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Standardization and Characterization of Oleo Resin of Pine obtained from Pinus roxburghii Sarg.

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ABSTRACT

The present study was design to standardize and develop scientific data for identification and quality control of Oleo Resin of Pine. The proximate analysis and nature of Oleo Resin of Pine (ORP) was confirmed by organoleptic, physiochemical characterization and fluorescence analysis. The preliminary phytochemical analysis was done by qualitative chemical tests. The total chemical components were confirmed by TLC and HPTLC analysis in suitable solvent system. Identification of chemical compounds, their molecular weight and structure were detected by GC-MS and the functional groups were detected by FTIR. Thermal analysis was carried out by DSC. The organoleptic and physiochemical data showed characteristic features of ORP. The preliminary phytochemical analysis of hydroalcholic extract of ORP showed presence of carbohydrates, glycosides, alkaloid, phytosterols, terpenes, saponins and phenols. The DSC thermogram of powder of ORP showed enthalpy of transition more than zero for all the 4 endothermic peaks. The GC-MS chromatogram of ORP revealed the presence of two compounds, Longicyclene at RT 21.11 and Longifolene at RT 22.03. TLC and HPTLC fingerprinting of ORP showed separation of components in Toluene: Ethyl acetate (93:7) mobile phase at 254, 366, 550nm.The FTIR spectrogram of ORP showed the seven characteristic bands assigned to different functional groups. The observations and results of the study have provided evidence based scientific data for standardization of ORP which will serve as reference standards to establish its identity, purity and also help to minimize adulteration and substitution

Keywords: Standardization; Oleo Resin of Pine; HPTLC; GCMS; FTIR; DSC.

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INTRODUCTION

Unani System of Medicine is the oldest traditional medicine and still it is gaining popularity worldwide but concerns over their safety and efficacy have certainly augmented the need of standardization. This can be achieved only if the single drugs and herbal products are evaluated and analyzed by using conventional and sophisticated modern techniques.[1] Resins have been used for a long time in Unani System of medicine to treat different ailments in a single dosage form or in combination with other herbal drugs as a Hypolipidemic, Aphrodisiac, Emmenagogue, Anti-fertility, Anti- obesity, Diuretic, Carminative etc.[2,3] *P. roxburghii* is commonly known as "Chir pine.[4] It is an important commercial species and famous for its resin, paper pulp and timer yield.[5] In Unani System of Medicine ORP is a drug of choice for chronic ulcers and internally it is used for gonorrhea and stomachic.[6] Literature survey has revealed that standardization of Oleo Resin of Pine (Pinus roxburghii) has not been done till date. Therefore, the present study was design to standardise and develop scientific data for identification and quality control of Oleo Resin of Pine.

MATERIALS AND METHOD

To accomplish the aims of the present study standard parameters of Oleo Resin of Pine has been determined by morphological, physiochemical, phytochemical and instrumental analysis.

Collection and identification of raw material

Oleo resin of pine was procured from the registered crude drug dealer in Kashmir valley. The identity of the drug was confirmed by Pharmacognostic, S. Noorunnisa Begum, Sr. Asst. Prof., Centre for Repository of Medicinal Resources (C-RMR), Trans Disciplinary University, (TDU), FRLTH, Bangalore. The sample of ORP was dried in shade, powdered and stored in airtight containers. A sample specimen was deposited in herbarium of National Institute of Unani Medicine with FRLHT Acc. No. 3895.

Organoleptic Evaluation of ORP [7]

Organoleptic Evaluation viz. colour, odour, taste were observed to check the characteristic nature of ORP.

Solubility test

ORP was evaluated for solubility in water, ethanol, petroleum ether, chloroform, benzene, and acetone as per I.P specifications. Solubility is expressed in terms of "parts" representing the number of milliliters (ml) of the solvent, in which 1 g of the test drug is soluble.[8, 9]

Determination of pH value [10]

pH was determined by using scientific portable Laquatwin HORIBA pH meter. Prior to the experiment the instruments was Calibrated by using Buffer solution of 4.00, 7.00 to ascertain accuracy of the instrument.

