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GC-MS ANALYSIS OF BIOACTIVE UNSAPONIFIABLE FRACTION FROM *SESBANIA SESBAN* L. (MERR)

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ABSTRACT

Analytical characterization was undertaken for the Unsaponifiable matter (USM) fractionated from the petroleum ether extract of *Sesbania sesban* using *GC-MS* technique. Perkin Elmer Autosystem XL Gas chromatograph equipped with Elite-5 capillary column and helium as carrier gas at a flow rate of 1ml/min was used for separation of the bioactive compounds. It was further analyzed using turbomass spectrometer operated in EI mode. Interpretation of mass spectrum was done using the database of National Institute Standard and Technology (NIST), WILEY8, and lipid library of American Oil Chemists' Society. The identification was made based on retention time, mass fragmentation pattern, peak intensity, molecular formula and molecular weight. GC-MS profile of bioactive fraction of unsaponifiable matter (USM), showed the presence of six major compounds including squalene, various sterol and phytol. The literature study revealed that the major identified phytosterols present in the fractions possess has cholesterol reducing agents, weak estrogenic activity and few of them were even used as precursor in the manufacture of many hormone used in contraceptives.

KEYWORDS: **GC-MS, Unsaponifiable matter, Phytol, Phytosterol, Squalene, *Sesbania sesban***

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INTRODUCTION

Sesbania sesban (L) Merr. , commonly known as Egyptian pea, Jayanti, Jait, Shewari belongs to the family leguminosae. It is a short-lived shrub or small tree up to 8 m tall, commonly grown as a shade plant for young seedlings grown during the hot season and as a wind break for sugarcane. The plant belongs to the genus *Sesbania* and is common throughout Africa and in Asian countries. It is commonly seen growing on the dikes between rice paddies, along roadsides and surrounding fields as a windshield[1]. The plant has got significant medicinal importance and is credited with various biological activities. Seeds of plant have shown control on female fertility in albino rats[2]. The leaf extract has been evaluated for its antiflammatory[3,4], antidiabetic activity[5] antioxidant[6] and antimicrobial activity[7]. Isolated bioactive compound from plant was evaluated for its spermicidal[8] activity. The plant is evaluated for cholinesterase[9] activity. Roots of this plant are evaluated for its antidiabetic activity[10]. According to Ayurveda, the leaves are purgative, maturant, demulcent and useful in all pains and inflammation. Juice of fresh leaves is credited with anthelmintic properties. The bark is useful in diarrhea and excessive menstrual flow. Juice of bark is given internally to cure skin diseases. Poultice of seeds is reported to be suppurative. Seeds are stimulant, emmenagogue, astringent, useful in diarrhea and reducing the enlargement of spleen. In the form of ointment, seeds are useful to cure itches and various skin irruptions[11].

Phytochemical study has identified the presence of oleanolic acid[12] and kaempferol trisaccharide[13] in the plant. Indole acetic acid has been isolated from the roots nodules of *Sesbania sesban*[14]. The pods are reported to contain lupeol, α-amyrin[15], galactomannan[16], stigmasta-5,24(28)-diene-3β-O-β-D-galactopyranoside[17], and β-sitosterol. GLC analysis of seed oil has shown the presence of linoleic acid, arachidic acid, myristic acid, palmitic acid, and oleic acid[18]. Literature review reveals that there are significant activities given by leaf extract of plant however not much investigation has been done on finding out the liposoluble or unsaponifiable secondary metabolites responsible for the biological activities of the plant. The present study was carried to identify the major bioactive compounds present in unsaponifiable fraction of leaf extract from *Sesbania sesban L.Merr* using GCMS technique. GC-MS is a hyphenated system, the combination of a chromatographic and spectrometric method which is widely being used in identification and characterization of bioactive components from plants. The approach exploits advantages of each, as Gas Chromatograph helps in separation of components from the sample and Mass spectrometer yields qualitative information about a 'pure' component. The combination of the two methods results in 3-D data providing both quantitative and qualitative information.

