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ANALYSIS OF PHYTOCONSTITUENTS AND CYTOTOXIC ACTIVITIES OF DIFFERENT PARTS OF *OCIMUM SANCTUM*

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Abstract

Phytochemical screening of extracts of *Ocimum Sanctum* revealed the presence of fatty acids, steroids, triterpenoid, phenolic compound, flavonoid, glycoside, quinines, and carotenoids. β -sitosterol and Ursolic acid were isolated from leaves of the plant by using column chromatography and identification of these compounds were performed with the help of melting points, Co-TLC and spectroscopic techniques such as IR, ^1H NMR and ^{13}C NMR. GC-MS of hexane fraction showed presence of pentanal, sotolone, hexane-3-one, hexane-2-ol, caryphyllene, benzene 1,2 di-carbolylic acids, isoeugenol, cavaacrol and eugenol compounds. The brine shrimp bio assay showed LC_{50} value 213.7, 97.72 and 21.87 for hexane, ethyl acetate and methanol extracts of leaves respectively, where as LC_{50} value 169.84, 141.25 and 151.35 for hexane, ethyl acetate and methanol extracts of stem respectively. All extracts are pharmacologically active.

Key words: β -sitosterol, Ursolic acid, spectroscopic techniques, brine shrimp bio assay.

Introduction

Nepal is rich in all three levels of biodiversity namely species diversity, genetic diversity and habitat diversity. There are thousands of plant species which are known to have medicinal value of the plants, plants extract and their active constituents. Among the 7000 species of medicinal plants recognized all over the world, more than 900 types of precious medicinal plants are found in Nepal (Joshi B. *et al.* 2011). The plant *Ocimum sanctum* (Tulsi) is one of the most important species of genus *Ocimum* which belongs to family *Lamiaceae*. The plant is widely cultivated in India and Nepal due to its specific aromatic fragrance and medical value. It is considered as the sacred plant in Indian subcontinent. In Aurveda, it has been well documented for its therapeutic potential and described as antiasthmatic and cough suppressant drug (Gupta SK, *et al.*, 2002; Singh N., 2005 & Xiaorui Z., 2002). It is very valuable medicinal plant for successful management of various diseases like bronchitis, bronchial asthma, dysentery, dyspesia, skin disease, chronic fever, reducing of blood glucose level as well as reduction of total cholesterol in blood (Prakash P. *et al.*, 2005). The plant shows various activities such as good antioxidant, anticancer preventive, antistress, immune modulator, chemo preventive, radio protective, antimicrobial, analgesic, anti-inflammatory antipyretic,

antifertility, antidiabetic, anti-thyroid, anti-toxic activity (Singh E *et al.*, 2012 & Prashar R *et al.*, 1998).

Materials and Methods

Sample collection and plant extraction

The fresh leaves and stems of plants were collected from Janakpur, Dhanusha districts of Nepal. The leaves and stems were shade dried and powdered separately. 250g of dried powder of leaves and stems were separately subjected to sequential extraction by soxhlet using different solvents such as hexane, ethyl acetate and methanol. The solvent was evaporated under reduced pressure and low temperature to get viscous extract.

Chemical and equipments

Silica gel (mesh 60-120) was used for column chromatography. Thin-layer chromatography was also performed on silica gel G coated TLC plates of E. Merck Company. Spots were visualised by exposure to iodine vapour, UV radiation and by spraying with ceric sulphate solution. All the solvents used in different experimental process were from E. Merck Company and Qualigen fine chemicals. Melting points were determined by using melting point apparatus from Griffin and George Company Limited. The ^1H NMR and ^{13}C NMR Spectra were scanned using 400 (MHz) NMR Spectrophotometer in CDCl_3 and with TMS as internal standard. GC-MS analysis was performed on a

gas chromatography mass spectrometer QP 2010, Shimadzu, Japan. Brine shrimp bioassay was carried out using brine shrimp from Yi Hu Fish farm Trading Pvt. Ltd, Singapore.

Phytochemical Screening

The phytochemical Screening was carried out according to Prof. I. Ciulei Procedure (Ciulei I., 2082).

