Original Article

Determination of antibacterial, antifungal activity and chemical composition of essential oil portion of unani formulation kulzam

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Kulzam is a popular unani, liquid formulation; indicated for several minor ailments like cough, cold, running nose, sore throat, insect bites, earache, tooth ache, etc. by the manufacturer. However, this over the counter formulation has not been scientifically evaluated for its claimed uses. Hence in the present study an attempt has been to check the chemical composition, antibacterial and antifungal activity as most of the above-mentioned conditions are underpinned by microbial activity. The antibacterial and antifungal activity of the formulation was carried out on human pathogenic bacteria *Pseudomonas aerogenousa*, *Escherichia coli*, *Staphylococcus aureus*, *Corynebacterium* and fungi *Candida albicans*, *Aspergillus fumigates* and was compared with standards ciprofloxacin and clotrimazole. Kulzam exhibited strong *in vitro* inhibition of growth against all the test micro-organisms at both 100 and 150 µl levels of undiluted formulation (test sample) and more than that of standard at 150 µl level. The chemical composition of essential oil of the formulation was determined by gas chromatography–mass spectroscopy (GC-MS) analysis. Thirteen compounds constituting about 93.56% of the essential oil were identified. The main components were Camphor, menthol, thymol, 2-propenal 3-phenyl-, eugenol, *trans*-caryophyllene, *p*-allylanisole, linalool, eucalyptol, L-limonene, 1-methyl-2-isopropylbenzene, and 1S-alpha-pinene. The outcome of this study shows that kulzam contain terpenes and their oxygenated derivatives, which are believed to be highly effective antibacterial, antifungal, analgesic, anti-inflammatory, antioxidant, spasmolytic and immunomodulatory agents. The formulation has been found to possess strong antibacterial and antifungal properties, and it becomes very difficult to pin point the specific compound responsible for studied activities. However, the study positively motivates the use of kulzam for common ailments.

Key words: Antibacterial, antifungal activity, GC–MS analysis, HPTLC analysis, kulzam

INTRODUCTION

It is a common practice in most of the Indian families to use household remedies for the treatment of minor ailments like cough, cold, running nose, sore throat, etc. If these alone do not give satisfactory relief, then people prefer to use OTC drugs for faster relief. Among the OTC drugs, majority prefer to use the formulations from alternative therapies like ayurveda, unani, siddha, etc., which are believed to be safe and affordable.

Kulzam is one such popular unani, liquid formulation; indicated for several ailments by the manufacturer. However, people prefer to use it for common ailments like cough, cold, sore throat, etc. Their experience speaks that, it works wonderfully in all aforementioned conditions and yields faster satisfactory relief.

In the present study, an attempt has been to check the chemical composition and antibacterial, antifungal activity as most of the above-mentioned conditions are underpinned by microbial activity, which is not yet been reported.

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MATERIALS AND METHODS

Formulation

Kulzam [Hamdard (WAKF) Laboratories Ltd., Ghaziabad, U.P.] was procured from local unani stores. The composition of the formulation as appeared on its label is as follows: Sat – e – Pudina – 80 mg, Sat0-e-Ajwain – 150 mg, Kafoor – 300 mg, Roghan baid majnoon – 0.18 ml, Roghan darchini – 0.18 ml, Roghan Zaitun – 0.03 ml, Roghan laung – 0.01 ml, colour red q.s. All the chemicals used were of highest purity, available commercially and were procured from himedia and qualigens fine chemicals.