Ash value [11]

Ash values such as total ash, acid insoluble ash, water soluble ash were determined using the following equations:

$$\text{Total ash value} = \frac{wt.\ of\ ash}{wt.\ of\ dried\ powder\ of\ sample} \times 100$$

$$\text{Acid insoluble ash} = \frac{wt.\ of\ \ acid\ insoluble\ ash}{wt\ of\ dried\ powder\ of\ sample} \times 100$$

$$\text{Water soluble ash} = \frac{wt.\ of\ water\ soluble\ ash}{wt.\ of\ dried\ powder\ of\ sample} \times 100$$

Moisture content [12]

Loss on Drying at 105^0c

This method was adopted as per British Pharmacopoeia. The percentage weight lost of the powder test drugs was calculated using following equation:

$$\text{Loss on Drying} = \frac{Initial\ weight - final\ weight}{initial\ weight} \times 100$$

Determination of extractive value [13]

Extractive value of ORP was carried out in Soxhlet apparatus by using various solvents like petroleum ether, chloroform, acetone and 50% hydroalchol and the percentage yield of test drug in each solvent was determined.

Micrometric properties of granules of Oleo Resin of Pine: [12, 14]

The Bulk and Tapped densities of powdered drugs were determined by standard methods. Powder compressibility (Carr's consolidation Index) and Hauser's ratio was determined by following formula:

Bulk Density = wt. of powder blend/ wt. of apparent volume

Tapped density = wt. of powder blend/ tapped volume

Bulkiness = 1/bulk density

Carr's index = Tapped density – Bulk density/Tapped density×100

Hauser's ratio= Tapped density/ Bulk density

Angle of repose is determined by the formula $\tan\theta = h/r$

Where, h = Height of pile of powder from the peak to the ground and r = Horizontal distance from the middle of the pile to the edge.

Determination of Foreign Matter

100 gram of drug sample was accurately weighed and spread on a thin layer. The foreign matter was detected by inspection with an unaided eye and also with the help of a lens (6x). The separated matter was weighed accurately and percentage of foreign matter was calculated.[15]

Preliminary Phytochemical analysis [8,16]

Preliminary phytochemical study was carried out on extracted samples. The extracts were subjected to various qualitative phytochemical tests such as alkaloids, glycosides, carbohydrates, phenolic compounds, tannins, phytosterols, fixed oils, flavonoids, proteins, amino acids and confirmation of presence or absence of gum was done by Ruthenium red test.

Test for inorganic constituents of ORP

Ash of the ORP was prepared first. To the ash 50% v/v hydrochloric acid and 50% v/v nitric acid were added and kept for an hour and then filtered. The tests were performed with the filtrate for the detection of inorganic constituents like sulphate, iron, phosphate, chloride, carbonate and nitrate.[17,18]

Florescence Analysis [8]

Powdered Resin samples were screened for florescence characteristics with or without chemical treatment. The observation pertaining to their colour under UV light and in day light were noticed and reported.

Chromatographic Analysis [7,19]

TLC and HPTLC were performed for the separation of different components present in ORP and the R_f values of detected components were noted.

The following Chromatographic conditions were used for the study:

Stationary phase: Pre-coated silica gel plates, Merck 60 F254; Mobile phase: Toluene: Ethyl acetate (93:7); Lamp: Deuterium; Wavelength: 254nm, 366nm and 550nm; Sample Application: CAMAG linomet 5; Spray gas- Inert gas; Sample solvent type: Methanol; Dosage speed: 150µl/s; Pre dosage volume: 0.2 µl; Scanner: CAMAG TLC Scanner 3 and Win CATS software.

Fourier Transform Infrared Spectroscopy (FTIR) [8]

Infrared spectra were measured by KBr – supported samples of ORP over the frequency range 4000$cm^{-1}$to 400cm^{-1} at resolution of 4 cm^{-1} using a model: 6700 FT-IR, Thermo fisher scientific, performed at Indian Institute of Science (IISc), Bangalore. The samples were thoroughly mixed with KBr, the dried mixture was then pressed to result in a homogenous sample/KBr disc.[75] The instrumental and experimental conditions were: Source: IR, Beam splitter: XT-KBr, Detector: DTGS KBr, No. of scans: 64, Sample gain: 8, optical velocity: 0.4747, Aperture: 80, Sample

preparation: KBr pellet method. The FTIR spectra was recorded and interpreted to find out the functional groups which were compared with reported reference.

DSC (Differential Scanning Calorimetry)[14,16]

Thermal properties like: Heat loss or gain, Melting temperature, and Phase transition temperature of Oleo Resin of Pine were characterized by using a Differential Scanning Calorimetry at heating rate of 10^0C/min in the thermal range of 30- 210^0C with the following methodology:

Instrument: METTLER TOLEDO DSC1 STARe system (chiller cooled), **Software:** STARe, **Heating rate:** 10 degrees per minute, **Temperature range of DSC machine:** -80deg to 550deg, **Purging gas and flow rate:** Nitrogen @ 40 mL/min, **Cell Temp:** Pt100, **Detection:** FRS-5 sensor, **Sample holder:** Aluminium Standard 40uL.

