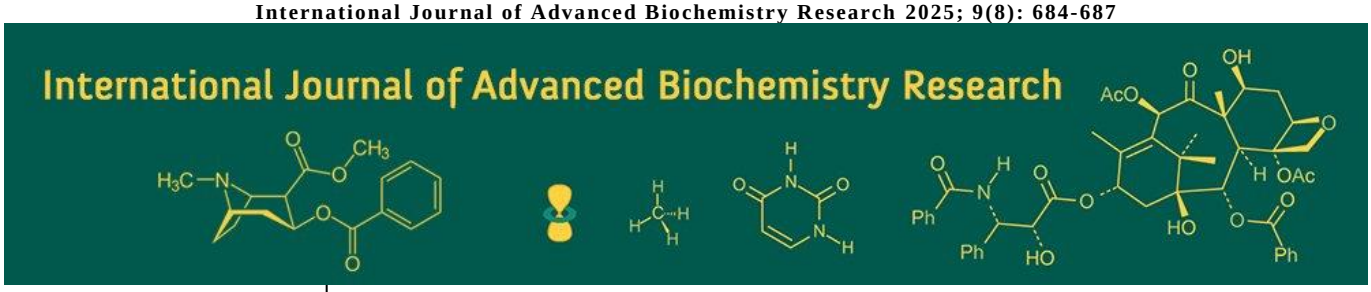

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Extraction, separation and identification of active compounds in *Rosemarinus officinalis* by using thin layer chromatographic (TLC) and gas chromatography and mass spectrometry studies

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Abstract
Sohxlet apparatus is used to prepare the plant extract from the leaves of *Rosemarinus officinalis* by using the solvent of hexane. TLC with hexane solvent of *R. officinalis* gave 5 spots, and the active compounds were isolated. *R. officinalis* spots was eluted through TLC employed in GCMS found the presence of 22 active compounds like Eucalyptol, Borneol, Camphor, trans-á-Terpinyl pentanoate, D-Verbenone, Bornyl acetate, Caryophyllene, à-Caryophyllene, Naphthalene, Cedrene, Copaene, trans-Z-à-Bisabolene epoxide, 1-Heptatriacotanol, n-Hexadecanoic acid, Heptamethyl-3-phenyl-1,4-cyclohexadiene, Trenbolone Acetate, 9,12-Octadecadienoyl chloride, (Z,Z)-, and other few unidentified compounds were identified.

Keywords: Soxhlet, TLC, GC-MS, *Rosmarinus officinalis*, phytochemicals, hexane

Introduction
Herbal medicinal products have long been a component of traditional medicine, providing therapeutic properties from plant-based sources. Growing worldwide interest in natural and complementary medicines has caused the demand for herbal products to escalate (Vishwakarma and Pandey (2025) [10]. Nowadays, rosemary is being grown worldwide but it is an important medicinal perennial shrub native to Mediterranean region and southern Europe. It has lot of medicinal values, and the extracts was widely used in traditional medicine. It is one among the family of Labiatae which is mainly used in cosmetics, because of its high abundance of antioxidant molecules (Gonzalez-Minero *et al.*, 2020) [5]. The three main subspecies of, *R. officinalis* are *R. officinalis* subsp. *officinalis*, *R. officinalis* subsps. *valentinus ferrer and R. officinalis* subsps. *palaui malag* (Carrubba *et al.*, 2020) [13].
Rosmarinus officinalis has been traditionally employed as an antispasmodic, mild analgesic, and remedy for neuralgia, migraine, headaches, insomnia, depression, and emotional disturbances. It possesses antimicrobial, antioxidant, anti-inflammatory, antitumor, antinociceptive, anti-apoptotic, and neuroprotective activities (Rahbardar & Hosseinzadeh, 2020) [11].
Rosemary is used as an antispasmodic in the treatment of renal colic and dysmenorrhea, for alleviating respiratory disorders, and for promoting hair growth. Its extract helps relax the smooth muscles of the trachea and intestines, and it also exhibits hepatoprotective, choleretic, and antitumor properties. The bioactive compounds in rosemary show therapeutic potential in the prevention or treatment of bronchial asthma, diabetes mellitus, peptic ulcers, spasm-related disorders, inflammatory conditions, ischemic heart disease, liver toxicity, atherosclerosis, cataracts, poor sperm motility, and certain types of cancer.
The rosemary plant exhibits distinct physical characteristics, including a dense, evergreen, and aromatic habit, with rigid branches and square stems. The leaves are small, pointed, sticky, and hairy, yielding essential oils valuable in traditional and modern medicine, aromatherapy, and the scent and flavor industries. In addition to its medicinal properties, rosemary has long been prized in culinary traditions and is recognized as both a functional food and a botanical nutraceutical.

