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Study of antibacterial activity of Essential Oil components obtained from pericarp of *Zanthoxylum rhetsa* (Indian origin) using HS-GCMS

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1. Introduction

Zanthoxylum rhetsa is a plant from *Rutaceae* family, commonly known as "Tirfal" in India. Its pericarp is used as a spice in Indian curry preparations due to its strong aroma and taste. The use of Essential Oil (EO) extracted from Tirfal is known for its antibacterial activity. However, its medicinal properties are not fully explored. The extracted oil from pericarp of Tirfal was subjected to Microbial assay, to check its Minimum Bactericidal Concentration (MBC). The compositional analysis of oil was performed using short transfer line headspace sampler HS-20 and Gas chromatograph-Mass spectrometer GCMS-QP2010 Ultra. The components responsible for antibacterial activity were studied by comparing the chromatographic responses of the 24 hours incubated vials containing EO with and without bacterial culture. The components of EO showing appreciable reduction in response could be responsible for its overall antibacterial activity.

Classification of *Zanthoxylum rhetsa*

Kingdom: Plantae
Division : Magnoliophyta
Class : Magnoliatae
Order : Sapindales
Family : Rutaceae
Genus : *Zanthoxylum*
Species : *rhetsa*

Fig. 1 Pericarp of *Zanthoxylum rhetsa* with seeds

2. Method of Analysis

Tirfal seeds with pericarp were collected from the local market of Mumbai in Maharashtra, India. After separation from the seeds, pericarp were grounded using a mixer. 20 g of grounded pericarp powder was mixed with 200 mL of deionized water and subjected to hydro-distillation for the extraction of oil[1]. The process was repeated 5-8 times to obtain sufficient quantity of EO.

2-1. GCMS analysis of EO

The EO extracted was analyzed using Shimadzu GCMS-QP2010 Ultra for identification of its components with the help of FFNSC (Flavour and Fragrance Natural and Synthetic Compounds) library[2], supported by 'Kovat' s Linear Retention Index' (LRI)[3] method.

2-2. Microbial Assay

The microbial assay of the EO was performed as given below:

Tube Assay: In Tubes, different concentrations of EO were prepared by mixing 10, 20 and 40 µL of oil with 490, 480, 460 µL of nutrient broth respectively.

↓

These tubes were inoculated with 500 µL of 24 hours old Staphylococcus aureus MTCC 96 culture, such that final concentration range of EO was 1%, 2% and 4%.

↓

All the tubes, along with positive , negative & media control were incubated for 24 hours at 37°C.

↓

Tubes were observed for turbidity after 24 hours of incubation. Results are shown in the Table 4.

↓

MBC Assay: From these tubes, loopful (5 µL) was withdrawn and spot inoculated onto a nutrient agar plate.

↓

The plate was observed after 24 hours of incubation at 37°C. (Fig. 4). Results are as shown in the Table 5.

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2-3. Analysis by HS-GCMS

For Headspace (HS) analysis, two sets were prepared. (i) 'Zero hours', (ii) 'After 24 hours'. Each set containing three HS vials, (A) 'Positive control' containing broth inoculated with culture, (B) 'Negative control' containing EO in broth and (C) 'Test' containing EO in broth inoculated with culture. From the microbial assay, the concentration showing antibacterial activity, was prepared in HS vial (C)'Test' of both the sets. HS-GCMS analysis of the sets,(i) and (ii) was performed. In set (ii), comparison between chromatograms of (C) 'Test' containing 2% EO in broth inoculated with culture & (B) 'Negative control' containing 2% EO in broth without culture was studied for the differences in their Total Ion Chromatogram (TIC).