Gas Chromatography Mass Spectroscopic Analysis (GC-MS) [4]

GC-MS analysis of ORP was carried out by using GC-MS modal thermo scientific TRAYS 1310 and MS TSQ 8000 with a capillary column TG5MS, 30 meter, 0.25 internal diameter. The sample was prepared by dissolving 5mg of powder in 5ml hexane and sonicated for 10 minutes followed by filtration. The filtrate was used as sample for injection under the following conditions: injection volume 1µl with split ratio 1:49, helium as carrier gas at 1.0ml/min constant flow mode, injector temperature 55^0C, oven temperature was 240^0C increasing from 5^o c ramp to 240^o c and detector was full scanned over 50-450 a.m.u range. Identification of compounds was carried out by comparing query mass spectra with NIST 2.2 library reference mass spectra.

Results and Discussion

Organoleptic character

The organoleptic properties are useful for proper identification of the crude drugs. The results of organoleptic characters of ORP are depicted in Table- 1.

Table 1: Physiochemical Properties

Parameters	Oleo Resin of Pine
Colour	Light cream
Taste and Odour	Bitter, strong characteristic odour
pH	5.24 ± 0.09
Moisture Content (%)	3 ± 0.00
Melting Point (^{0}C)	85-110
Solubility	insoluble in water, Soluble in ethanol, Benzene, and chloroform
Total Ash Values (%)	12 ± 1.73
Acid Insoluble Ash Values (%)	8.68 ± 1.06
Water Soluble Ash Values (%)	0.66 ± 0.85
Foreign organic matter (%)	0.92 ± 0.00

The values are expressed as mean ± SD, N=3

Physiochemical properties

Table 1 shows some of the physiochemical characteristics of ORP. For the proper identification of plant and its constituents, organoleptic and physicochemical parameters provide useful information. Moisture content is a key parameter which determines the quality, efficacy and shelf life of the plant derived drugs and confirmed by Loss on drying method. The moisture content of ORP in the study is found to be less (3%) which indicates that ORP is suitable for formulations containing moisture sensitive drugs.[16] The pH of ORP was found to be slightly acidic. Melting point of the crude drugs is an important parameter for standardization because the crude drugs from plant origin may contain the mixed chemicals. The melting point of ORP was determined as 85-110 ^{0}C. The ash values can be used for detection of impurities, adulteration and substitution of Oleo Resin of Pine.[13] the percentage of foreign organic matter was found to be less than 1% which is in accordance with the limits described by WHO, i.e. less than 2%.

Determination of extractive value

The extracts were prepared with various solvents like 50% Hydroalcholic, Petroleum Ether, Chloroform, and Acetone by standard methods. Percentage yield of extracts obtained are 6.50%, 1.34%, 3.30%, and 2.50% respectively. Extractive value is an important parameter to check the quality of the crude drugs. It indicates an approximate measure of the chemical constituents present in the drug. ORP shows higher extractive value in hydroalcholic extract which indicates presence of both polar and non polar compounds. [8] The results obtained are given in **Table 2.**

Table-2: Different Extractive Values of Oleo Resin of Pine

Extracts	Extractive value (Oleo Resin of pine)
Hydroalcholic (50-50)	6.50%
Petroleum Ether	1.34%
Chloroform	3.30%
Acetone	2.50%

Micrometric properties of ORP

Micrometric properties like Bulk density, Tapped Density, Bulkiness, Compressibility Index %, Hauser's ratio and Angle of Repose (^{0}) were ruled out for ORP. The results are given in Table 3. Bulk Density of a granulation is primarily dependent on particle size, particle size distribution and particle shape. It is an indirect measure of granule flow. Hauser's ratio of ORP was observed to be 1.2000% which confirms that granules have a

good flow property as Hausener's ratio less than 1.25 indicates good flow and the values greater than 1.25 indicate poor flow.[16,20]

Table 3: Micrometric study data of Oleo Resin of Pine (ORP)

Parameters	ORP Values
Bulk Density (gm/ml)	0.4545
Tapped Density (gm/ml)	0.7143
Bulkiness (ml/gm)	2.200
Compress. Index %	36.3636
Hauser's ratio %	1.5714
Angle of Repose (^{0})	48.74

Phytochemical screening

The Phytochemical screening of ORP was carried out and the results obtained are depicted in Table 4. The presence of phytoconstituents makes the plant and its parts useful for treating different ailments of human beings. Presence of phenolic compounds and saponins in ORP reveals that it may possesses antioxidant, hypotensive and cardio depressant properties [21] which may be helpful for the management of Heart failure, cardiomyopathy [22] and presence of alkaloids may proves that it has promising potential of anti-hyperglycemic and anti-inflammatory.[23]

