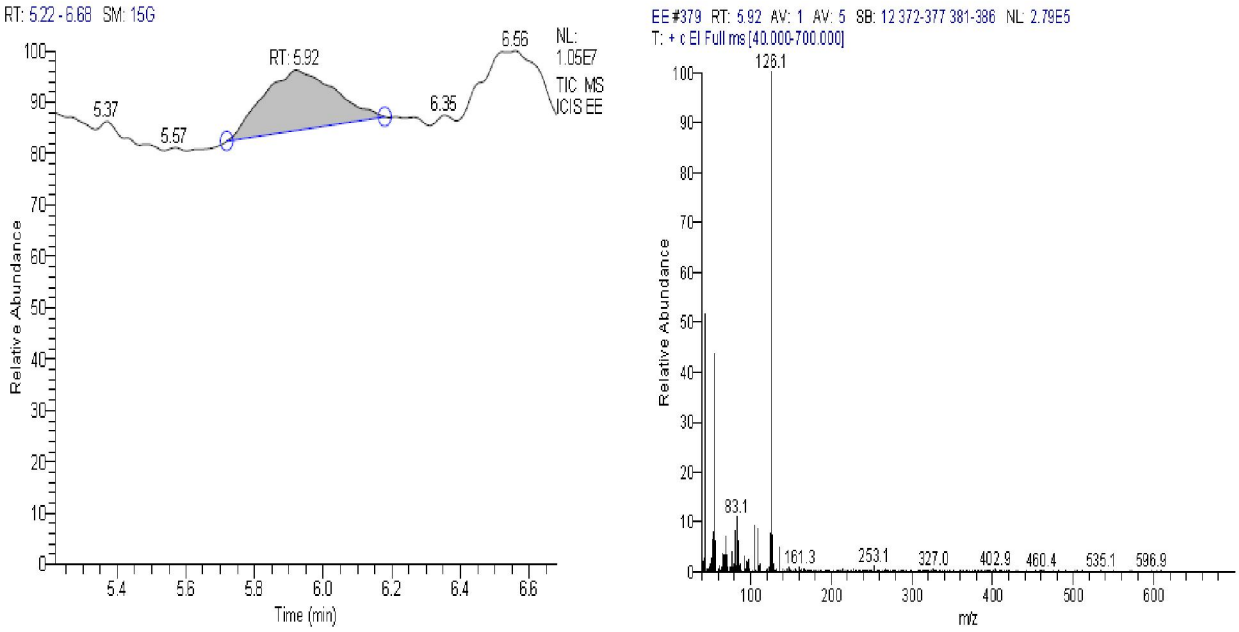


Fig -2. TLC Image for fraction second isolate (F_3Y)

3.3.2.1 Gas Chromatogram and Mass Spectrum of isolate F_3Y



Library Search Results Table

Compound Name	RT	Molecular Formula	Cas #
Cholestan-22(26)-epoxy-3,16-dione	5.92	C27H42O3	NA
1H-Azonine, octahydro-1-nitroso-	5.92	C8H16N2O	20917-50-4