Rosemary possesses notable insect repellent properties, making it a useful herb in the prevention of pest-related damage. The plant's adaptability for pruning and to shaping also renders it fitting for topiary purposes. In light of its numerous applications and bioactive compounds, the current study aims to identify the active constituents available in the R. officinalis plant extract (Encyclopedia of Food and Health, 2016) [14].

Materials and Methods

Preparation of plant extracts

The leaves of R. officinalis were collected from HRS, Ooty and it was clearly washed in running tap water and it was kept for two weeks for shade dry in room temperature. The fine powder made by grounding the shade dried leaves. The powder then packed in the air tightened polythene bags and stored in room condition for further use. For the extraction purpose the Soxhlet apparatus was used. 25 g of the medicinal plants' (*E. citriodora*, *T. vulgaris*, *A. squamosa*, *R. officinalis* and *C. citrates*) leaves were powdered and weighed separately into the 200 ml of chloroform or hexane for 24 hours percolation. Temperature control at 40 ºC (hexane) and 60 ºC (chloroform) was established and maintained with an electric heating mantle. The resultant solvent extract was collected, evaporated, and the residual extract was subsequently stored at 4 °C in airtight containers. (Torres-Rodriguez *et al.*, 2024) [9]

The per cent extractive values were computed with following formula,

$$\text{Per cent extractive} = \frac{\text{Weight of the dried extract}}{\text{Weight of dried plant material}} \times 100$$

Separation of compounds through TLC

A 10 µl aliquot of the sample was applied to pre-activated, ready-made TLC plates (10 × 10 cm) and developed for 45 minutes using a solvent system composed of chloroform, methanol, glacial acetic acid, and ethyl acetate in a 50:40:05:05 ratio. After development, the plates were dried and examined for coloured spots, indicative of the presence of phenolics, terpenes, alkaloids, or flavonoids. All spots were visualized under UV light, and the Rf values were determined using the formula described by Manimegalai *et al.* (2010) [7].

$$\text{Rf value} = \frac{\text{Distance (cm) spot travelled}}{\text{Distance (cm) solvent travelled}}$$

Gas Chromatography-Mass Spectrometer analysis (GCMS)

The eluted active compounds through TLC with hexane solvent was analysed by Single Quadrapol Thermo-DSQ1 equipped with a column of DB5 (30 m×0.25 mm ID×1micrometer) with the carrier gas as helium. Column oven temperature maintained was 60 °C. Injection temperature was maintained at 250 °Celcius and flow rate of the Helium was maintained as one ml per minute. Injection was performed in the volume of 1 µL. The voltage of ionization was 70eV.

The essential chemical components were picked out by matching of their mass spectra with reference spectra from the National Institute of Standards and Technology (NIST) mass spectral library. The relative abundance of each component was calculated as the percentage of its peak area in relation to the total chromatographic peak area.

Results and Discussion

TLC is used to separate the active compounds present in the *R. officinalis* plant extract with Rf value of 0.83, 0.75, 0.92, 0.83, 0.90 respectively. The TLC plate of *R. officinalis* was shown in Figure-1.