Isolation and characterization of compound

The compound was isolated by using column chromatography and characterized with the help of melting point, CO-TLC, spectroscopic data such as IR, ¹HNMR, ¹³CNMR and comparing these data with the authentic sample.

Analysis of Hexane Fraction for GC-MS

On eluting column with 2% ethyl acetate in hexane, multiple spots were obtained which was further analyzed from GC-MS.

Brine shrimp bioassay

The brine shrimp bioassay was carried out according to Mayer *et al.* procedure (Mayer *et al.*, 1982).

Result and Discussion

Yield of Extract

The yields obtained from the leaves and stems are presented in Table 1. The table shows that the polar solvents gave higher yield than non polar solvents.

Table 1: Yields of different solvent extracts

Part of Plant	Hexane (g)	Ethyl acetate (g)	Methanol (g)
Leaf	8.62	12.39	25.47
Stem	4.05	7.32	12.41

Phytochemical Screening

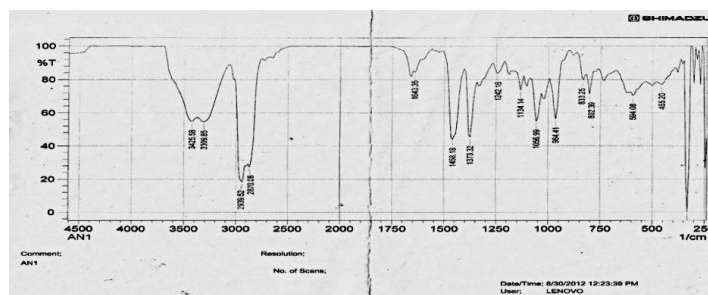
The results obtained from the phytochemical screening of leaves and stems of plants are shown in Table 2. The result showed the presence of Tannins, coumarine derivative in leaves extract but not in stem extract and Anthocyanoside, Anthracenoside flavonic glycoside were present in stem extract but not in leaves extract. Phytoconstituents like terpenoid, flavonoids, reducing sugar, fatty acid, glycoside, coumarine etc. were present in both the leaf and stem whereas quinines present only in leaves and carotenoid was present only in stem of the plant.

Isolation and Characterization of Compound

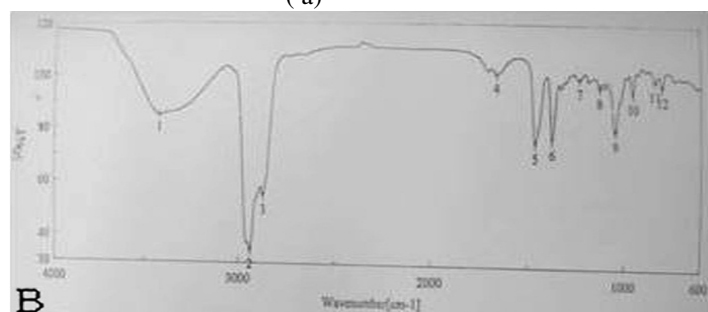
First Compound

The compound was isolated from hexane extract by column chromatography with 5% ethyl acetate in hexane as white needle shaped crystal. Its melting point was found to be 132 °C. It showed positive Salkowski test with reddish color indicating the presence of tetracyclic triterperoid. The compound gave single spot with 20% Ethyl acetate in hexane on TLC with Rf values 0.45.

The IR Spectrum showed broad absorption peak at 3425 and 3309cm⁻¹ indicating the presence of -OH group. The peak at 2939 and 2870cm⁻¹ indicated the presence of aliphatic C-H stretching. The absorption peak at 1643cm⁻¹ indicated the unsaturation that is C=C absorption peak and peak at 1056cm⁻¹ indicated the presence of C-O stretches. The absorption band at 1485cm⁻¹ indicated the presence of C-H bending of CH₂ and absorption band at 1373 and 1348cm⁻¹ is due to C-H bending of gem-dimethyl group. The absorption peak at 965 and 802cm⁻¹ is due to C=C-H group. The absorption peak at 1242cm⁻¹ is due to C-C stretching and peak at 725cm⁻¹ indicated mono substitution in aromatic ring. The IR spectrum of the isolated compound was compared which IR spectrum of authentic sample (β -Sitosterol) which are identical and these spectra are shown in Fig. 1.