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Table 1: Antibacterial and Antifungal activity of kulzam by cup plate diffusion technique

Test organisms	Zone of inhibition (in mm)			
	Kulzam		Ciprofloxacin	Clotrimazole
	100 µl	150 µl	500 µg/ml	1 mg/ml
P. aeruginosa	30	32	22	–
E. coli	36	36	22	–
S. aureus	20	28	20	–
Corynebacterium	20	20	24	–
Candida albicans	40	40	–	27
A. fumigatus	40	40	–	27

For each zone an average of three independent determinations were noted

Table 2: Volatile compounds as detected by GC–MS from unani herbal marketed formulation kulzam

Peak	Retention time	Compound	%Matching with Wiley library
1	6.312	1S-alpha-pinene	99
2	7.589	Beta-pinene	99
3	8.976	Benzene, 1-methyl-2(1-methylethyl)-	99
4	9.112	1-Limonene	99
5	9.264	Eucalyptol	99
6	9.487	Dipropylene glycol	99
7	9.614	1-propanol, 2-(2-hydroxypropoxy)-	99
8	9.733	Dibutylene glycol	99
9	9.956	Gamma terpinene	99
10	11.200	Linalool	99
11	11.394	Bicyclo (3.1.0) hexan-2-one, 4-methyl-1-(1-methylethyl)-, (1. Alpha., 4.beta., 5.	99
12	11.853	2-(1-Cyclopent-1-enyl-1-methylethyl)cyclopentanone	99
13	12.665	Camphor	99
14	13.017	Isoborneol	99
15	13.255	Borneol	99
16	13.454	L-Menthol	99
17	13.870	3-Cyclohexene-1-methanol, alpha. Alpha., 4-trimethyl-	97
18	13.928	Endo-isocamphonone	99
19	15.955	2-Propenal, 3-phenyl	99
20	16.202	Benzene, 1-methoxy-4-(2 propenyl)	99
21	16.389	Phenol, 5-methyl-2-(1-methylethyl)	99
22	20.273	Alpha-Humulene	99
23	22.268	Cinnamaldehyde propylene glycol acetal	99
24	22.441	2(1H)-Naphthalenone, 3,4-dihydro-	98
25	23.050	Caryophyllene oxide	97
26	26.651	Benzyl benzoate	99

Test Organisms

The human pathogenic bacteria *Pseudomonas aerogenousa, Escherichia coli, Staphylococcus aureus, Corynebacterium* and fungi *Candida albicans, Aspergillus fumigatus* were obtained from the central Laboratory, Department of Microbiology, Navodaya Medical College Hospital and Research Centre, Raichur, Karnataka.

Antibacterial and Antifungal Studies

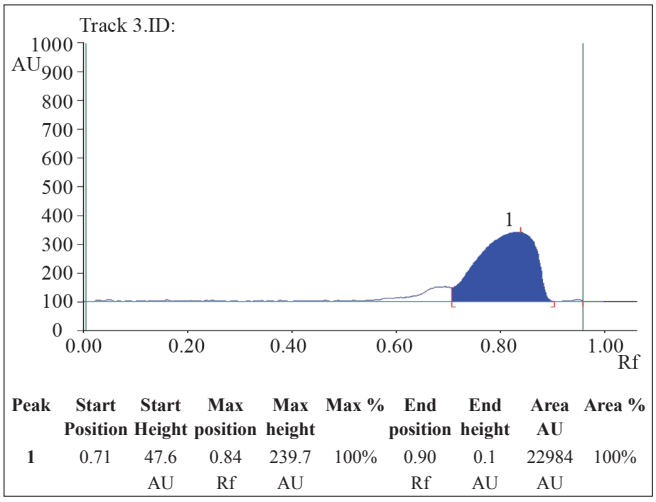
The formulation was subjected to antibacterial and antifungal studies and was carried out by agar cup plate method. Ciprofloxacin (500 µg/ml) and clotrimazole (1 mg/ml) were used as standards. The antibacterial and antifungal activity evaluated and employed for the study was Muller Hinton agar medium and Sabouraud dextrose agar medium. The medium was sterilized by autoclaving at 120° (1511b/in^2). About 30 ml of the molten nutrient agar medium inoculated with respective strains of bacteria and fungi (6 ml of inoculum to 300 ml of nutrient agar medium) was transferred asceptically into each sterilized petri dish (10 cm diameter). In each plate, three wells of 5 mm diameter were made using a sterile borer. Accurately 100 µl, 150 µl of the test and 100 µl of standard solution were transferred to cups aseptically and labelled accordingly. The plates were maintained at room temperature for 2 h to allow the diffusion of the solutions in to the medium. The Petri dishes used for the antibacterial screen were incubated at 37±1°C for 24 h, while those used for antifungal activity were incubated at 28°C for 48 h. The diameter of zone of inhibition surrounding each of the wells was recorded.[1]