MATERIALS & METHODS

Chemicals and solvents

Petroleum ether (60-80^{o}) was procured from Merck Specialties Pvt. Ltd. Mumbai. Potassium hydroxide and Silica Gel (60-120#) was purchased from Rankem, RFCL limited, New Delhi. All other solvents which are used in this project were of Analytical grade. Glass wares used were of class I grade glass.

Collection of plant and Authentication of plant material

The plant material was collected from the village Khadakjam of Dist. Nasik, Maharashtra, in the month of November 2012. At the time of collection, plant including pods, leaves, and flowers were collected. The plant was authenticated by Dr. S.R.Kshirsagar, Taxanomist of Department of Botany, S.S.V.P.S College of Science, Dhule, Maharashtra India.

(i) Extraction of Plant Material

The leaves were separated, washed with the water, followed by 95 % ethanol, and further dried under shade. It was pulverized and 100g powder leaves was extracted with Petroleum ether (60-80^{0}C) using Soxhlet apparatus and percent yield was calculated.

(ii) Preliminary Phytochemical screening

The petroleum ether extract was subject to qualitative preliminary screening using various chemical reagents to analyze the chemical compounds present in the extract as per the procedures in standard text[19].

(iii) Separation of Unsaponifiable Matter (USM) from Petroleum Ether Extract

The standard process for evaluation of plant sterols by GC-MS analysis requires the extraction of lipid extract with a non polar compound and further saponification for separation of unsaponifiable matter and its purification to get major yield of phytosterol in fraction. The saponification process of petroleum ether extract was carried out as per the IP-96[20]. Five gram of petroleum ether extract was transferred in to 250 mL round bottom flask fitted with a reflux condenser. A solution of 2 gm KOH in 40 mL ethanol (95%) was added to the extract and refluxed for one hour with frequent shaking. The content of the flask is transferred to the separating funnel with the aid of 100 mL of hot water while the liquid was still warm. It is shaken carefully with several quantities each of 100 mL of peroxide free solvent ether. All ether extracts are combined and transferred to second separating funnel and it is swirled with of water and allowed to separate. Aqueous part is rejected and again ether extract is washed with 3% W/V solution of KOH, followed by successive quantities of water until aqueous layer is free from basic alkali. The solvent ether layer is concentrated by distillation under reduced pressure dried and weighed to give unsaponifiable matter. The unsaponifiable matter was further purified and fractionated using silica gel (60-120#) as stationary phase in column using dichloromethane, petroleum ether and chloroform as eluents. The purified fraction of unsaponifiable matter was pooled together and further subjected to preliminary phytochemical identification of major chemical compounds that may be present in the fraction.

(i) Preliminary Phytochemical screening of USM

The purified fraction of unsaponifiable matter was subjected to preliminary screening to confirm the presence of major bioactive groups according to standard referred procedures[19].

(ii) GC-MS Analysis

Analytical characterization was undertaken for the Unsaponifiable matter (USM) fractionated from petroleum ether extract of *Sesbania sesban* using GC-MS technique. Perkin Elmer Autosystem XL Gas chromatograph comprising an AOC-20i auto-sampler equipped with Elite-5MS (5% diphenyl / 95% dimethyl poly siloxane) capillary column (30 × 0.25 µm ID × 0.25 µm) was utilized. An injection volume of 1 µl bioactive fraction of USM was employed at a split ratio of 10:1 and helium (99.999%) was used as carrier gas at a flow rate of 1ml/min for separation of the bioactives. The injector temperature was maintained at 250 °C, the ion-source temperature was 230 °C, and the initial oven temperature was maintained at 80 °C for 5min, with an increase of 10 °C/min upto 290 °C, and was hold for 15 minutes. The mass-detector used in this analysis was Turbo-Mass Perkin-Elmer, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-4.1. Mass spectra were taken at 70 eV; with a mass range from 30 to 650 AMU. The total run time of sample at GC-MS was 40 min. Relative percentage amount of each phytoconstituent was measured as the average peak area to the total areas.

