Elucidation of Secondary Metabolites from Thyme Oil and Comparing its Antimicrobial Potential with Conventional Antibiotics against Uropathogens

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***Abstract*—GC-MS analysis of thyme oil showed the presence of various secondary metabolites comprising 13 components representing approximately 99% of the oil. The major components were carvacrol, thymol, terpinene-4-ol. The antimicrobial activity of thyme oil was assessed by disc diffusion method against uropathogens like *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, P. mirabilis and Staphylococcus aureus.* The MIC was determined by broth microdilution method. The ZOI and MIC (%) were in the range of 19±3 mm and 0.08% (v/v equivalent) for *E. coli*; 14±4 mm and 0.25% for *S. aureus*; 16±4 mm and 0.04% for *K. pneumoniae*; 18±3mm and 0.04% for *Proteus mirabilis*; 18±3 mm and 0.04% for *Proteus vulgaris.* Thyme oil showed low MIC values and high growth inhibition in comparison to 16 broad spectrum antibiotics tested. The result of the bioassay showed potent bactericidal properties of the oil.**

***Index Terms*—Thyme oil, secondary metabolites, carvacrol, terpinen-4-ol, uropathogens, bactericidal.**

I. Introduction

In recent decades alternative and complementary medicines have enjoyed increased popularity due to increasing incidences of adverse side effects associated with overuse of antibiotics and consequent antibiotic selection pressure in current clinical use. This has necessitated searching for new antimicrobial compounds from vegetable species with diverse chemical structures and novel mechanism of action to combat multiresistant microorganisms. The study of natural products from vegetable origin presenting antimicrobial activity has been shown in many perspectives mainly because of the presence of terpenes, alkaloilds, saponins and tannins. This illustrates the importance of natural products as source of new antimicrobial agents [1]. Research in essential (volatile) oils has attracted increased attention from both academic and industrial circles due to a growing interest in green consumerism, world-wide. The antiseptic, antiviral, antimycotic, antitoxigenic, antiparasitic, and insecticidal properties, of aromatic and medicinal plants and their extracts have been documented since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s [2] [3]

The genus *Thymus*, belonging to the family *Lamiaceae*, is a aromatic perennial herb, native of Mediterranean region and southern Europe. The essential oil of common thyme is made up of 20-50% thymol, which is an antiseptic. *Thymus* spp. is well known as medicinal plants because of their biological and pharmacological properties. It is used as excellent tonic and herbal tea, and used in the treatment of tonsillitis, gum diseases, rheumatism, arthritis and fungal infections. Thymus oils are widely used in pharmaceutical, cosmetic and perfume industry.

In light of this, the objective of the present study was to perform phytochemical prospection of thyme oil by GC-MS and to validate its antimicrobial effect against uropathogens.

II. Materials and Methods

A. Collection of Uropathogens

In the present study, 182 urinary isolates, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *P. mirabilis and Staphylococcus aureus* were collected from various Pathology laboratories of

Manuscript received September 15, 2012; revised December 25, 2012

doi: 10.12720/jolst.1.1.29-32

Nagpur, India. The cultures were maintained on Trypticase Soya Agar (M990) and stored at 4˚C.

B. Reference Cultures

The reference cultures of *Escherichia coli* ATCC 25922 (beta-lactamase negative), *Pseudomonas aeruginosa* MTCC 741 , *Klebsiella pneumonia* MTCC 432 , *Proteus vulgaris* MTCC 426 , *P. mirabilis* MTCC 425 *and Staphylococcus aureus* MTCC 96 were collected from Institute of Microbial Technology, Chandigarh (MTCC), India.

C. Antimicrobial Agents

The essential oil of *Thymus vulgaris* was procured from Dr. Urjita Jain's Forest Herbals Pvt. Ltd. Mumbai.