Table 4:- Results of Phytochemical Screening of Oleo Resin of Pine

Test	Oleo Resin of Pine
Test for carbohydrate (Molish,s and Benedict's test)	**+ve**
Test for Gums (Ruthenium Red)	-ve
Test for Glycosides (Keller-killaini test)	**+ve**
Test for Alkaloids (Dragendroff's, Wagner's, Hager's, Mayer's test)	**+ve**
Test for flavonoids (Alkaline reagent & Lead acetate tests)	-ve
Test for Tannins (Ferric chloride and Lead acetate test)	-ve
Test for phytosterols /Terpenes (Salkowski's test, Hosse's reaction test, Liebermann Burchard's reaction)	**+ve**
Test for Fixed oils (Filter paper test and Tinc. Alkane test)	-ve
Test for saponins (Froth test, Foam test)	**+ve**
Test for Phenols (Lead acetate test)	**+ve**
Test for proteins and amino acids(Ninhydrin test, Burette's reaction, Millions reaction, Xanthroprotenic reaction)	-ve
Test for sulphate	**+ve**
Test for Iron (potassium ferrocyanide test, Potassium thiocyanate test)	**+ve**
Test for Phosphate	-ve
Test for Chloride	**+ve**
Test for Carbonate	-ve
Test for Nitrates	**+ve**

Florescence Analysis

In order to avoid substitution and adulteration the florescence analysis of the powder drugs is necessary[13] The results of Florescence analysis of ORP are presented in Table 5.

Table 5: Florescence Analysis of Oleo Resin of Pine

	Oleo Resin of Pine	
Treatment	**Visible light**	**Ultraviolet (U.V)**
Powder	Light cream	Green yellow
Powder + water	Khaki 1	Khaki 2
Powder + 5% sodium Hydroxide	Golden Brown	Hunter green
Powder + conc. sulphuric acid	Chocolate Brown	Dark Coffee
Powder + hydrochloride acid	Maize yellow	Neon
Powder + benzene	Canary Yellow	Mint Green
Powder + Acetone	Deep Gold	Fluorescent lime
Powder + Hexane	LT Gold	Lime green

Results are described according to the online standard color charts

Chromatographic analysis

High Performance Thin Layer Chromatography is applicable for the separation, detection, qualitative and quantitative analysis of phytochemical [24] Chromatographic fingerprinting gives an ideal about the presence of active constituents in the drug.[13] TLC and HPTLC were carried out on Hydroalcholic extract of ORP. The most suitable solvent system was found to be a combination of Toluene and Ethyl acetate. A maximum number of 11 spots were observed in Toluene: Ethyl acetate (93:7) solvent system in both the tracks of ORP at 366nm; 7 spots in tract 1(T1) and 8 spots in tract 2 (T2) at 254nm and 8 spots in tract 1(T1) and 7 spots in tract 2 (T2) at 550nm were observed for ORP. The results are depicted in Table 6. & figure 1.

Table 6: Results of HPTLC of ORP

Track	**Mobile phase**	**254nm**		**366nm**		**550nm**	
		No. of spots	**R_f**	**No. of spots**	**R_f**	**No. of spots**	**R_f**
ORP T_1	Toluene (93):Ethyl acetate (7)	7	0.04, 0.10,0.36,0.54,0.62,0.71, 0.79	11	0.04, 0.06, 0.13, 0.17, 0.18, 0.23, 0.32, 0.36, 0.42, 0.54, 0.82	8	0.02, 0.09, 0.35, 0.52, 0.61, 0.69, 0.81, 0.95,

ORP T_2	Toluene (93):Ethyl acetate (7)	8	0.03, 0.10, 0.35, 0.45, 0.53, 0.61, 0.70, 0.79	11	0.03, 0.05, 0.12, 0.16, 0.22, 0.26, 0.32, 0.36, 0.40, 0.53, 0.81	7	0.02, 0.08, 0.34, 0.52, 0.60, 0.68, 0.80

