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Chemical Analysis (GC-FID-MS) and Antimicrobial Activity of *Parmotrema perlatum* essential oil Against Clinical Specimens

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ABSRTACT

The major study of this work was to evaluate the chemical composition and the inhibitory components of *Parmotrema perlatum* against the clinically isolated specimens of *Staphylococcus*, *Streptococcus*, *Escherichia, Pseudomonas* and *Candida*. The GC-FID-MS by using a Trace GC Ultra apparatus MS DSQ II detectors and FID-MS splitter under the operating conditions of a polar capillary column was employed to determine the chemical composition of *Parmotrema perlatum* essential oil. Amino acids viz (38.1%) and cymene (29.1%) were the most adequate constituents in this oil. The antibacterial activity of *Parmotrema perlatum* oil was tested against standard strains of bacteria and clinical strains isolated from patients with infections of the oral cavity, abdominal cavity, respiratory, genitourinary tracts, skin, and from the hospital environment. Kirby-Bauer disc diffusion technique was employed to determine the susceptibility of the organism towards the *Parmotrema perlatum* essential oil exhibited very strong antimicrobial activity to inhibit the growth of the clinical specimens. Application of natural antimicrobial based essential oil from *Parmotrema perlatum* can be potential source in the treatment and prevention of different types of infections as an alternative to the chemical - based antibiotics based on the investigation.

Keywords: Chemical analysis, Antimicrobial activity, *Parmotrema perlatum* oil, Essential oil, Clinical specimens.

INTRODUCTION

Parmotrema perlatum is commonly known as black stone flower or kalpasi, is a species of lichen used as a spice in India[1]. This spice has the potential to be an effective antimicrobial therapy for

many pathogens especially against opportunistic *Gram-negative* bacteria which has become as one of the major challenges for the present environment due to the rise in multi-drug resistant mutant organisms developed as a result of antibiotic abuse.[2-3] *Gram-positive* cocci such as *Staphylococcus* species has recently shown the significant rise in the infection and this essential oil is one of the choices for the safest & effective medicine that could be to treat bacterial infections[4-9]. The *Parmotrema perlatum* essential oil also has antifungal activity against the medically important yeast infections caused by *Candida* species[10-11]. The antibacterial components such as phenolic serves as a good antibacterial agents in the *Parmotrema perlatum* essential oils proved to be a very important weapon versus multidrug-resistant strains of bacteria and there is no proof of the rise of resistant type of bacteria[9,12]. This has proved once again that nature has blessed with plenty of natural resources available towards almost all types of infections and it will be our needs which will drive closer towards them. Thus, the need of the hour is to perform these types of studies to discover the hidden natural resources to resurrect the ancient effective forgotten techniques to treat several infections as a combat against antibiotic abuse causing the germination of mutant multidrug-resistantt organism. *Parmotrema perlatum* essential oil chemical analysis was done by GC-FID-MS to find out its chemical ingredients which inhibits infectious organisms by the antimicrobial components present[1,9,11,13-14].

MATERIALS AND METHODS

Material and reagents

Parmotrema perlatum essential oil, Clinical sample from a patients, Standard strain of the bacterium and different types of Hi Media culture plate were used.

Specimen collection

The standard strains, *Staphylococcus*, *Streptococcus*, *Escherichia*, *Pseudomonas* and *Candida* species were employed against the study of the clinical specimens of *Staphylococcus*, *Streptococcus, Escherichia*, *Pseudomonas* and *Candida* species which were collected from the various Jeddah, hospitals[7-8,15].

Identification of bacterial strain

Isolation and purification of the clinical specimens

Staphylococcus specimen was identified by using the standard microbiological techniques [9,14,16] by culturing on blood culture media with 5% of sheep's erythrocytes and also culturing on mannitol salt agar. The ability of the bacterium to produce catalase and coagulase was determined by slide test. The Gram staining showed *Gram-positive* cocci in clusters.