Antibiotics with varying concentrations Ampicillin (10mcg), Kanamycin (30mcg), Streptomycin (10mcg), Tobramycin (50mcg), Norfloxcacin (10mcg), Co-trimoxazole (25mcg), Chloramphenicol (30mcg), Colistinmethane sulphonate (100mcg), Gentamycin (10mcg), Nalidixic acid (30mcg), Trimethoprim (5mcg), Tetracycline (100mcg), Amoxycillin (30mcg), Cephatoxime (30mcg) were supplied by Hi-media Laboratories, Mumbai.

D. Antimicrobial Activity

In vitro antiuropathogenic activity of thyme oil was evaluated by paper disc diffusion method [4]. For this, sterilized blank Whatman filter paper discs of size 6mm were used. These discs were impregnated with 15 µg of thyme oil. A lawn culture of test strain on Mueller-Hinton agar was exposed to the discs of oil. All the plates were then incubated at 37˚C for 24 hrs. After incubation, results were noted by measuring zone of growth inhibition in mm using zone reader and average values of three replicates were calculated for each isolate and recorded. Conventional antibiotics like Ampicillin (10mcg), Kanamycin(30mcg), Streptomycin (10mcg), Tobramycin(50mcg), Norfloxcacin10mcg), Co-trimoxazole(25mcg), Chloramphenicol (30mcg), Colistinmethane sulphonate (100mcg), Gentamycin (10mcg), Nalidixicacid (30mcg), Trimethoprim (5mcg), Tetracycline (100mcg), Amoxycillin (30mcg), Cephatoxime (30mcg) were used as positive standards in order to test the sensitivity of the isolated uropathogens against thyme oil.

The oil was also assayed for MIC determination using broth microdilution method. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth of the bacteria. The thyme oil was dissolved in Tween-20 (0.5%). The density of the bacterial suspension was standardized by McFarland 0.5 standard.

E. Gas Chromatography and Mass Spectroscopy (GC-MS) Studies

The GC-MS studies were performed using Shimadzu QP-2000 GC/MS instrument at 70eV (unless otherwise specified) equivalent to OV-1, fused silica capacity - 0.25 mm X 50 M with film thickness - 0.25 micron. The entry on the GC- MS trace such as 100-6-10-250 means that the initial temperature was 100˚C for 6 min and then heated at the rate of 10˚C per minute to 250˚C. Carrier gas (helium) flow: 2ml per minute. Identification of GC-MS spectra is based on the direct comparison of Kovates index and mass.

III. Result and Discussion

A. GC-MS Prospection of Thyme Oil

Phytochemical prospection of thyme oil indicated the presence of 13 compounds by GC-MS. The main constituents present in *Thymus vulgaris* were Terpinene-4-ol (32.7%) followed by Thymol (18.1%), α-Terpinene (7.4%), Carvacrol (5.6%) α-pinene (3.5%), but the levels of other compounds were low. (Fig's 1 to 3 and Table I). The identification is based on the direct comparison of Kovates index and mass spectra. Variation in chemical composition of EO's may be observed due to the origin, the environmental conditions, and the developmental stage of the collected plant materials. Antimicrobial activity of EO is attributed mainly to its major components, although the synergestic or antagonistic effect of one compound in minor percentage of mixture has to be considered [5]. Therefore antimicrobial activity may vary based on the variations in the chemical compositions.

TABLE I. Identification of Molecular Mass of Different Compounds Present in the Essential Oil of *Thymus Vulgaris* Using Gc-Ms Analysis

Peak#	Scan No.	Compound	Retention Time (Min)	% Area	Identification
1	2	α-pinene	0.03	3.5	MS
2	10	p-cymene	0.3	1	MS
3	364	Unkhown	12.1	2.8	
4	372	Unknown	12.36	3.7	
5	385	Carvacrol methyl ether	12.8	9.7	MS
6	395	α-Terpinene	13.13	7.4	MS
7	1004	Terpinene-4-ol	33.43	32.7	MS
8	1049	Carvacrol	34.93	3.7	KI,MS
9	1136	Thymol	37.83	18.1	KI,MS
10	1162	Carvacrol	38.7	5.3	KI,MS
11	1168	Carvacrol	38.9	5.6	KI,MS
12	1208	Carvacrol	40.23	4.6	KI,MS
13	1237	Carvacrol	41.2	1.9	KI,MS
			Total	100%	