(i) Identification of Phytocomponents in USM

Separated chromatograms of different phytoconstituents were further subjected to mass fragmentation to get the spectral data. Interpretation of mass spectrum was done using the database of National Institute Standard and Technology (NIST), WILEY8, and lipid library of American Oil Chemists' Society. The identification was made based on peak area, molecular formula, molecular weight and mass spectrum.

RESULTS & DISCUSSION

(i) Extraction yield

The percent yield of petroleum ether extract from leaves of *Sesbanian sesban* was found to be 2.4% and the total yield of unsaponifiable matter (USM) in petroleum ether extract was found to be 39%.

(ii) Preliminary Phytochemical screening

Preliminary phytochemical evaluation of petroleum ether extract from leaves of *Sesbanian sesban* showed the presence of lipids, carbohydrate, wax, steroidal glycosides whereas the unsaponifiable matter showed majorly the presence of carbohydrate, lipids, oil and steroid groups as given in Table 1. The appearance of USM was waxy and the color was orange red may be due to the presence of carotene and xanthophylls.

Table 1

Phytochemical screening of Petroleum ether & USM from Sesbania sesban L.Merr

Sr. No.	Phytochemical groups	*Sesbania sesban* leaves extract	
		Petroleum ether Extract	USM
1	Carbohydrates	+	+
2	Alkaloids	-	-
3	Glycosides	+	+
4	Steroids & triterpenoids	+++	+++
5	Tannins	-	-
6	Phenolic compounds	-	-
7	Flavonoids	+	-
8	Fats & fixed oils (copper sulphate solution)	+++	+
9	Volatile oil	-	-
10	Amino acids	-	-

'+' refers to be present, '+++' refers to be abundantly present and '-'refers to absence

(iii) GC-MS Analysis

Gas chromatogram of bioactive fraction of USM showed the presence of nine peaks with six major peaks at retention time 20.46, 26.11, 28.73, 30.69, 31.56 and 33.17 minutes amounting to 80% of USM fraction as shown in figure 1.