Streptococci specimen was identified by culturing on Blood culture media with 5 percent of sheep's erythrocyte-forming beta-hemolytic colonies. The inability of the bacterium to give coagulase & catalase was determined by a slide test. The Gram staining showed *Gram-positive* cocci in chains.

Escherichia specimen was identified by culturing on Mac Conkey culture media forming pink lactose fermenting colonies and were identified by biochemical methods with motility test by hanging drop method to differentiate from other *Gram-negative* entero-bacteria and determined by the enzymatic test of oxidase negative todifferentiate from *Pseudomonas*.

Pseudomonas specimen was identified by culturing on Mac Conkey culture media forming characteristic green pigmented non-lactose fermenting colonies and were identified by biochemical methods with motility test by hanging drop method to differentiate from other *Gram-negative* entero-bacteria and determined by the enzymatic test of oxidase-positive to differentiate from *Escherichia.*

Candida specimen was identified by culturing on Sabaraud's dextrose culture media forming pale white oval colonies and determining with a rapid screening germ tube test by inoculating

in the serum of the sheep and incubating at 37^{0}C for 3 h to check its virulence factor. The Gram staining result shows *Gram-positive* oval yeast budding cells. The chlamydospore formation testwas done on cornmeal agar incorporated with tryptophan as a confirmatory test.

***Parmotrema perlatum* essential oil preparation**

100.00 of *Parmotrema perlatum* grounded using mortar and pestle. The grounded powder dissolved in 400.00 ml distilled water and boiled at 100^{0}C for about 1 hour. The solution is filtered using filter paper[1]. Further this oil was analyzed by GC-FID-MS by utilizing a GC.

Antimicrobial susceptibility testing

The following three methodologies were employed[9,14,16] to determine the antimicrobial susceptibility testing for the *Parmotrema perlatum* essential oil against isolated and purified collected clinical isolates along with the standard strains. Different types of sstandard antibiotics were used for the study to compare antimicrobial susceptibility results to correlate with the *Parmotrema perlatum* essential oil.

Kirby-Bauer method or disc diffusion method

The disk diffusion method (Kirby-Bauer) is more suitable for routine testing in a clinical lab where a large number of isolates are tested for susceptibility to antimicrobial effects. A standardized antibiotic disc was prepared and incubated at 37^{0}C soaked overnight in the *Parmotrema perlatum* Essential oil and incorporated on *Mueller-Hinton* culture media. The overnight incubation at 37^{0}C shows drug susceptibility of microorganisms This method provides a quick way to determine the efficacy of the antimicrobial properties. The comparative study was performed by incorporating all the standard strains respectively by the same *Mueller-Hinton* agar plate method by repeating the procedure. The results were observed and recorded.

Minimum inhibitory concentration-Tube dilution method

Minimum concentration required by *Parmotrema perlatum* essential oil to inhibit the clinical isolates was determined by this MIC test by using different sets for each clinical specimens inoculated in peptone water along with the antimicrobial agents and after overnight incubation at 37^{0}C in an incubator where a serial dilution of the respective *Parmotrema perlatum* essential oil is prepared and inoculated with the inoculum The MIC values were observed with the last tube with turbidity determining the bacteriostatic effect of the respective essential oils and were recorded.

Minimum bactericidal concentration method

The minimum bactericidal concentration (MBC) was performed to determine the lowest concentration of all the *Parmotrema perlatum* essential oils required for the bactericidal effect on the clinical isolates by dividing the appropriate culture plate for each isolate into six portions representing each dilution of MIC. The sample from each dilution was inoculated on the each designated portions for the respective dilutions for all the *Parmotrema perlatum* essential oil MIC dilutions separately. The inoculated plates were incubated overnight at 37^{0}C to evaluate the bactericidal activity of the *Parmotrema perlatum* essential oils against the respective isolate.

Note: Sabaraud's media was used for the demonstration of the anti-fungal activity of *Candida* against *Parmotrema perlatum* essential oil by following the above methodology[1,10].