(a)



(b)

Fig. 1: The IR spectrum of the isolated compound (a) and IR spectrum of β -Sitosterol (b)

Table 2: Results of phytochemical screening

S.N.	Groups of compound	LEAVES			STEM		
		Hexane	Ethyl Acetate	Methanol	Hexane	Ethyl Acetate	Methanol
1.	Fatty acids	+	+	-	+	-	-
2.	Volatiles oils	-	-	-	-	-	-
3.	Alkaloids	-	-	-	-	-	-
4.	Carotenoids	-	-	-	+	+	-
5.	Flavanoids	-	-	-	-	-	-
6.	Emodins	-	-	-	-	-	-
7.	Quinones	+	-	+	-	-	-
8.	Coumarins	+	-	+	-	+	-
9.	Sterols and Triterpenes	+	+	-	+	+	-
10.	Tannins	-	+	+	-	+	-
11.	Glycosides	+	-	+	-	-	+
12.	Reducing compounds	-	-	+	-	-	+
13.	Anthocyanosides	-	-	-	-	-	+
14.	Polyoses	-	-	-	-	-	-
15.	Saponins	-	-	-	-	-	-
16.	Anthracenosides	-	-	-	-	-	+
17.	Flavone Glycoside	-	-	-	-	-	+

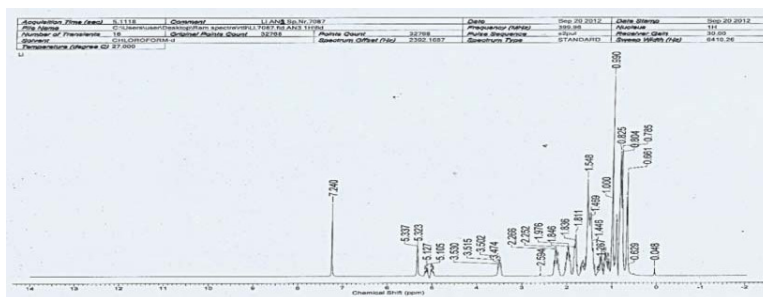
The ^1H NMR spectrum of compound displayed two singlets at $\delta 1.00$ and $\delta 0.66$ indicated the methyl proton of C-19 and C-18 respectively. The proton NMR spectrum also exhibited one olefinic double bond proton (C-6) as a doublet at $\delta 5.33$. Other multiplets at $\delta 3.51$ equivalent to singlet proton was assigned for the proton of C-3. Two upfield signals also doublet at $\delta 0.80$ and $\delta 0.78$ respectively due to presence of secondary methyl group at position C-26 and C-27 of skeleton representing the presence of isopropenyl group. The very upfield chemical shift at $\delta 0.82$ as a triplet was assigned for terminal methyl group of C-29. Similarly, other upfield chemical shift at $\delta 0.94$ as a doublet was assigned for methyl group at position C-21 of molecular structure. Three multiples equivalent to two protons each appeared at $\delta 1.83$, $\delta 1.97$ and $\delta 2.26$ were assigned for 3CH_2 group. The remaining protons have appeared at a range of $\delta 1.01$ to $\delta 1.7$. The weak signal of chemical shift at $\delta 5.1(\text{m})$ and $5.12(\text{m})$ were also obtained due to mixture of stigma sterol. The ^1H NMR spectrum of the isolated compound was compared which ^1H NMR spectrum of authentic sample

(β -Sitosterol) which are identical and these spectra are shown in Fig. 2.

The ^{13}C NMR spectra of the compound revealed the presence of 29 carbons. The chemical shift at $\delta 140.75\text{ppm}$ and $\delta 121.70\text{ppm}$ represented carbons of alkene conjugated that is C5 and C6 carbon. Similarly, the chemical shift at $\delta 71.810\text{ppm}$ represented C_3 carbon due to presence of OH group at this position. The chemical shift at $\delta 20.01\text{ppm}$ and 23.12ppm represented the C18 and C19 angular carbon.

The chemical shift at $\delta 138.29$ and $\delta 129.27$ represent the conjugation which was due to possibility of mixture of stigmasterol. The value of ^{13}C NMR spectrum of the isolated compound was identical with β -Sitosterol compound which is shown in Fig. 3.