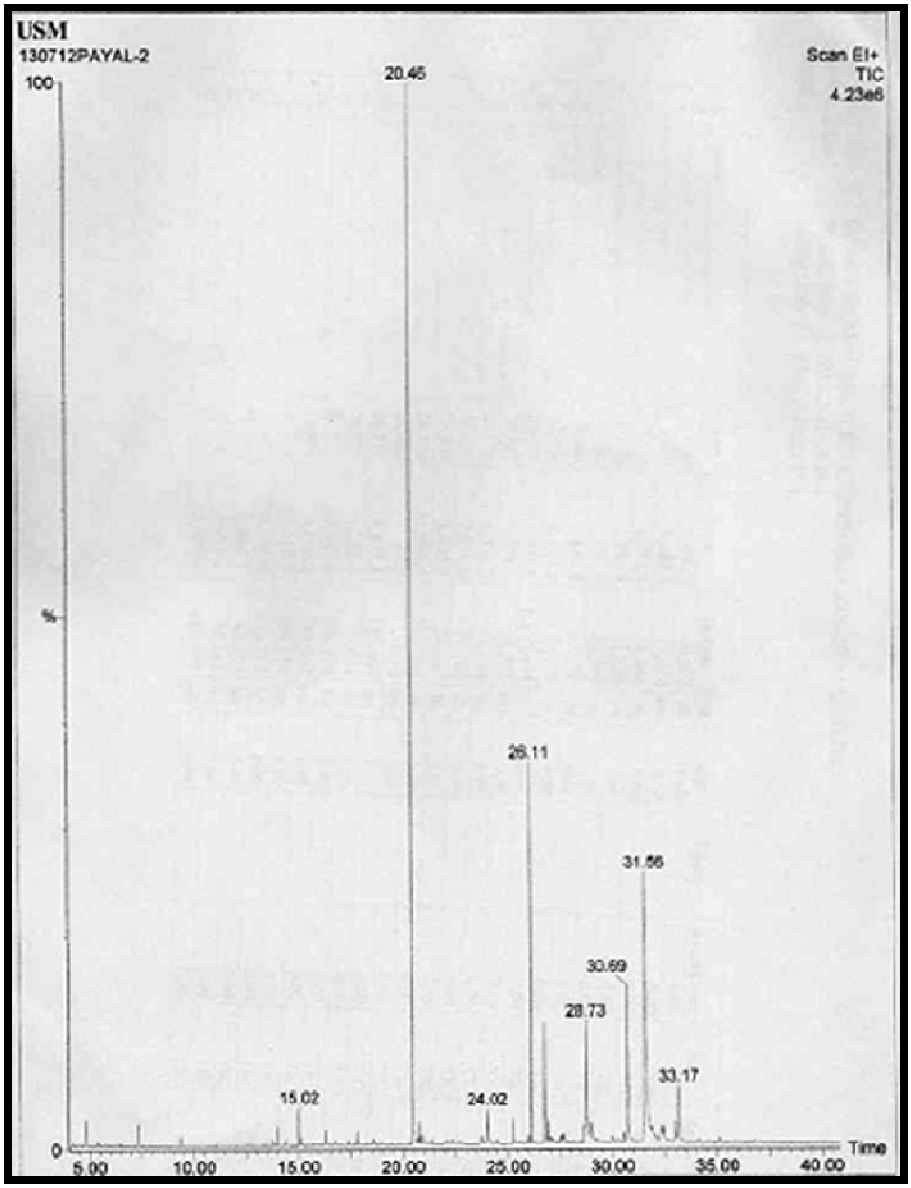

Figure 1
GC-MS Chromatogram of purified bioactive fraction of USM from Sesbania sesban L.Merr.

The mass fragmentation patterns of major compounds were compared to the mass spectrum of available database from NIST, WILEY8, and lipid library of American Oil Chemists' Society. On comparing their spectrum, based on molecular weight, peak area and intensity of MS fragmentation ion and M+ ion peak, the six major compounds were identified as Phytol (31%), Squalene (8%), Campesterol (7%), Stigmasterol (9%), Stigmasta-5, 22-dien-3-ol acetate (22%), and cycloartenol (3%) as shown in table 2.

Table 2
Major Phytoconstituents identified in purified fraction from USM of Sesbania sesban L.Merr. using GC-MS analysis

Sr. No.	RT in min	Name of Compound	Molecular Formula	Major MS fragmentation ions (m/z) with M+ ion	MW	Real % Area
1	20.46	Phytol	296	41,43,55,57, 71(100), 81, 83, 109,111, 123, 137,196, 263, 278, 296	$C_{20}H_{40}O$	31.63
2	26.11	Squalene	410	55,69(100), 81, 95,121,136, 149, 203, 231, 259, 299, 325, 341, 367, 391, 410	$C_{30}H_{50}O$	8.81
3	28.73	Campesterol	400	41, 43(100), 55, 57, 67, 69, 71, 81, 95, 107, 145, 161, 173, 199, 213, 231, 255, 273, 289, 315, 367, 382, 400	$C_{28}H_{48}O$	7.01
4	30.69	Stigmasterol	412	41, 43, 55(100), 57, 67, 69, 81, 83, 91, 95, 105, 107, 110, 119, 123, 133, 145, 147, 159, 173, 185, 199, 213, 255, 271, 300, 351, 398, 412	$C_{29}H_{48}O$	9.52
5	31.56	Stigmasta-5, 22-dien-3-ol, acetate (3β)-	454	41, 43(100), 55, 57, 69, 81, 83, 91, 93, 95, 105, 119, 133, 145, 159, 213, 255, 282, 351, 394, 454	$C_{31}H_{50}O_2$	22.06
6	33.17	Cycloartenol (9,19 cyclo-9β-lanost-24 – en-3β-)	440	41,55, 69(100), 73, 81, 93, 95, 107, 110, 121, 135, 147, 161, 175, 189, 203, 218, 229, 255, 339, 366, 394, 409, 412, 440	$C_{31}H_{52}O$	3.81