RESULTS AND DISCUSSION

Chemical components analyzed from the tested *Parmotrema perlatum* essential oil.

The chemical components analysis of the Essential oil derived from the tested *Parmotrema perlatum* was found to meet all the requirements to be a good antimicrobial substance. The content of tested *Parmotrema perlatum* essential oil constitutes Alkaloids Carbohydrates, Phytosterols, Fixed oils, fats, Phenolic compounds, Tannins, Proteins, Amino acids, Gums, mucilage,-Saponin, carvacrolas etc. listed (Table 1).

Table 1: Analysis of Chemical Components Procured from the tested *Parmotrema perlatum* essential oil by GC-FID-

Compound	Total oil %	R. indices
Alkaloids	0.60	932.00
Carbohydrates	1.90	936.00
Camphene	1.20	950.00
Phytosterols	1.00	962.00
Cineole	2.10	1024.00
Limonene	0.20	1025.00
Fixed oils and fats	5.20	1051.00
Ketones	0.10	1082.00
Terpene camphor	0.60	1023.00
Tanins	1.30	1164.00
Proteins	0.30	1176.00
Methyl ether	1.30	1215.00
g-Terpinene	1.00	1226.00
Borneol acetate	0.30	1270.00
Amino acids	38.10	1267.00
Carvacrol	2.30	1278.00
Phenolic compounds	0.20	1329.00
Gums and mucilage	0.10	1356.00
Copaene	0.20	1379.00
Carvcrol	0.10	1386.00
Caryophyllene	3.10	1421.00
Saponin	0.10	1509.00
Humulene	0.10	1455.00
Muurolene	0.30	1474.00
Coumarins	0.10	1488.00
Cuparene	0.10	1498.00
p-Cymene	0.60	1507.00
Calamenene B	0.20	1517.00
Caryophyllene oxide	0.30	1520.00
Cadinene	0.10	1534.00
Monoterpene alcohols	0.50	1578.00
Sesquiterpene alcohols	0.10	1618.00
Aldehydes	0.10	1650.00
Ethers	0.10	1659.00

Test for antimicrobial susceptibility

The clinical specimens were collected from different sources from the hospital and compared with that of standard strains of the reciprocals to determine the antimicrobial susceptibility towards the *Parmotrema perlatum* essential oil[7,10,18-19]. The clinical bacterial specimens collected were *Staphylococcus* species, *Streptococcus* species, *Escherichia* species, *Pseudomonas* species and the fungal yeast *Candida* species due to its clinical importance. A collection of standard antibiotics was used to compare the antimicrobial activity with that of the *Parmotrema perlatum* essential oil. The methodology employed was Kirby-Bauer disc diffusion method to determine the susceptibility of each organism by means of zone formations which determines sensitivity of the organism[14]. The minimum dilution required for the *Parmotrema perlatum* essential oil was determined by using the technique of Minimum Inhibitory-Bactericidal concentration methods using different sets for each specimen. The observation of the results were interpreted and recorded. The results showed a significant values of antimicrobial compounds towards the collected clinical specimens[9,20]. The results achieved were exemplary as described (Tables 2-5).

Table 2: Antimicrobial susceptibility of *Staphylococcus* isolates

		Antimicrobial Susceptibility														
Specimen	MIC of oil dl/ml	*FOX*	*E*	*CC*	*F/M*	*VA*	*TEC*	*TE*	*C*	*CIP*	*SXT*	*FA*	*LZD*	*R*	*I*	*S*
Nasal specimen	0.5	-	-	_	+	+	+	+	*	-	+	+	5	1	6	
Otitis specimen	0.75	-	-	-	-	+	+	_	+	-	-	_	+	8	0	4
Skin	0.75	+	+	+	-	+	+	_	+	+	+	-	+	3	0	9
Wound specimen	0.5	_	-	-	_	+	+	-	+	*	-	-	-	7	1	4
Abdominal Cavity Exudate	0.25	-	-	-	-	+	+	-	+	-	+	+	+	6	0	6
Ulceration	0.75	-	+	-	-	+	+	+	+	-	*	+	+	4	1	7
Groin	0.75	+	+	+	+	+	+	+	+	+	-	+	+	1	0	11
Abscess	0.25	-	-	-	+	+	+	+	+	-	-	+	+	7	0	5
Urine specimen	0.75	*	-	+	+	+	+	+	+	-	+	+	-	4	1	7
Drain	0.5	+	+	+	—	+	+	+	+	+	+	+	+	1	—	11