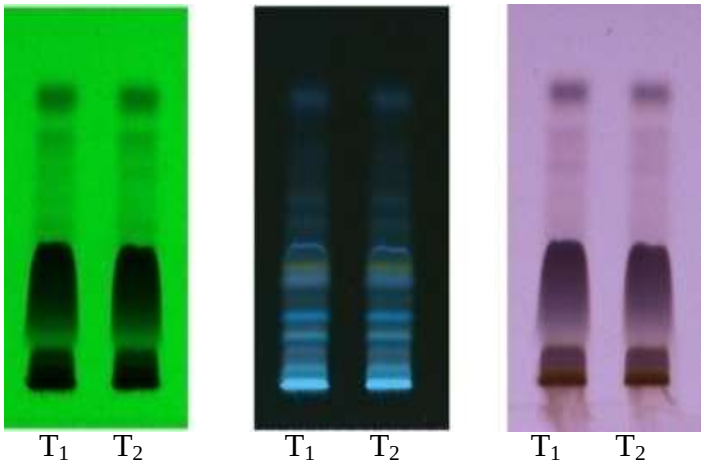


Figure 1:- TLC profile of ORP

Differential Scanning Calorimetry (DSC)

The DSC thermo gram of ORP showed enthalpy of transition (delta H) more than zero for all the 4 peaks, one at 59.45^{o}C with the delta H of 1.14J/g, second at 64.95^{o}C with the delta H of 28.16J/g, third at 78.440C with a delta H of 42.04J/g and fourth at 96.80^{o}C with delta H of 66.8J/g, it means that ORP has gained heat at 4 phases therefore all the resultant peaks are endothermic peaks with release of energy expressed in J/g.[25] The DSC thermo gram of ORP is shown in the figure 2.

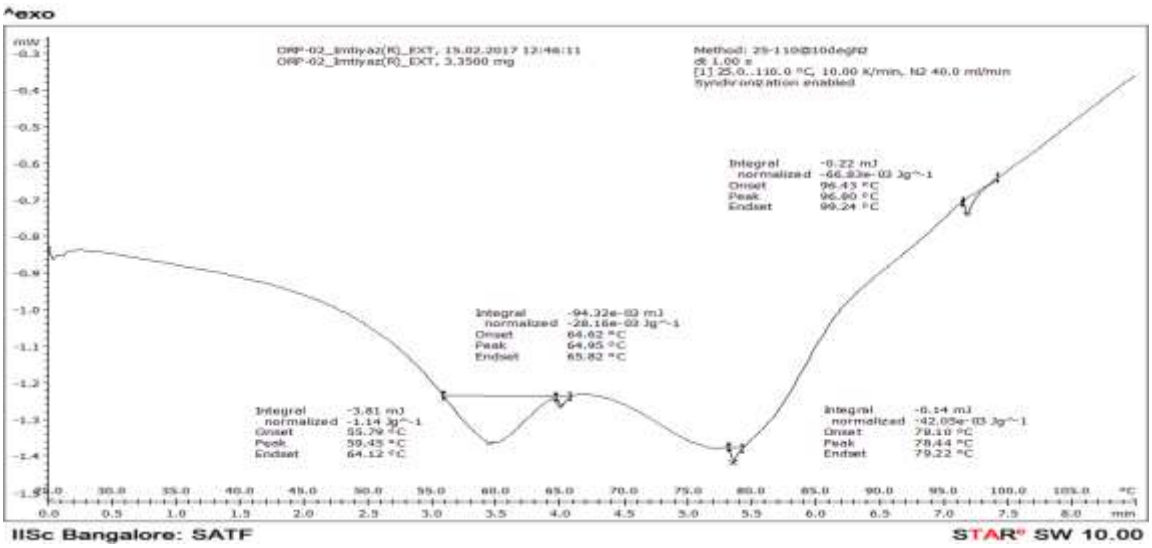


Figure 2: DSC of Oleo Resin of Pine

Fourier Transform Infrared (FTIR)

FTIR spectroscopy is commonly used to determine the functional group present in the sample and is an important tool for qualitative analysis of organic and organometallic compounds. The wavelength of various IR absorption bands are characteristics of particular types of chemical bonds. Therefore, it is used to confirm the identity of a particular compound and also determines the newly synthesized molecule (Banu K S et al., 2015).In the present study the FTIR spectra was recorded and interpreted to find out the functional groups and were compared with reported reference.[26] The FTIR spectrum of ORP showed the characteristic alcoholic O–H stretch at 3476.04cm^{-1}, C-H stretch at 2949.14 cm^{-1}, C-H stretch at 2866.72, O-H stretch at 2534.10, C=O stretch at 1698.12cm^{-1}, C=C stretch at 1465.58 and -C-H bending at 1386.10. (Shown in Figure. 3)