Plant sterols and stanol, are present in various legume plants in the form of free fatty alcohol, ester of fatty acids or in complex form attached with glycosides. They are present in large proportion in unsaponifiable matter of plants. They are included in food ingredients as “Generally Recognized As Safe” (GRAS) products and are known to reduce the risk of coronary heart disease. They are absorbed in trace amount, and are recognized as cholesterol reducing agents[21]. They inhibit the absorption of cholesterol from the intestine by competing with absorption of cholesterol from the diet and further inhibiting re-absorption of circulating bile cholesterol[22, 23]. Recent studies on beneficial effect of cholesterol reducing plant sterols have increased research interest and lead to potential finding about sterols. Gas Chromatography, hyphenated with mass spectrometry, is known to be the most excellent and extensively used tool for the chromatographic separation, identification, and quantification of phytosterols. GC-MS analysis of unsaponifiable fraction led to identification of important phytosterols from *Sesbania sesban* leaves. Among the major phytosterols identified in USM, phytol was found to be present in abundance. Study reveals that phytol, is an acyclic diterpene alcohol, which has a floral delicate balsamic odor[23]. It is used as an ingredient of fragrance in soap, perfurmes, shampoos, cosmetic & toiletries preparations. It has significant effect on diabetes and related complications[25]. The effect of phytol was suggested to be due to bioactive phytanic acid that induced differentiation of white and brown adipose tissue[26].

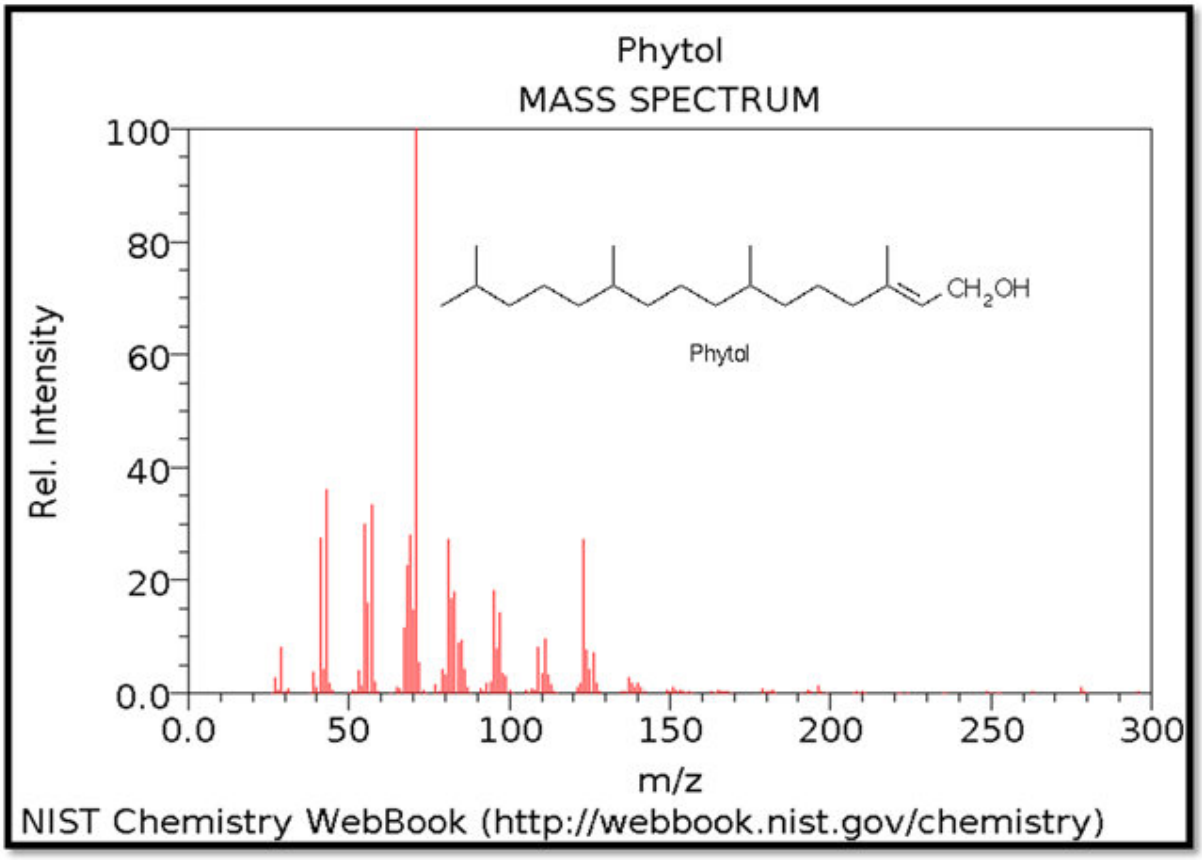

Figure 2
Mass spectrum of Phytol[27]