Fig. 2 HS-20 coupled with GCMS-QP2010 Ultra by Shimadzu

Table 1 HS-GCMS analytical parameters

Headspace parameters			
Mode	: Loop		
Oven Temp.	: 90°C		
Sample Line Temp.	:110°C		
Transfer Line Temp.	:120°C		
Shaking Level	:3		
Equilibrating Time	:30 .0 min		
Injection Time	:1.0 min		
Chromatographic parameters			
Column	: Rtx-5Sil MS (60 m × 0.32 mm × 1.0 µm)		
Column Flow	: 2 mL/min		
Split Ratio	: 5.0		
Carrier gas	: Helium		
Column oven program	: Rate°C /min	Temperature°C	Hold time (min)
		50.0	0.0
	3.0	280.0	10.0
Mass Spectrometry parameters			
Ion source temperature	: 200°C		
Interface temperature	: 260°C		
Ionization mode	: EI		
Acquisition mode	: Scan		

3. Results

3-1. GCMS analysis of EO

The chromatographic separation was obtained by direct liquid injection of EO in GCMS. Identification of individual components was done using 'FFNSC library' & LRI method. The TIC of EO is shown in Fig. 3. Also, the identified components accounting for more than 0.3% of total area are enlisted in the Table 3.

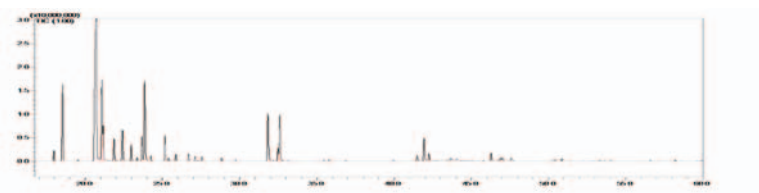

Fig. 3 TIC of EO obtained from Tirfal pericarp

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Table 3 Tirfal pericarp EO composition by GCMS Analysis with LRI method:

Sr. No.	Name	Retention Time (min)	Area %	Retention Index
1	Tricyclene	17.98	0.98	909
2	Pinene <alpha->	18.54	8.47	921
3	Sabinene	20.72	33.16	965
4	Pinene <beta ->	21.08	11.40	973
5	Dec -1-ene	21.87	2.16	989
6	Phellandrene <alpha ->	22.42	3.04	1000
7	Terpinene <alpha ->	22.97	1.58	1011
8	Cymene <para ->	23.37	0.36	1019
9	Limonene	23.68	2.61	1025
10	Phellandrene <beta ->	23.86	9.40	1029
11	Ocimene <(E) -, beta ->	24.25	0.49	1037
12	Terpinene <gamma ->	25.17	2.54	1056
13	Octanol <n->	25.40	0.31	1061
14	Sabinene hydrate <cis->	25.87	0.68	1070
15	Terpinolene	26.70	0.71	1087
16	Linalool	27.11	0.49	1096
17	Sabinene hydrate <trans->	27.56	0.49	1105
18	Menth-2-en-1-ol <cis-,para->	28.83	0.37	1131
19	Terpinen-4-ol	31.83	5.66	1192
20	Terpineol <alpha->	32.48	1.42	1205
21	Decanal <n->	32.60	5.32	1208
22	Cubebene <beta ->	41.50	0.55	1394
23	Elemene <beta ->	41.94	2.49	1404
24	Dodecanal <n->	42.26	0.78	1412
25	Amorphene <gamma ->	46.29	0.86	1504
26	Bisabolene <(Z) -, gamma ->	46.92	0.38	1520
27	Zonarene	47.59	0.48	1536

3-2. Microbial Assay

The results obtained in the 'Tube assay' are shown in Table 4. According to the observation, the minimum concentration of Tirfal EO required to inhibit the bacterial growth was found to be 2%. The activity of 2% EO was further confirmed by performing MBC assay. The results of MBC assay are shown in Fig. 4. According to the observation mentioned in Table 5, the MBC of Tirfal EO against S.aureus MTCC 96 strain was 2%. This concentration was further selected for HS-GCMS analysis.

Table 4 Tube Assay

EO Concentration	Result
1%	+
2%	-
4%	-
Positive control *	+
Negativecontrol **	-
Mediacontrol ***	-

Table 5 MBC Assay

EO Concentration	Result
1%	+
2%	-
4%	-
Positive control	+

Note:

+	Growth	-	No growth

* To check viability of bacterial culture used
** To check sterility of Essential Oil
*** To check sterility of broth

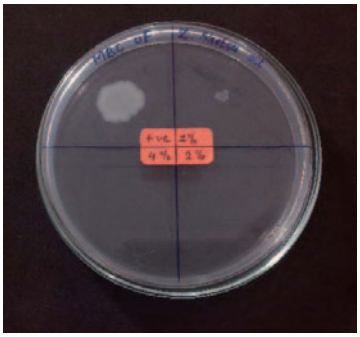

Fig. 4 MBC assay

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3-3. Analysis by HS-GCMS

The two vials from set (ii), i.e. (B) and (C) were analyzed using HS-GCMS. Their TIC were compared for checking the difference in the area of individual components. The comparison of two chromatograms is shown in Fig. 5.