Thus on the basis of melting points, CO-TLC, IR, ^1H NMR, ^{13}C NMR data and comparison of these data with literatures (Bag *et al.*, 2012, Arjun *et al.*, 2010 and Anooj K *et al.*, 2010) the isolated first compound was identified as β -sitosterol. The structure of this compound is shown in Fig. 4.



(a)



(b)

Fig. 2: The ¹H NMR spectrum of the isolated compound (a) and ¹H NMR spectrum of β –Sitosterol (b).

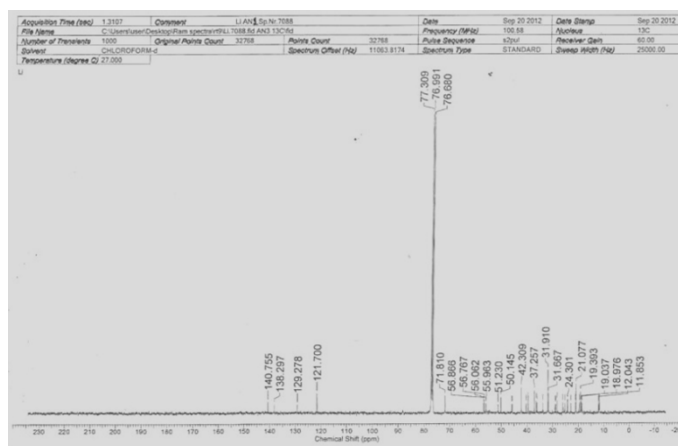


Fig. 3: ¹³C NMR spectrum of the isolated compound sample (β –Sitosterol)

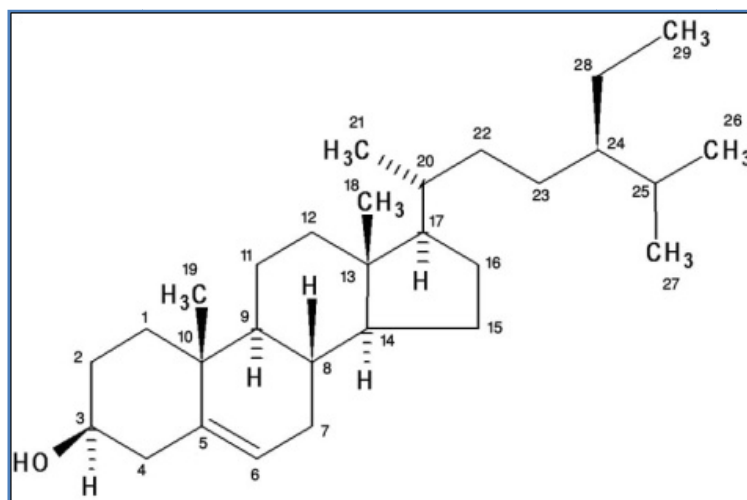


Fig. 4: Structure of β-sitosterol

Second compound

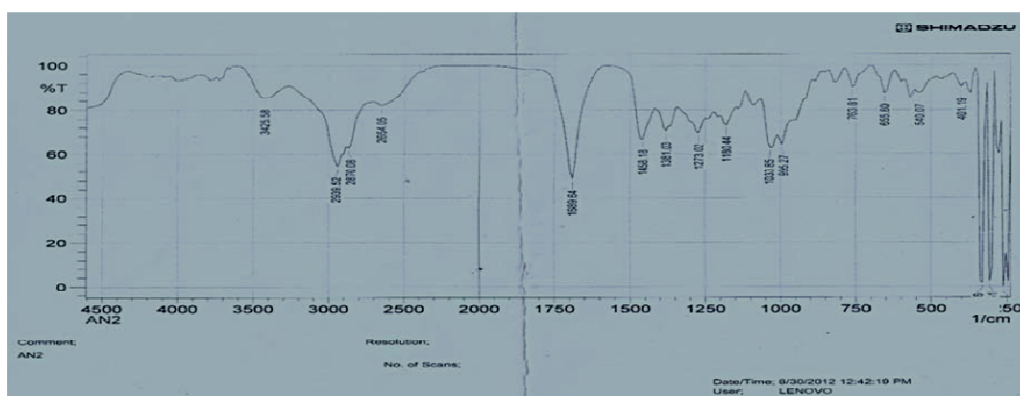
The compound was obtained from hexane extract by column chromatography with 10% ethylacetate in hexane as colourless crystal having m.pt.225 ° C. It showed effervescences with sodium bicarbonate solution. It also gave positive salkowski test with reddish colour indicating the presence of triterpenoid. The compound gave single spot showed with Rf values 0.64.in 30% ethyl acetate in hexane.