Chemical Analysis by Gas ChromatographyMass Spectroscopy (GC–MS)

The samples were analyzed using Shimadzu GC–MS-QP2010 Plus apparatus equipped with quadrapole detector and split injection system. The GC was fitted with a ZP-624 capillary column (30 mm×1.4 mm, film thickness 0.25 µm). The temperature programmed was as follows: injector temperature 220°C, initial oven temperature at 50°C for 2 min, then rise to 250°C at the rate of 10°C/min for 25 min, transfer line temperature 220°C. Helium was used as carrier gas at 35.6 kPa pressure with flow 2.5 ml/min and electronic pressure control being kept on. The EM voltage was 952.9 V with lower and upper mass limits set at 30 and 350 *m*/*z*. Samples were solved in *n*-hexane and injected automatically. MS spectra of separated compounds were compared with one from Wiley 7 Nist 05 mass spectral database. The identity of the spectra above 95% was needed for the identification of compounds.

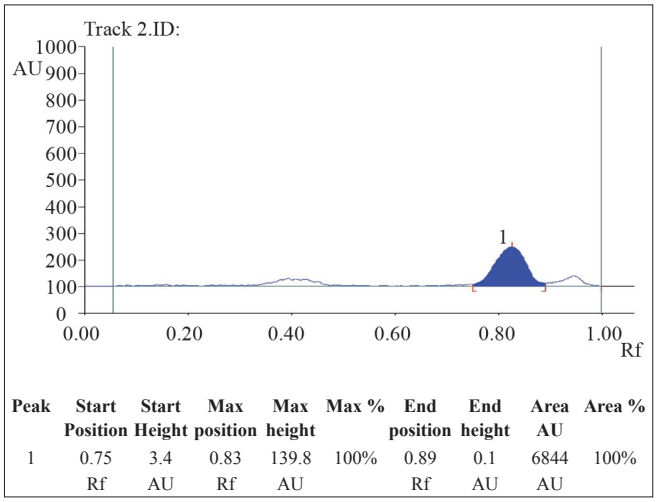
Table 3: Phytoconstituents and their reported biological actions

Compound	Biological actions	Uses claimed by manufacturer
Eucalyptol	Anti-inflammatory[3], cough suppressant, in treatment of non-prulent rhino sinusitis[4], Anti-nociceptive[5], spasmolytic activity[6]	Headache, toothache, earache, cough, cold and catarrh
Menthol	Analgesic[7,8], antifungal activity[9], antimicrobial activity[10]	
Thymol	Anti-inflammatory activity[11], Antioxidant[12], antimicrobial activity[13]	Insect bites, burns and scalds
alpha-humulene	Anti-inflammatory activity[14]	
(−)-*trans*-caryophyllene	Anti-inflammatory activity, antimicrobial activity[15]	Itching and scabies
3-phenyl-2-Propenal	Antimicrobial activity[16]	
α-pinene	Antimicrobial activity[15]	Nose bleeding
Linalool	Antimicrobial activity[17], spasmolytic activity[18]	
Eugenol	Antimicrobial activity[19]	Pneumonia and Lumbago
1-Methyl-2-isopropylbenzene	–	Stomach troubles: indigestion, flatulence, loud eructation loose Motion, vomiting, nausea, dysentery cholera, plague and other epidemics
L-limonene	Immunomodulatory activity[20], spasmolytic activity[21], antinociceptive[22]	
Camphor	Analgesic[23], antimicrobial activity[24]	
p-Allylanisole	–	