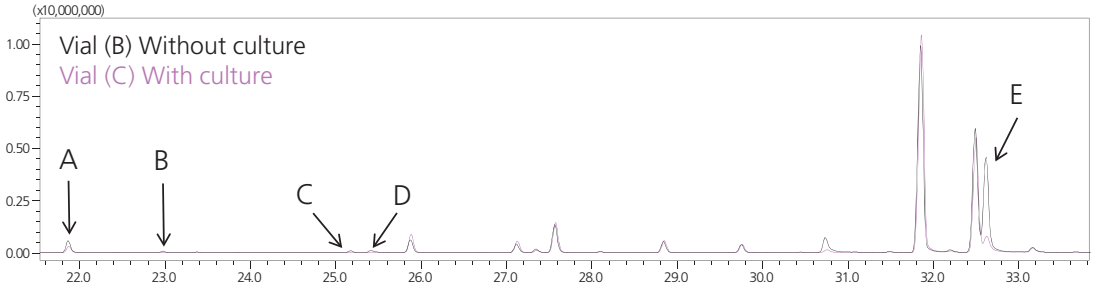
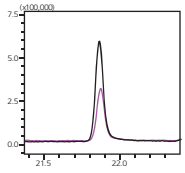
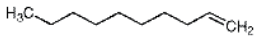
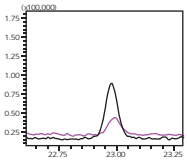
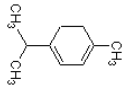
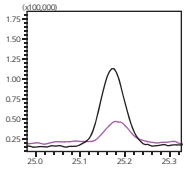
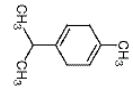
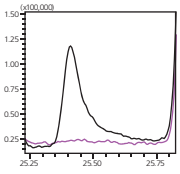
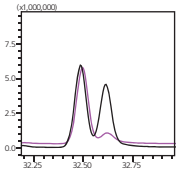
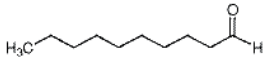


Fig. 5 Comparative TIC of Tirfal pericarp EO between Vial (B) and (C)

On comparison, it was observed that some of the components showed substantial decrease in the area. These components are enlisted in Table 6, along with their 'Magnified Chromatograms' and '% Decrease in Area'.

Table 6 List of components showing substantial decrease in area

ID	Retention Time (min)	Magnified Chromatograms	Name and Molecular Structure	% Decrease in Area
A	21.86		Dec-1-ene 	49.39
B	22.98		Terpinene <alpha -> 	71.08
C	25.17		Terpinene <gamma -> 	73.91
D	25.41		Octanol <n-> 	100.00
E	32.61		Decanal <n -> 	79.84

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4. Conclusion

- The identification of components present in EO obtained from the pericarp of Zanthoxylum rhetsa was done using FFNSC library and was further confirmed with LRI method.
- The antibacterial activity of 2% Tirfal EO against S.aureus MTCC 96 strain was confirmed.
- The components responsible for the antibacterial activity of Tirfal EO could be Dec-1-ene, Terpinene <alpha>, Terpinene <gamma>, Octanol <n> and Decanal <n>; as they showed substantial reduction in their area. There exist a lot of scope for research on these components as one of the natural sources of medicines.
- Shimadzu Headspace sampler HS-20 coupled with GCMS-QP2010 Ultra can be used efficiently to perform similar studies with other essential oils and natural products.

5. References

[1] Rui-Xue Zhu, Kai Zhong, Wei-Cai Zeng et al., Essential oil composition and antibacterial activity of Zanthoxylum bungeanum, African Journal of Microbiology Research Vol. 5(26), 4631-4637, 2011.
[2] FFNSC- MS Library (Chromaleont, Messina, Italy) ver. 2, 2012.
[3] E. Kovats, Adv. Chromatogr. (1) 229, 1965 .

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