Table 3: Antimicrobial susceptibility of *Streptococcus* isolates

		Antimicrobial Susceptibility																	
Specimen	MIC of oil dl/ml	*AM*	*C*	*CIP*	*E*	*FOS*	*F/M*	*GM*	*IPM*	*LNZ*	*P*	*S*	*SYN*	*TE*	*TEC*	*VA*	*R*	*I*	*S*
Urine specimen	0.75	+	+	+	+	+	+	+	+	*	-	+	-	-	-	-	5	1	9
Wound specimen	0.5	+	+	_	+	+	+	_	+	-	-	_	-	-	-	-	9	0	6
Ulceration	0.5	+	+	_	+	+	+	_	+	+	+	-	-	+	+	+	4	0	11
Bile	0.5	+	+	-	+	+	+	-	+	*	-	-	-	_	-	-	8	1	6
Bedsore Swab	1.5	+	+	-	+	+	+	-	+	-	+	+	-	-	-	-	7	0	8
Pharynx specimen	0.5	+	+	+	+	+	+	+	+	-	*	+	-	-	+	-	4	1	10
Health care worker	0.5	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	2	0	13
Drain	0.25	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	5	0	9
Shelf	0.25	+	+	+	+	+	+	+	+	-	+	+	-	*	-	+	3	1	11
Disinfection dispenser	0.25	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	1	0	14
Blood sample	0.25	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	4	0	11
Hospital bed	0.25	+	+	+	+	+	+	+	+	-	*	+	-	+	+	+	2	1	12

Table 4: Antimicrobial susceptibility of *Escherichia* isolates

Specimen	MIC of oil	Antimicrobial susceptibility																				
	d dl/ml	*AMC*	*CF*	*CZ*	*CXM*	*GM*	*AM*	*NOR*	*F/M*	*FOX*	*CIX*	*CAZ*	*ATM*	*IPM*	*CIP*	*NET*	*NN*	*C*	*TE*	*SXT*	*R*	*I*	*S*
Pharyngeal specimen	0.75	+	_	+	*	-	+	-	-	-	-	_	+	_	+	+	+	+	*	10	2	7	
Bronchial secretion	0.5	+	_	+	-	-	_	-	-	-	-	-	-	+	-	+	+	_	+	-	14	0	5
Groin specimen	0.25	+	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	_	+	+	7	0	12
Abdominal exudation	0.52	+	-	+	*	-	-	-	-	_	-	-	_	+	_	+	+	-	+	*	11	2	6
Anal specimen	0.5	+	-	+	-	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-	11	0	8
Bedsore specimen	0.75	+	+	+	-	*	+	-	-	-	+	-	-	+	-	+	+	+	+	-	7	1	11
Ulceration specimen	0.75	+	+	+	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	5	0	14
Wound specimen	0.5	+	+	+	-	-	+	-	-	-	-	+	-	+	-	+	+	+	+	-	8	0	11
Urine specimen	0.75	+	+	+	-	+	+	-	+	*	-	+	-	+	-	+	+	+	+	-	6	1	12