KI = Kovates index; MS =Camparison of mass spectra

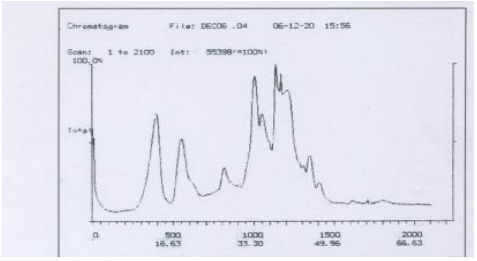

Figure 1. GC-MS chromatogram of thyme oil

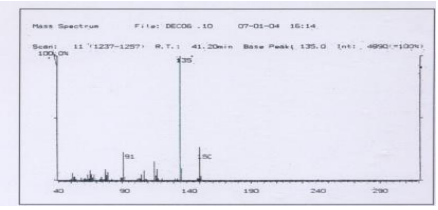

Figure 2. Mass spectrum of carvacrol

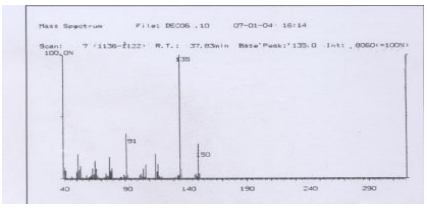

Figure 3. Mass spectrum of thymol

Similar secondary metabolites were also reported by Sara Burt 2007 [5]. The antimicrobial properties of thyme are due to its main components: Thymol, carvacrol [6]. Besides, there are several chemo types for thyme, such as: Linalool, α-Terpineol, Thymol, Carvacrol-cymene, Terpinene-4-ol, and 1, 8-cineole, most of them are reported to show varying degree of antimicrobial activity.

B. Assessment of Inhibition of Bacterial Growth

The antibacterial activity of Thyme oil against six uropathogens which were considered in this study was assessed by evaluating the ZOI and MIC values. Results (Table II and Table III) showed that thyme oil have great potential of antibacterial activity against all the tested uropathogens except *P.aeruginosa.* The ZOI and MIC for bacterial strains which were sensitive to the thyme oil were in the range of 14-23 mm and 0.04 - 0.25% (except *Pseudomonas spp.* which is resistant to the oil). Compared to positive antibacterial standards (Table IV) thyme oil and its components have a stronger antibacterial activity. These results are in concordance with the findings of Gustafson et al, 1998[7], Carson et al., 1995[8]. The MIC of thyme oil for E.coli is 0.08%, presented in Table III is in general agreement with previously reported studies on volatile oils [9],[10], [11], [12]. However, it was observed that thyme oil failed to inhibit the growth of *P. aeruginosa* [8]

TABLE II. ZONE OF INHIBITION RECORDED WITH THYME OIL AGAINST DIFFERENT UROPATHOGENS

Sr. No.	Uropathogen	Mean	SD	Min	Max	Remark
1	*E. coli*	19	±3	15	22	S
2	*S. aureus*	14	±4	10	20	S
3	*P. aeruginosa*	5	±1	4	8	R
4	*K. pneumoniae*	16	±4	10	23	S
5	*P. mirabilis*	18	±3	14	21	S
6	*P. vulgaris*	18	±3	15	22	S

S: Susceptible; **R**: Resistant

TABLE III. DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF THYME OIL AGAINST ROPATHOGENS

Uropathogens	MIC of thyme oil (0.5% v/v equivalent)
Proteus vulgaris	0.04%
Proteus mirabilis	0.04%
Staphylococci aureus	0.25%
Escherichia coli	0.08%
Klebsiella pneumonia	0.04%
Pseudomonas aeruginosa	Resistant