The IR spectrum displayed characteristics absorption band for hydroxyl group at 3425cm⁻¹. The absorption peak at 2654 cm⁻¹ and 1689cm⁻¹ indicated the presence of carboxyl group. The absorption band at 1033 cm⁻¹ and 996cm⁻¹ indicated the -C-OH band and another peak at 2939 and 2870cm⁻¹ indicated the presence of aliphatic C-H stretching. The IR spectrum of the isolated compound compared with IR spectrum of authentic sample (Ursolic acid) which are identical and shown in Fig. 5.

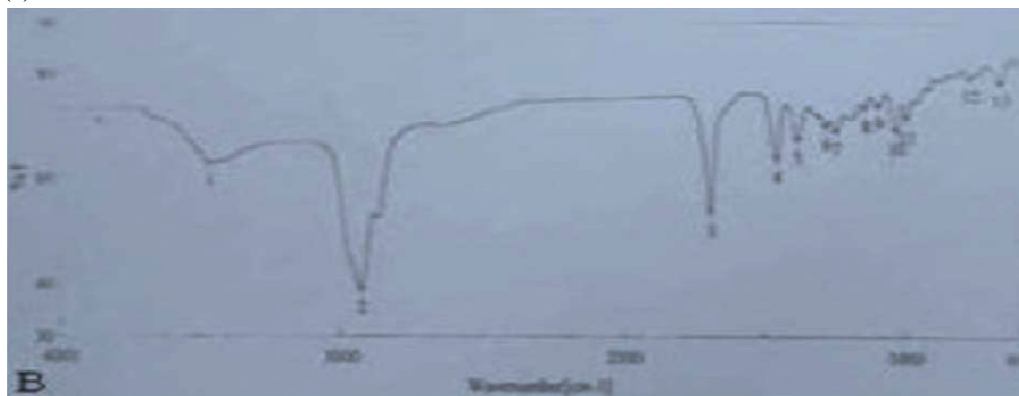
The ¹HNMR spectrum of the compound showed one proton broad signal at δ5.26 assigned to vinylic H-12 protons. One proton double doublets (triplet) at δ3.214

assigned to α-oriented carbinol H-3 proton. One proton doublet at δ2.789 was ascribed to C-18 proton. Five three proton broad signal at δ0.86, 0.70, 1.113, 0.966, 1.1 were associated correspondingly with tertiary C-23, C-24, C-25, C-26, C-27 methyl protons respectively and three proton doublets at δ0.75 and 0.908 corresponds to C-29 and C-30 methyl protons. The remaining methane and methylene protons resonated from δ2.60 to 1.23. The ¹HNMR spectrum of the isolated compound and ¹HNMR spectrum of authentic sample (Yamaguchi H. *et al.*, 2008) are shown in Fig. 6.

The ¹³CNMR spectrum of compound displayed 30 carbons atoms and important signals appeared for carboxyl group at δ182.92ppm (C-28). The chemical shift at δ143.57 and δ122.625 represented carbons of alkene conjugated that is C-12 and C-13 carbon. The chemical shift at δ79.008 represented carbinol carbon that is C-3 carbon. The ¹³CNMR spectrum of the isolated compound compared with ¹³CNMR spectrum of authentic sample (Ursolic acid), which are identical and shown in Fig. 7.