The next major phytoconstituents found were Stigmasta-5, 22-dien-3-ol acetate (22%) and Stigmasterol (9%) and Campesterol. Stigmasterol is an unsaturated plant sterol occurring in plant fats or oils of soybean, calabar bean, and rape seed, and in a number of medicinal herbs. It was used as a precursor in the synthesis of Progesterone[28]. It also finds its use in the synthesis of corticosteroids, estrogen, androgen, and vitamin D3 as an intermediate[29]. Stigmasterol is found to reduce the plasma cholesterol level and also inhibited the hepatic synthesis and absorption of same from intestine[30].

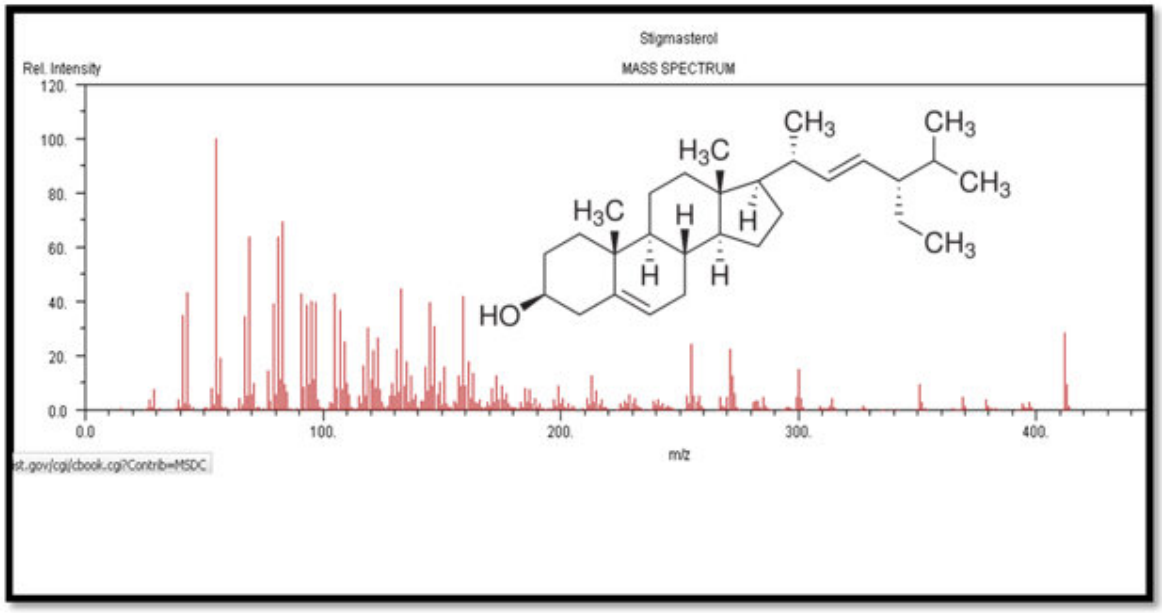

Figure 3
***Mass spectrum of Stigmasterol*[27]**

Stigmasterol has also shown its inhibitory effect on several pro-inflammatory and matrix degradation mediators that are characteristically involved in OA-induced cartilage degradation and thus possess potential antiosteoarthritic activity[31]. A recent study conducted on Stigmasterol has revealed its potential effect on reducing the T3 and T4 thyroid hormone, antioxidative and hypoglycemia[32]. Stigmasterol & its derivatives have been investigated for its antidiabetic, antifeedant, antitumor, antimutagenic & antioxidant activity[33]. Campesterol like other plant sterol helps in reducing the total and LDL cholesterol levels[34].