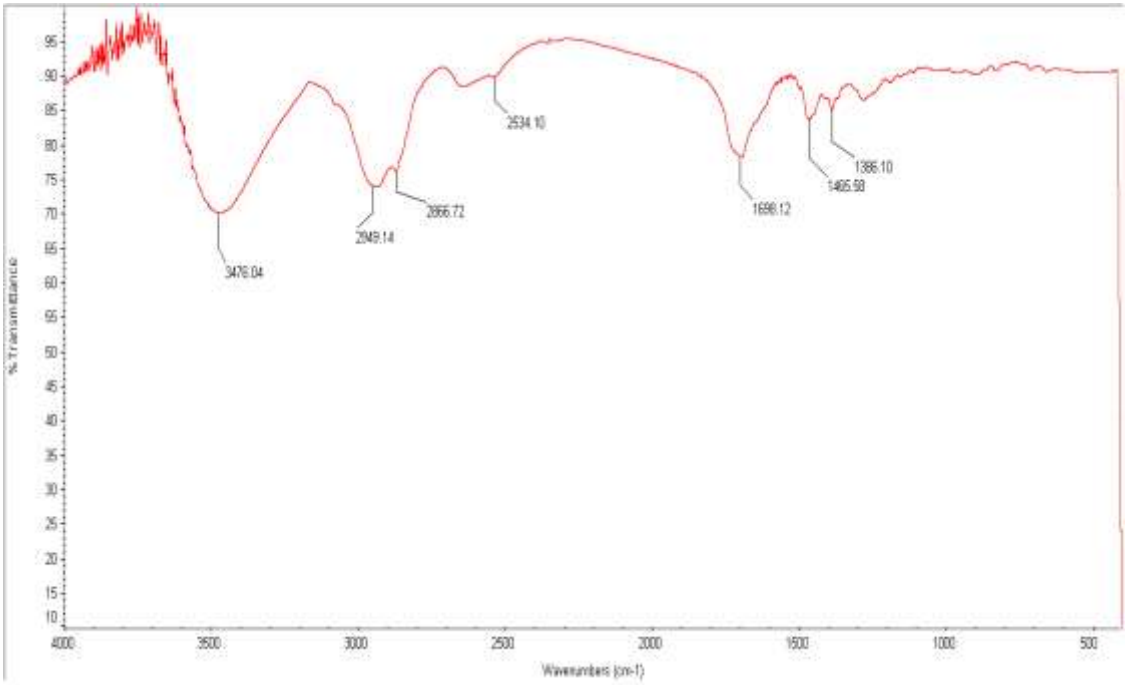


Figure 3: FTIR of ORP

Gas Chromatography- Mass Spectroscopy (GC-MS)

The GC-MS was done on Oleo resin of pine for identification and quantification of components present in the sample. GC analysis of ORP was found to contain 2 main peaks one at RT 21.11 with an area percentage of 29.95 and another component at RT 22.03 with an area percentage of 70.05 (figure-4, table-7). In mass spectroscopy these 2 components were identified by comparing query mass spectra with reference mass spectra in Chemical Abstract Service (CAS) & National Institute of Standard and Technology (NIST) library via spectral matching and the components were detected as Longicycline and Longifolene. The synonym of Longicyclene is 1,2,4-Methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1α,2α,3aβ,4α,8aβ,9R)] with Molecular Formula $C_{15}H_{24}$ and Molecular Weight: 204 Exact Mass: 204.1878 CAS#: 1137-12-8 NIST#: 413540 ID#: 14358 DB: replib. The Molecular Formula of Longifolene: $C_{15}H_{24}$, Molecular Weight: 204 Exact Mass: 204.1878 CAS#: 475-20-7 NIST#: 62059 ID#: 1406 DB: replib. (Shown in figure: 5 & 6).

Table7: Results of GC-MS chromatogram

Apex RT	Area	%Area	Identification
21.11	8933250.657	29.95	Longicyclene
22.03	20895801.09	70.05	Longifolene