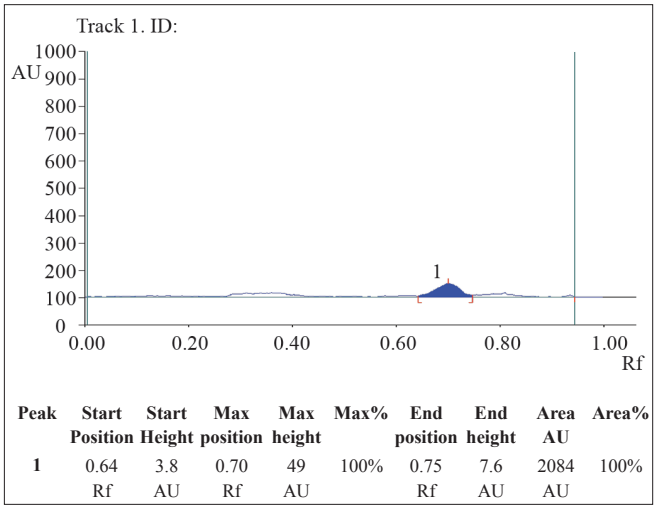
Peak	Start Position	Start Height	Max position	Max height	Max %	End position	End height	Area AU	Area %
1	0.71	47.6 AU	0.84 Rf	239.7 AU	100%	0.90 Rf	0.1 AU	22984 AU	100%

Figure 1: Chromatogram of standard menthol



Peak	Start Position	Start Height	Max position	Max height	Max %	End position	End height	Area AU	Area %
1	0.75 Rf	3.4 AU	0.83 Rf	139.8 AU	100%	0.89 Rf	0.1 AU	6844 AU	100%

Figure 2: Chromatogram of standard thymol

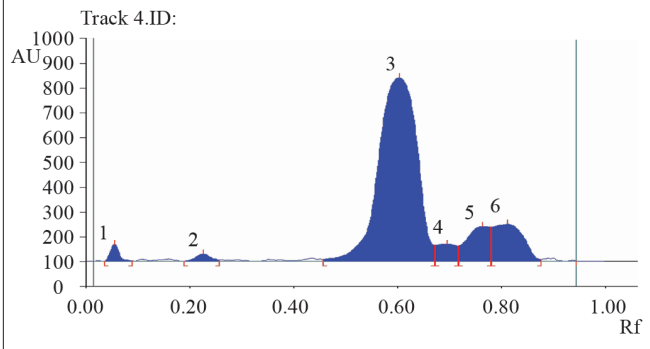


Peak	Start Position	Start Height	Max position	Max height	Max%	End position	End height	Area AU	Area%
1	0.64 Rf	3.8 AU	0.70 Rf	49 AU	100%	0.75 Rf	7.6 AU	2084 AU	100%

Figure 3: Chromatogram of standard camphor

Fingerprinting Studies by High Performance Thin Layer Chromatography Analysis

All the chemicals used in the study were of analytical grade (SD fine chemicals Pvt. Ltd. Mumbai) obtained from the central storehouse of the institution. Thymol, menthol, camphor was obtained from Lobei Chem. Pvt. Ltd., Mumbai. High Performance Thin Layer Chromatography (HPTLC) was performed on 20 cm×10 cm aluminum backed plates coated with silica gel 60F254 (Merck, Mumbai, India). Standard solutions of Thymol, menthol, camphor and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100-l Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28±2°C), with hexane: ethyl acetate 9:1 (v/v), as mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then scanned at 200, 250 and 300 nm with a Camag TLC Scanner with WINCAT software, using the deuterium lamp.