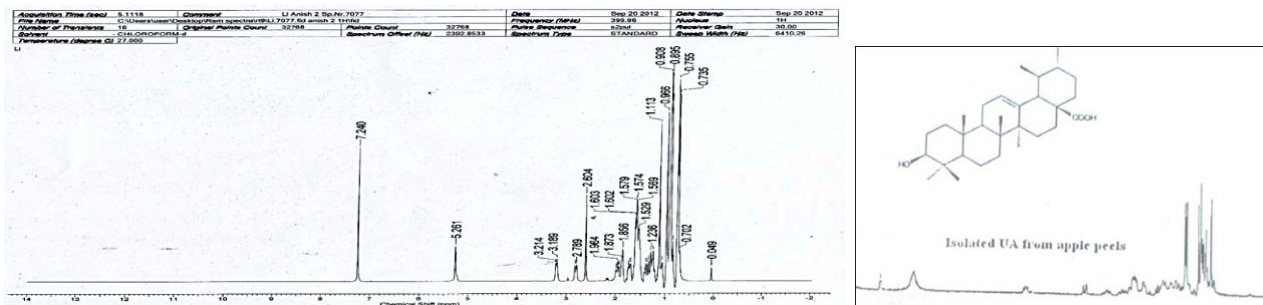


(a)

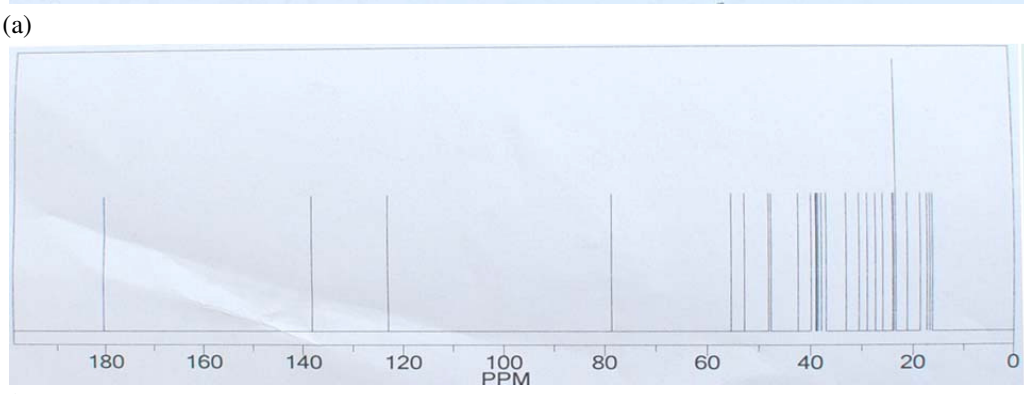
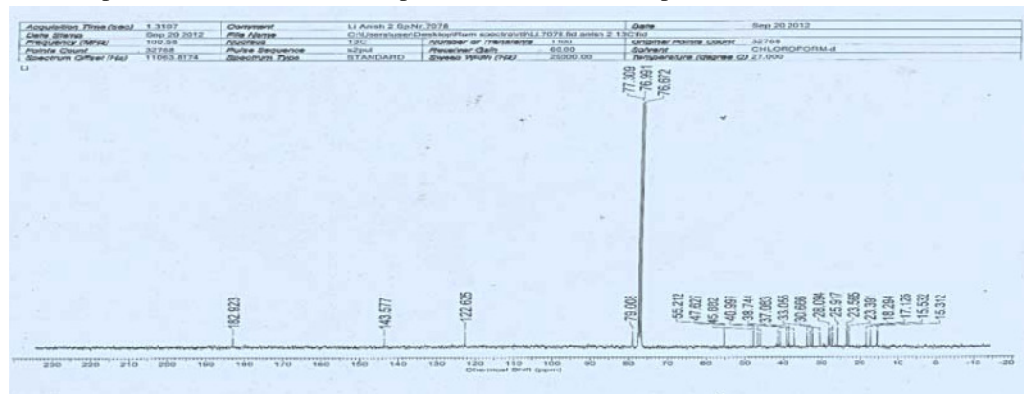


(b)

Fig. 5: The IR spectrum of the isolated compound (a) and IR spectrum of Ursolic acid (b).



(a) (b)
 Fig. 6: The ¹H NMR spectrum of the isolated compound (a) and ¹H NMR spectrum of Ursolic acid (b).