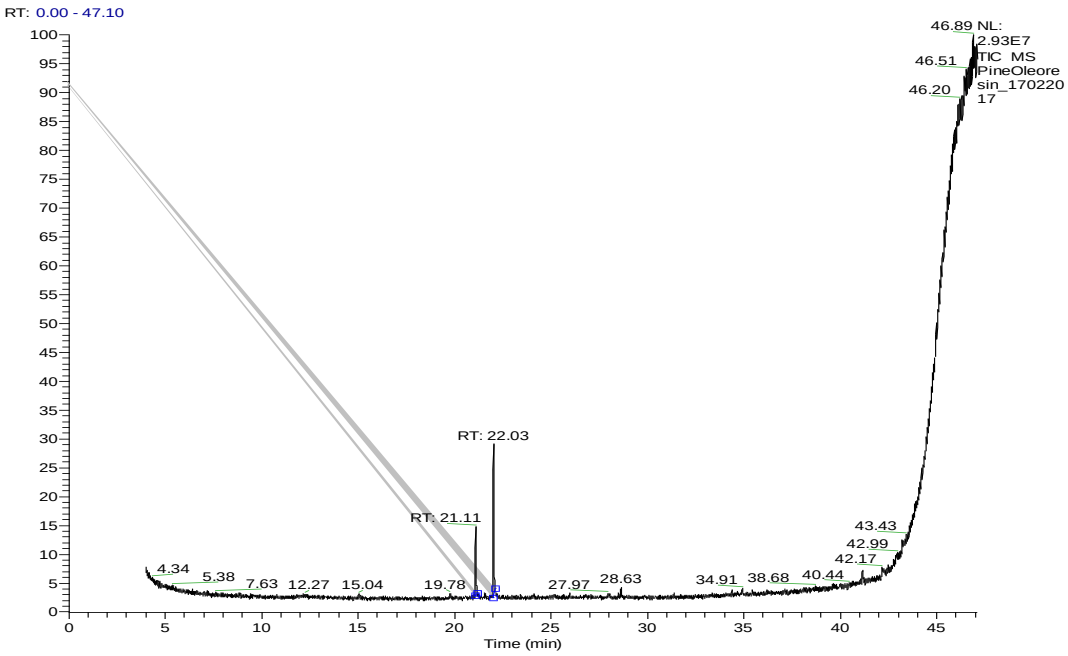
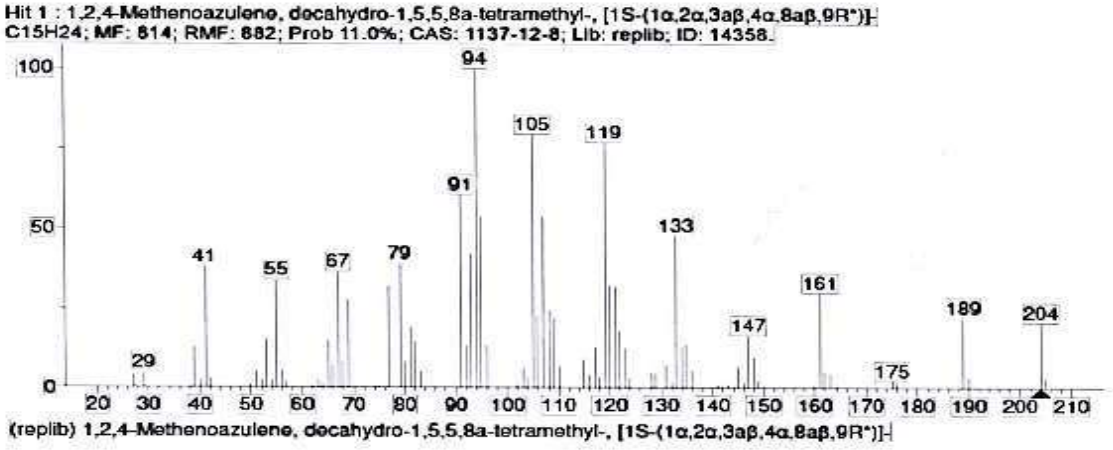
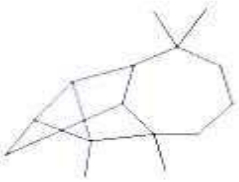


Figure 4: GC- MS Chromatogram





Name: 1,2,4-Methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1α,2α,3aβ,4α,8aβ,9R*)]-
Formula: $C_{15}H_{24}$
MW: 204 Exact Mass: 204.1878 CAS#: 1137-12-8 NIST#: 413540 ID#: 14358 DB: replib
Other DBs: Fine, EINECS
Contributor: NIST Mass Spectrometry Data Center
InChIKey: WCEIQUQVIOGRBF-UHFFFAOYSA-N Non-stereo
Synonyms:
1.Longicyclene
2.1,2,4-Methenoazulene, decahydro-1,5,5,8a-tetramethyl-, [1S-(1α,2α,3aβ,4α,8aβ,9R)]-

Experimental RI median±deviation (#data)
Semi-standard non-polar:1374±3 (21)
Standard non-polar: 1371±5 (11)
Polar: 1554±26 (11)

Figure 5: Mass spectra showing Logicyclene (Total Ion Chromatogram)