Table 5: Antimicrobial susceptibility of *Pseudomonas* isolates

Specimen	MIC of oil	Antimicrobial susceptibility																	
	d dl/ml	*MZ*	*PP*	*CAZ*	*GM*	*NN*	*AMC*	*TZP*	*CTX*	*ATM*	*IPM*	*MEM*	*NET*	*CIP*	*SXT*	*C*	*CL*	*R*	*I*	*S*
Ear Specimen	1.2	-	-	-	-	_	+	_	+	+	+	*	-	+	-	-	11	1	4	
Pharyngeal Specimen	2.25	-	-	-	-	-	-	+	-	+	_	+	-	-	_	-	-	12	0	3
Groin specimen	1.5	-	-	+	+	+	-	+	-	+	_	+	+	+	-	-	+	6	0	10
Toe specimen	1.75	-	-	_	-	-	_	+	_	+	-	+	*	-	-	-	-	13	0	3
Anal specimen	2.25	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	-	11	0	5
Sputum	1.5	-	-	-	+	-	-	+	-	+	+	+	-	*	+	-	-	9	1	6
Bronchial secretion	2	-	-	+	+	+	+	+	-	+	+	+	+	-	+	-	+	5	0	11
Bedsore specimen	1.75	-	-	-	-	+	-	+	-	+	+	+	-	-	+	-	+	9	0	7
Wound specimen	1.25	-	+	*	-	+	-	+	-	+	+	+	-	+	+	-	+	5	1	10
Ulceration specimen	2.0	+	+	+	-	*	+	-	-	+	+	+	+	+	+	-	+	4	1	11
Urine specimen	2.25	+	+	+	-	*	+	-	-	+	-	+	-	+	+	-	+	6	1	9

Abbreviations used in Tables 2-5

Antibiotic Symbols: Cefoxin-FOX; Erythromycin-E; Clindamycin-CC; Nitrofurantoin- F/M; Vancomycin-VA; Teicoplanin-TEC; Tetracycline-TE; Chloramphenicol-C; Ciprofloxacin-CIP; Trimethoprim/ Sulfamethoxazole-SXT; Fisidic acid-FA; Lmezolid-LZD; Ampicillin-AM;, Fosfomycin-FOS; Gentamycin-GM; Imipenem-IPM;, linezolid- LZD; Penicillin-P; Streptomycin-S; Synercid-SYN; Amoxicillin/ clavulanic acid-AMC; Cefalotin-CF; Cefazolin-CZ; Cefuroxie-CXM; Norfloxacin-NOR; Cefotaxime-CTX; Ceftazidime-CAZ; Aztreonam-ATM; Netilmicin-NET; Tobramycin-NN; Mezlocillin-MZ; Piperacillin-PIP; Piperacillin/tazobactam-TZP; Meropenem-MEM.;

Resistant- (-) ; Intermediate-(*) ; Sensitive-(+)[9].

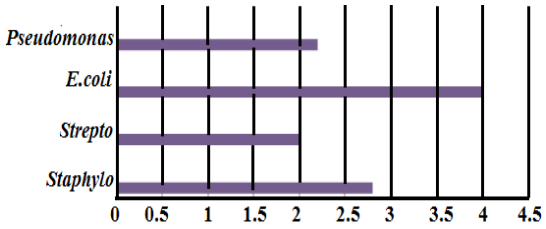

Fig. 1. Comparative chart-antimicrobial minimum inhibitory concentrations-clinically isolated specimens

The *Parmotrema perlatum* essential oil constitutes of phenolic compounds along with other antimicrobial agents listed (Table 1) analysed from the GC-FID-MS by using a Trace GC Ultra apparatus MS DSQ II detectors and FID-MS splitter under the operating conditions of apolar capillary column to determine the chemical composition which shown as a potential antimicrobial agent due to its chemical properties. The phenolic compounds are excellent antimicrobial substances which were prepared chemically to disinfect bolus of microbial load[2-3]. The phenolic content of the *Parmotrema perlatum* essential oil may serve as one of the major factor to eliminate the infections as an alternate to the hazardous chemicals. The *Parmotrema perlatum* essential oil has the potency to eliminate the infection with no serious side effects. The *Parmotrema perlatum* is a lichen found in the southern parts of India which was used in ancient traditional therapies to treat the infection. This study was an attempt to resurrect the natural traditional methods to treat infections with the nature blessed products rather than seeking the hazardous chemical components produced by the pharmaceutical companies by destroying the eco-system as well as the human immune system[5]. The *Parmotrema*