(a) (b)
 Fig. 7: The ¹³C NMR spectrum of the isolated compound (a) and ¹³C NMR spectrum of Ursolic acid (b).

Thus, on the basis of melting points, IR, ¹H NMR, ¹³C-NMR spectral data and comparison of these data with authentic sample, the compound was identified as Ursolic Acid. The structure of Ursolic acid is shown in Fig. 8.

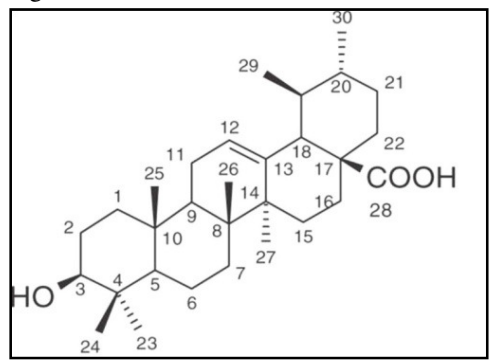


Fig. 8: The structure of Ursolic acid

Analysis of GC-MS Spectrum of Hexane Fraction

The fraction obtained from column with 2% ethyl acetate in hexane was further analyzed by GC-MS qualitatively, which are shown in Fig. 9. The spectra revealed the presence of 9 compounds such as pentanal, sotolone, hexane-3-one, hexane-2-ol, caryphyllene, benzene 1,2 dicarbolylic acids, isoeugenol, cavracrool and eugenol, which are given in Table 3. The compounds isoeugenol, cavracrool, Benzene1,2 dicarbolylic acids and eugenol were found to be major compounds in this fraction.

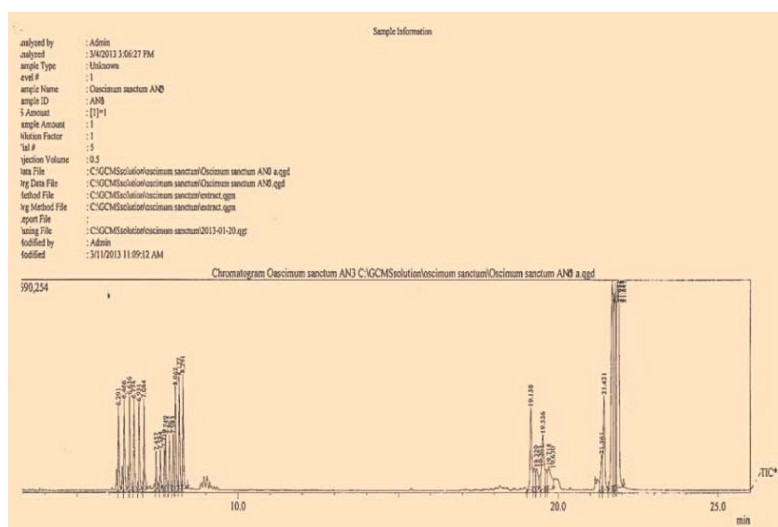


Fig. 9. GC-MS spectrum of 2% ethyl acetate in hexane.

Table 3: GC-MS Spectrum of 2% of ethyl acetate in hexane fraction

S.N	RT	COMPOUND	MW	Area (%)
1	8.602	Pentanal	86	4.82
2	8.177	sotolone	112	4.92
3	19.150	Hexane-3-one	100	7.09
4	19.526	Hexane-2-ol	102	6.22
5	21.431	Caryophyllene	162	7.70
6	21.681	Benzene 1, 2 di-carboxylic acids	166	16.33
7	21.716	Isoeugenol	164	12.32
8	21.784	Cavracrol	150	14.06
9	21.841	Eugenol	164	26.55

Brine Shrimp Bioassay

The Brine Shrimp bio assays of leaves showed that LC_{50} values of hexane, ethyl acetate and methanol fractions were 213.79, 97.72 and 21.87 respectively. The Brine Shrimp bioassay of stem showed that LC_{50} values of hexane, ethyl acetate and methanol fractions 169.84, 141.25 and 151.35 respectively. Among the entire fraction, methanol fraction of leaves showed lowest LC_{50} value and ethyl acetate fraction of stem showed lowest LC_{50} value. However, all the fractions were found to be bioactive.

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