Fig 1: TLC plate of *R.officinalis*

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the hexane extract of *Rosmarinus officinalis* leaves revealed the presence of twenty-two bioactive compounds. These compounds, along with their retention times (RT), molecular formulas, molecular weights (MW), and detection probabilities, are detailed in Table 1. The chromatogram illustrating the twenty-two peaks identified in the GC-MS analysis is presented in Figure 2.

Among the compounds detected, the following showed the highest probabilities of presence:

- 2-Phenanthrenol,4b,5,6,7,8,8a,9,10-octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans) (81.83%)
- Eucalyptol (75.34%)
- α-Caryophyllene (73.28%)
- n-Hexadecanoic acid (61.21%)
- 1,2-Benzenedicarboxylic acid, diisooctyl ester (61.20%)
- Heptamethyl-3-phenyl-1,4-cyclohexadiene (48.92%)
- Benzo[b]furan,3-(4-methoxyphenyl)-2,6-dimethyl- (35.41%)

These findings suggest that *R. officinalis* is particularly rich in these major bioactive constituents.

In Iranian *R. officinalis*, major compounds include 1,8-cineole (5.63-26.89%), camphor (1.66-24.82%), and α-pinene (14.69-20.81%) (Conde-Hernández *et al.*, 2017) [4]. Egyptian rosemary varieties contain higher proportions of camphor (38.6-44.8%), α-pinene (14.5-21.1%), and eucalyptol (13.1-15.5%) (Bousbia *et al.*, 2009) [3].

Abirami and Rajendran (2012) [1] reported that the methanol extract of *Vernonia cinerea* primarily contained n-hexadecanoic acid (42-88%), 1,2-benzenedicarboxylic acid, diisooctyl ester (23.00%), and squalene (11.31%).

Begam *et al.* (2013) [2] described the primary constituents of rosemary essential oil as camphor (5.0-21%), 1,8-cineole

(15-55%), α-pinene (9.0-26%), borneol (1.5-5.0%), camphene (2.5-12%), β-pinene (2.0-9.0%), and limonene (1.5-5.0%), with variability based on growth stage and climate.

Dongmei *et al.* (2015) [2] identified 1,8-cineole (51.783%) and α-pinene (13.508%) as the predominant compounds in rosemary essential oil.

According to Conde-Hernández *et al.* (2017) [4], the Italian variety of rosemary contains 1,8-cineole (23.39%), α-pinene (13.14%), camphor (13.02%), and camphene (6.54%). The French variety, on the other hand, features 1,8-cineole (15.69%), α-pinene (37.5%), camphene (4.64%), and verbenone (6.61%).

Khan *et al.* (2023) [6] reported that the key constituents in rosemary essential oil include α-pinene (21.52%), camphene (6.27%), verbenene (0.39%), β-pinene (3.49%), β-myrcene (1.63%), p-cymene (2.98%), 1,8-cineole (15.17%), camphor (6.80%), borneol (8.61%), verbenone (8.56%), and borneol acetate (6.07%).

Soliman *et al.* (2024) [8] found that the major constituents of essential oil from dried aerial parts of *R. officinalis* included verbenone, α-pinene, 1,8-cineole, and camphor.

Table 1: Active Compounds Identified in *R. officinalis* Hexane Extract via GC-MS