Peak	Start position	Start height	Max position	Max height	Max %	End position	End height	Area AU	Area %
1	0.04 Rf	0.5 AU	0.05 Rf	67.9 AU	5.68%	0.09 Rf	1.2 AU	986.2 AU	1.54%
2	0.19 Rf	0.1 AU	0.23 Rf	28.9 AU	2.42%	0.26 Rf	5.1 AU	751.8 AU	1.17%
3	0.46 Rf	7.1 AU	0.60 Rf	739.1 AU	61.81%	0.67 Rf	65.4 AU	47398 AU	73%
4	0.67 Rf	65.4 AU	0.70 Rf	69.9 AU	5.84%	0.72 Rf	62.1 AU	2270 AU	3.54%
5	0.72 Rf	62.4 AU	0.79 Rf	140.8 AU	11.77%	0.78 Rf	137 AU	5332 AU	8.31%
6	0.80 Rf	138.0 AU	0.83 Rf	149.3 AU	12.48%	0.88 Rf	7.2 AU	7437 AU	11.59%

Figure 4: Chromatogram of unani formulation Kulzam

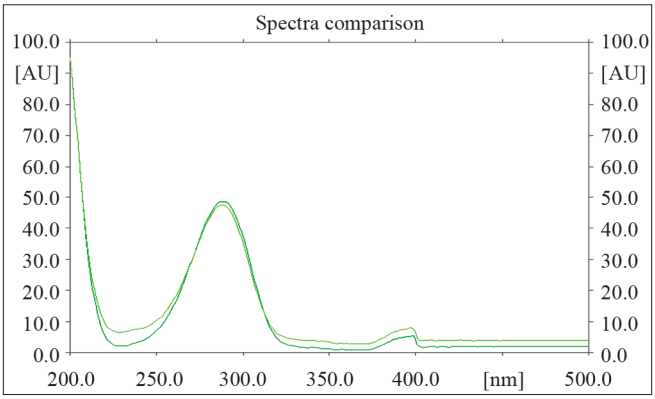


Figure 5: *In-situ* UV spectra of camphor standard and camphor from sample

Preparation of Standard Stock Solution

A stock solution of thymol, menthol and camphor (1000 µg/ml) was prepared by dissolving 25 mg of accurately weighed above samples in methanol and diluting to 25 ml with methanol in a standard volumetric flask. 0.1 ml of above stock solutions are again diluted to 10 ml in standard volumetric flask with methanol to get working standard solution of concentration 10 µg/ml.

Preparation of Sample Solution

The sample was sonicated for 10 min and the contents of the flask were filtered through Whatman No. 1 paper (Merck, Mumbai, India) to remove particulate matter. Accurately measured 10 µl of undiluted unani marketed formulation kulzam was taken for TLC analysis.