3.3.2.2 ^{1}H-NMR Spectrum of isolate F_3Y

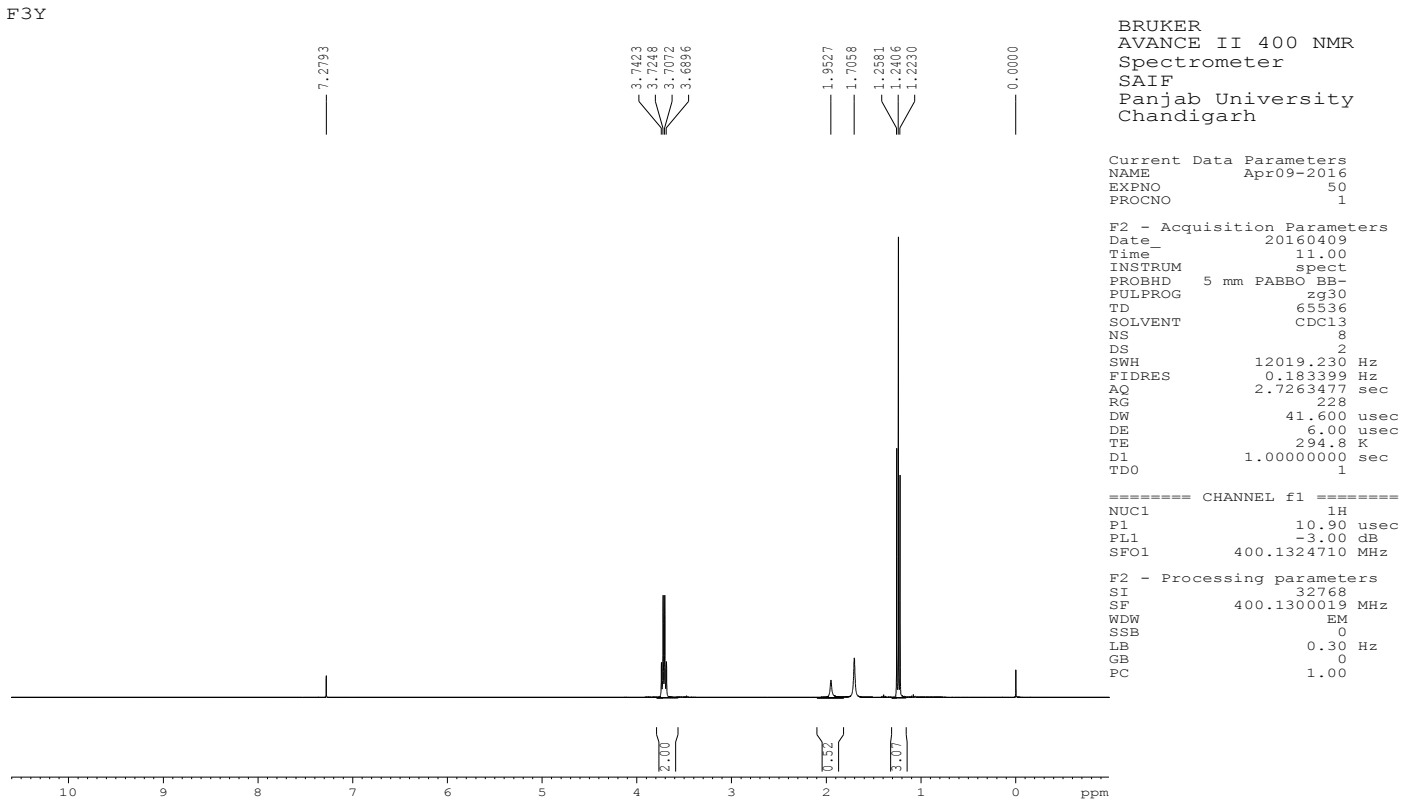


Fig- 3. ^{1}H-NMR Spectrum of isolate F_3Y

The ^{1}H-NMR spectrum in Fig-3 shows the peak at δ 1.22- 1.95 correspond to the protons of the cyclic ring, which appeared as multiplet due to overlap of signals, The N-CH_2 gave signals near δ 3.68-3.74 as doublet

3.3.2.3 IR Spectrum of isolate F_3Y

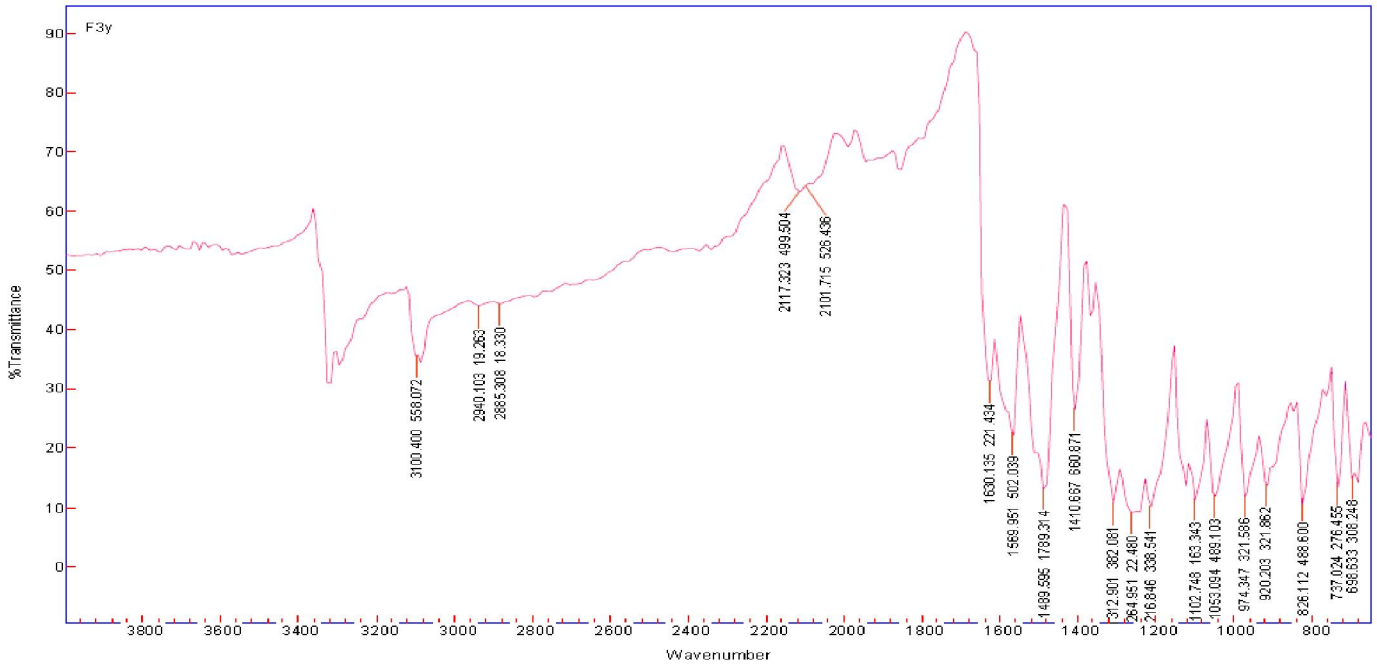
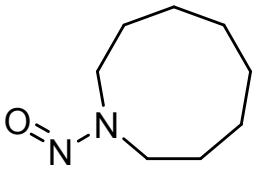