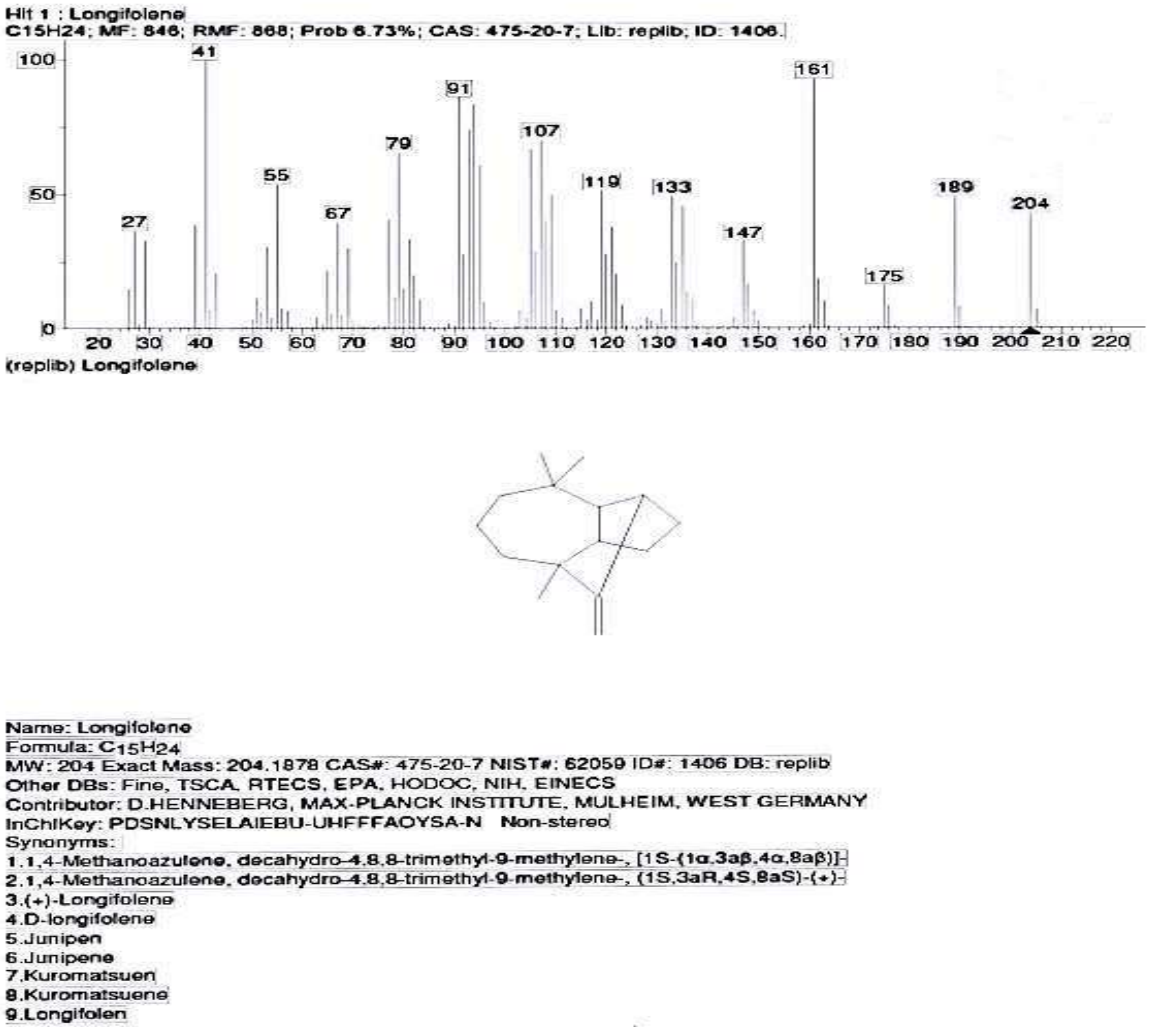


Figure 6: Mass spectra showing Molecular Structure of Logifolene

CONCLUSION

A number of resins have been used in Unani System of Medicine as therapeutic agents. Most of the resins are similar looking which may lead to confusion and create a lot of problem in their identification. Therefore, the present study was design to standardize Oleo Resin of Pine used in Unani System of Medicine. The organoleptic and physiochemical parameters obtained from this study will be useful to set the standards for establishing the authenticity of ORP and can prevent the accidental misuse and adulteration to the greater extent. The results analyzed by TLC and HPTLC fingerprinting profiles of ORP could be used as a valuable analytical tool in the routine quality control and standardization of ORP. The presence of organic constituents like phenols, saponins and alkaloids in ORP revealed by preliminary phytochemical analysis indicates that it possess antioxidant, hypotensive and cardio depressant properties and may be useful as therapeutic agent to cure different diseases after additional cavernous analysis and investigations of all the

active chemical constituents to substantiate the clinical efficacy. So, in the light of the observation, results and discussion it can be concluded that the standard parameters obtained from the current study will serve as standard tool or reference for identification of ORP and will also help to minimize the adulteration and substitution in future.

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