Sl. No.	Compound	Molecular Formula	Molecular Weight (g/mol)	RT (min)	Probability (%)
1	Eucalyptol	$C_{10}H_{18}O$	154	3.97	75.34
2	Camphor	$C_{10}H_{16}O$	152	7.76	9.22
3	Borneol	$C_{10}H_{18}O$	154	8.57	15.79
4	trans-α-Terpinyl pentanoate	$C_{15}H_{26}O_2$	238	9.55	1.21
5	D-Verbenone	$C_{10}H_{14}O$	150	10.10	1.16
6	Bornyl acetate	$C_{12}H_{20}O_2$	196	12.83	17.55
7	Caryophyllene	$C_{15}H_{24}$	204	16.88	21.26
8	α-Caryophyllene	$C_{15}H_{24}$	204	17.92	73.28
9	Naphthalene	$C_{15}H_{24}$	204	18.74	10.28
10	Cedrene	$C_{15}H_{24}$	204	19.82	8.05
11	Copaene	$C_{15}H_{24}$	204	20.14	2.39
12	trans-Z-α-Bisabolene epoxide	$C_{15}H_{24}O$	220	23.81	35.87
13	1-Heptatriacotanol	$C_{37}H_{76}O$	536	31.49	17.60
14	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	31.79	61.21
15	Heptamethyl-3-phenyl-1,4-cyclohexadiene	$C_{19}H_{26}$	254	32.61	48.92
16	Trenbolone Acetate	$C_{20}H_{24}O_3$	312	32.84	8.13
17	9,12-Octadecadienoyl chloride, (Z,Z)-	$C_{18}H_{31}ClO$	298	33.85	5.46
18	2-Phenanthrenol, octahydro-4b,8,8-trimethyl-1-(1-methylethyl)-, (4bS-trans)-	$C_{20}H_{30}O$	286	35.26	81.83
19	Spirost-8-en-11-one,3-hydroxy(3α,5α,14α,20α,22α,25R)-	$C_{27}H_{40}O_4$	428	35.89	6.14
20	Squalene	$C_{30}H_{50}$	410	36.20	1.43
21	Benzo[b]furan,3-(4-methoxyphenyl)-2,6-dimethyl-	$C_{17}H_{16}O_2$	252	36.40	35.41
22	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	36.65	61.20

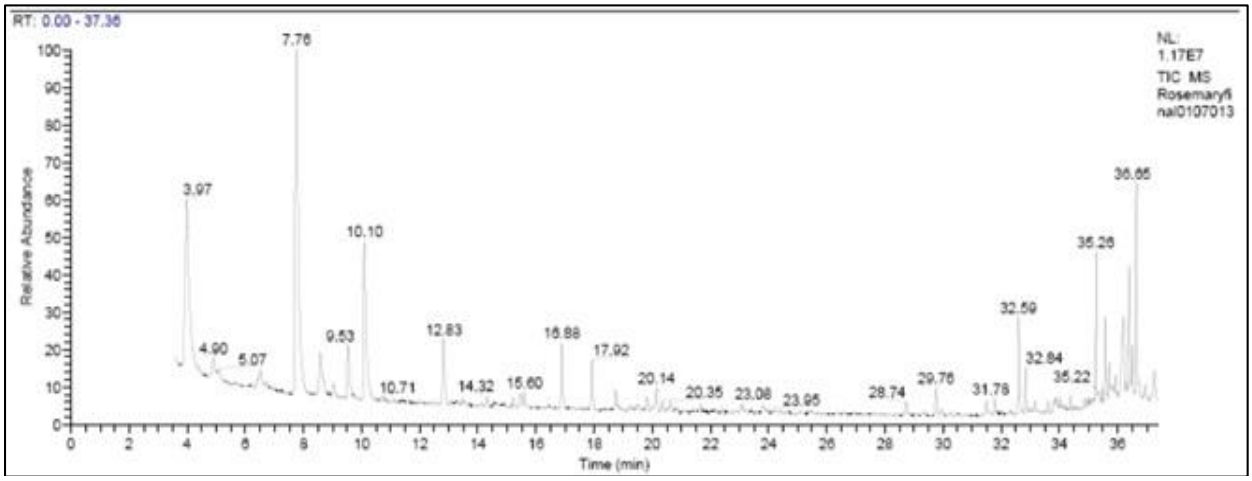

Fig 2: Total ion chromatogram (TIC) of *R. officinalis*

Conclusion

This study identified twenty-two chemical constituents within Hexane extracts of R.officinalis through gas chromatogram-mass spectrometry (GC-MS) analysis.

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