Fig-4. IR Spectrum of isolate F_3Y

The IR spectral analysis of isolate **F_3Y** -1is showed the presence of following absorption band.

Sr.No	Absorption cm^{-1}	Assignment for group	Literature value cm^{-1}
1	2940.103	C-H	2840-3000
2	1489.595	C-C	1400-1500
3	1312.901	C-N	1000-1350
4	1569.951	N=O	1515-1570
5	2117.323	N-N	2110-2160

On the basis of above spectral data of GC-MS,^{1}HNMR and IR the assign structure of isolate F_3Y is



1HAzonine, octahydro1nitroso

$C_8H_{16}N_2O$
Exact Mass: 156.13
Mol. Wt.: 156.23

4. RESULT AND DISCUSSION

Phytochemical evaluation is to confirm the presence of various chemical constituent present in plant. Phytochemical analysis listed in Table No.1. Due to higher polarity of methanolic extract show revealed presence of maximum phytochemical composition these phytoconstituents independently responsible for the broad range of medicinal properties.

GC-MS chromatogram analysis of the Methanolic extract of *Echinops echinatus* Fig-1 showed major 10 peaks which indicating the presence of various phytochemical constituents. On comparison of the mass spectra of the constituents with the NIST library. The various phytochemicals which contribute to the medicinal activities like antimicrobial, antifungal, antiviral and antioxidants.(H.Wagner, S. Bladt, 1996).

GC-MS chromatogram analysis of the methanolic extract of *Echinops echinatus* showed high percentage of 1,30 Triacontanediol is a nitro compound like alkaloid. After stage wise analysis we succeed in isolation of pure component from crude extract of *Echinops echinatus* leaves is further confirmed by GC-MS,^{1}HNMR and FTIR. The results of all these revels the presence of **1HAzonine, octahydro1nitroso** which is confirmed in structural elucidation by 1HNMR.

The result of antibacterial screening was carried out against six bacterial strains. *Echinops echinatus* is listed in TableNo.3. Methanolic extract of *Echinops echinatus* shows most efficient inhibition bacterial growth against gram positive bacterial strain due to presence of some bioactive phytochemicals like 1HAzonine, octahydro1nitroso act as Nitro compound, Phytol is Diterpene and Quinic acid.

The radical scavenging activity of the *Echinops echinatus* leaf extract was tested using stable free radical DPPH (Deep purple colour) as DPPH has the advantage of being unaffected by certain side reaction Graph-1 show the DPPH radical scavenging activity of *Echinops* extract with ascorbic acid as reference where the IC_{50} value for the *Echinops* extract was calculated by using graph pad prism non linear curve fit Sigmoidal, 4PL, X is log(concentration) and it was found to be IC_{50}= 82.06 lower than 100. The lower the IC_{50} values show the higher antioxidant activities.

5. CONCLUSION

The presence of various bioactive compounds in the *Echinops echinatus* justifies the use of whole plant for various ailments by traditional practitioners. However, isolation of individual phytochemical constituents we succeed in isolation of pure component from crude extract of *Vernonia elagenfolia* leaves is further confirmed by GC-MS,^{1}HNMR and FTIR and subjecting it to the biological activity will definitely give fruitful results. From the results, it could be concluded that *Echinops echinatus* contains various bioactive compounds. Therefore, it is recommended as a plant of phytopharmaceutical importance.

6. ACKNOWLEDGEMENT

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