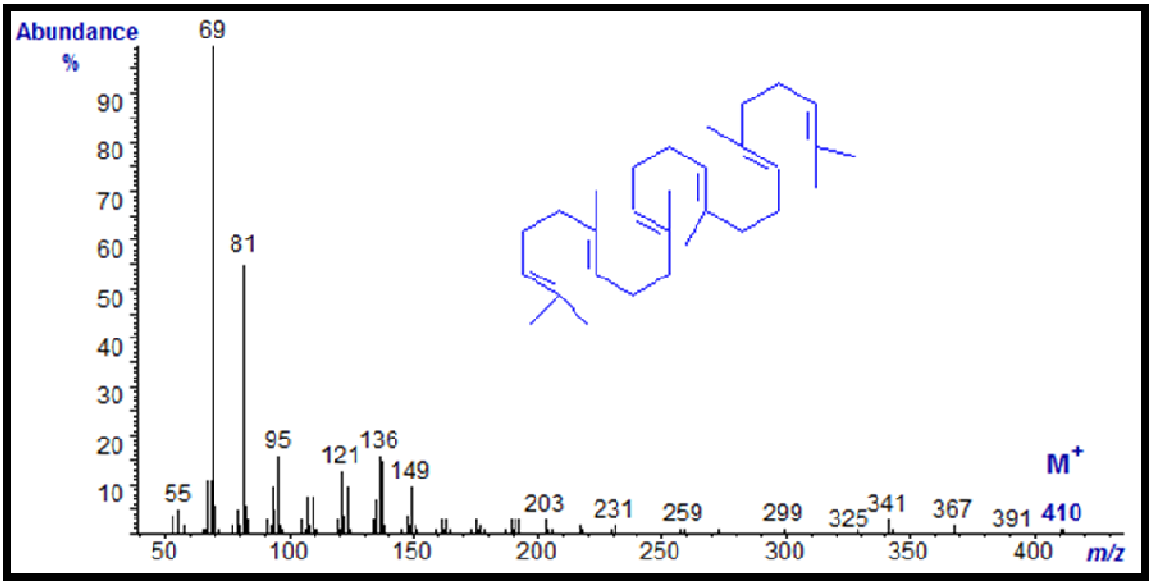

Figure 4
***Mass Spectrum of Squalene*[35]**

Another important compound identified in USM is squalene, a natural hydrocarbon and a triterpene, which is considered to be a vital initial material for the synthesis of cholesterol, steroid hormones, and vitamin D in the human body[36]. Squalene is reported to possess anticancer[37], antitumor, antioxidant, pesticidal and antimicrobial activity. It is also used in cosmetics for its sunscreen effect, and recently was investigated for its use as an immunologic adjuvant in vaccines[37]. It is believed to be main chemical agent active against pathogens causing diseases in plants & human beings. Squalene is found to potentiate the effects of some cholesterol-lowering drugs[36, 37]. It plays a complimentary role in cholesterol reduction when added with β-sitosterol[37]. The fraction also showed the presence of 1-octasanol, a free fatty alcohol which is used in treating parkinson's disease[38]. Cycloartenol act as a precursor compound in the synthesis of sterol and stanol in plants[39]. The presence of these compounds justifies the ethnomedicinal claim of this plant to be used in various diseases.

CONCLUSION

The GC-MS analysis of bioactive fraction of unsaponifiable matter from petroleum ether extract of *Sesbania sesban* revealed the presence of important secondary metabolite such as Phytol (31%), Squalene (8%), Campesterol (7%), Stigmasterol (9%), Stigmasta-5, 22-dien-3-ol acetate (22%), and cycloartenol (3%) that possess potential antidiabetic, anticancer, antioxidant, antimicrobial, and cholesterol reducing activity. Further investigation can be made on isolated phytoconstituents to substantiate its use in specific diseases. The above study helps in confirming the presence of important bioactive compounds that confirms medicinal importance of this plant in curing various ailments.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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