perlatum essential oil is eco-friendly as well as also boosts our immune system to combat the potentially dangerous drug resistant clinical pathogens. In our study, the clinical specimens were collected from different sources from the hospital and compared with that of standard strains of the reciprocals to determine the antimicrobial susceptibility towards the *Parmotrema perlatum* essential oil. The clinical bacterial specimens collected were *Staphylococcus* species, *Streptococcus* species, *Escherichia* species, *Pseudomonas* species and the fungal yeast *Candida* species due to its clinical importance. A collection of standard antibiotics was used to compare the antimicrobial activity with that of the *Parmotrema perlatum* essential oil. The methodology employed was Kirby-Bauer disc diffusion method to determine the susceptibility of each organism by means of zone formations which determines sensitivity of the organism. The minimum dilution required for the *Parmotrema perlatum* essential oil was determined by using the technique of Minimum Inhibitory-Bactericidal concentration methods using different sets for each specimen. The observation of the results were interpreted and recorded. The results shown a significant values of antimicrobial compounds towards the collected clinical specimens. The results achieved were exemplary and were proved that even the clinical specimens which were resistant to the antibiotics were also sensitive towards the Parmotrema perlatum essential oil and the MIC values required to inhibit the growth of the clinical infectious specimens was much lesser compared to that of standard antibiotics values. The interpretation of results was done by the comparative values of standard antibiotics with that of *Parmotrema perlatum* essential oil. The interpretation shows that the antimicrobial activity of the *Parmotrema perlatum* essential oil is exemplary with that of standard antibiotics (Fig. 1). The clinical specimen of *Pseudomonas* species which was least susceptible among the bacteria to the standard antibiotics shown susceptibility towards the *Parmotrema perlatum* essential oil. The clinical specimen of *Escherichia* species shown excellent susceptibility among all the bacterial clinical specimens. The clinical specimens of the *Staphylococcus* species and *Streptococcus* species shown promising values with *Parmotrema perlatum* essential oil when compared to the standard antibiotics. The clinical specimen of the fungal yeast *Candida* species was also attempted to check its susceptibility towards *Parmotrema perlatum* essential oil which shown satisfactory effects and no antifungal drug was used in the study for the comparison. The satisfactory results of the antifungal activity of the *Parmotrema perlatum* essential oil has open a step forward to study more about the antifungal properties of such essential oils which can help to combat many other fungal infections especially in the prophylaxis of *Candidiasis* in the diabetes and also in the prophylaxis of vaginitis infections caused by this yeast[10,11]. Elaborate study about the antifungal activity of *Parmotrema perlatum* essential oil yet to be studied but the preliminary investigations has proved promising results. This study about the *Parmotrema perlatum* essential oil was an attempt to resurrect and awaken the world about the potential antimicrobial properties present in this types of essential oils as an alternative to the chemical compounds in the antibiotic which are toxic to the living cells to combat the dangerous infection. The major advantage of administering *Parmotrema perlatum* essential oil is that it will not cause the recurrent infections with no side effects.

CONCLUSION

The study on *Parmotrema perlatum* essential oils is an attempt to revoke and resurrect the ancient forgotten technologies to treat the potentially hazardous infections from the microbes by the administration of abundant natural antimicrobial components derives from the gifted nature with no side effects. The WHO has warned that the time with the antibiotics is running out due to our misuse of them which has given rise to the multi-drug resistant organisms and the essential oils such as *Parmotrema perlatum* essential oil can be an excellent alternative resource.

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Conflict of interests

The authors declare no conflict of interest.

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