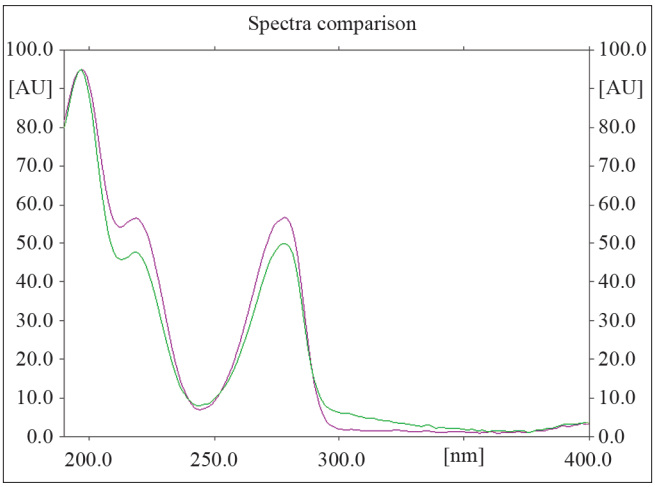


Figure 6: *In-situ* UV spectra of menthol standard and menthol from sample

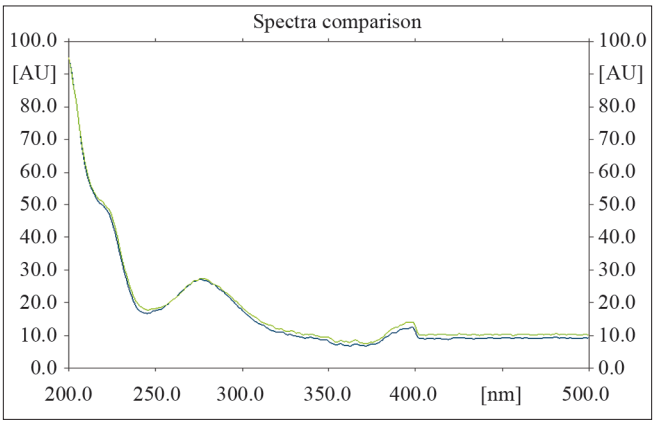


Figure 7: *In-situ* UV spectra of thymol standard and thymol from sample

RESULTS AND DISCUSSION

A perusal of the Table 1 reveals that, the kulzam exhibited strong *in vitro* inhibition of growth against all the test microorganisms at both 100 and 150 µl levels of undiluted formulation (test sample). It also draws attention that, gram-negative micro-organism are more susceptible to inhibitory action than gram-positive organisms. Documented reports quote that; gram-negative bacteria are more sensitive to the antibacterial activity of most of the essential oils.[2] The formulation studied also contains essential oils, which could be responsible for the above observation.

The zone of inhibition exhibited by the formulation against *P. aeruginosa* and *E. coli* at both the levels is significantly more than that exhibited by standard ciprofloxacin; however the zone of inhibition against *S. aureus* at 100 µl level is at par with the standard and more than that of standard at 150 µl level. On the other hand, the zone of inhibition against *corynebacterium* is only moderate.

Against fungi, *Candida allbicans* and *Aspergillus fumigatus*,

the formulation exhibited very significant zone of inhibition compared to that of standard clotrimazole.

The formulation was analyzed for the detection of chemical components by GC–MS technique. It was revealed that, the phytoconstituents identified belonged to different chemical classes. Thirteen compounds, constituting about 93.56% of the essential oil composition of the formulation, were identified and presented in Table 2. The main components were Camphor, menthol, thymol, 2-propenal 3-phenyl-, eugenol, *trans*-caryophyllene, *p*-allylanisole, linalool, eucalyptol, L-limonene, 1-methyl-2-isopropylbenzene, and 1S-alpha pinene. Most of the phytoconstituents detected have been reported by several workers to possess biological actions, which have been tabulated in Table 3.

HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials. It allows the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC analysis of the marketed unani formulation kulzam was carried out with the solvent system—hexane: ethyl acetate 9:1 (v/v). Menthol, thymol and camphor were used as reference standards. The amount of thymol and camphor found in the formulation was 1086 μg/ml and 1089 μg/ml, respectively. Though menthol was detected *in situ* UV absorption spectra, the Rf value of standard menthol did not match any Rf value in the sample finger print. HPTLC fingerprinting of unani formulation kulzam, gave six spots at the following Rf values: 0.05 Rf (1.54%), 0.23 Rf (1.17%), 0.60 Rf (73%), 0.70 Rf (3.54%), 0.79 Rf (8.31%), 0.83 Rf (11.59%). The HPTLC chromatograms are presented in Figures 1–7 and can be used regularly in routine quality control of this herbal formulation.[25]

Most of the ingredients incorporated in the formulation and chemical compounds detected by GC–MS have been reported to possess strong antibacterial, antifungal, analgesic, anti-inflammatory, immunomodulatory, cough suppressant and spasmolytic properties. These compounds may be responsible for the uses claimed by the manufacturer. However, further pharmacological studies in experimental animals are very much necessary. This study concludes that, the formulation contains therapeutically important phytochemicals, possesses strong antibacterial and antifungal activity and hence positively motivates the use of Kulzam for common ailments.

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