TABLE IV. ANTIBIOTIC RESISTANCE SHOWN BY DIFFERENT UROPATHOGENS

Sr. No.	Antibiotics	% of Resistant samples of *E. coli* (*N*=70)	% of Resistant samples of *P. mirabilis* (*N*=12)	% of Resistant samples of *P. vulgaris* (*N*=19)	% of Resistant samples of *P. aeruginosa* (*N*=35)	% of Resistant samples of *S. aureus* (*N*-=16)	% of Resistant samples of *K. pneumoniae* (N=30)
1	Ampicillin	85.7	75.0	78.9	80	87.5	63.33
2	Streptomycin	60.0	50.0	47.9	68.57	62.5	53.33
3	Chloramphenicol	42.8	33.3	52.6	57.1	56.2	46.66
4	Tetracycline	34.2	33.3	47.9	51.4	68.7	50
5	Tobramycin	31.0	41.6	42.3	57.1	56.2	40
6	Gentamycin	68.5	33.3	47.9	51.4	62.5	46.66
7	Kanamycin	72.8	41.6	42.3	48.5	68.7	36.66
8	Nitrofurantoin	28.0	33.3	36.8	42.8	25.0	30
9	Norfloxacin	28.0	25.0	26.0	28.5	31.2	23.33
10	Co-trimethprim	47.1	58.3	36.8	28.5	62.5	53.33
11	Nalidixic acid	31.0	41.6	42.3	40.0	50.0	36.6
12	Erythromycin	71.4	Nt	Nt	Nt	75.0	Nt
13	Colistin	42.8	Nt	Nt	68.5	56.2	Nt
14	Sulphamethoxazol	45.7	Nt	Nt	57.1	50.0	Nt
15	Cefuroxime	28.0	Nt	Nt	Nt	Nt	Nt
16	Cephatoxime	Nt	25.0	26.0	28.5	62.0	40

N- No. of samples, Nt – Not tested

It was reported by Nenad et al., 2007 [13] that the main advantage of natural agents is that thyme do not enhance the antibiotic resistance, a phenomenon commonly encountered with the long-term use of synthetic antibiotics. There are reports of the active principles of EO's from various plants with antibacterial

or antifungal activity. The antimicrobial activity of EO's is assigned to a number of small terpenoides and phenolic compounds which in pure form demonstrate high antibacterial activity [14]. Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity [15].

Several studies have focused on the antimicrobial activity of the essential oils of thyme in order to identify the responsible compounds [5],[16]. Essential oils comprise a large number of components and it is likely that their mode of action involves several targets in the cell. The hydrophobicity of essential oils enables them to partition in the lipids of the cell membrane, rendering them permeable and leading to leakage of cell contents. Physical conditions that improve the action of essential oils are low pH, low temperature and low oxygen levels. Synergism has been observed between carvacrol and its precursor *p*-cymene [5]. The essential oils and their components are known to be active against a wide variety of microorganisms, including Gram-negative and Gram-positive bacteria. Gram-negative bacteria were shown to be generally more resistant than Gram-positive ones to the antagonistic effects of essential oils because of the lipopolysaccharide present in the outer membrane, but this was not always true [17], as also is reported here.

IV. Conclusion

The data presented confirms the antibacterial potential of *T. vulgaris* essential oil. The essential oil tested represents an inexpensive source of natural antibacterial substances for use in pathogenic systems to prevent the growth of bacteria. The phytochemical prospections of the thyme oil have demonstrated the diverse class of compounds and these compounds are related to known biological activities (as antibacterials). The oil presented best activity against uropathogenic strains of *Proteus vulgaris, Proteus mirabilis, E.coli,* and *Klebsiella pneumoniae.*

Hence a paradigm shift in the treatment of infections and diseases is necessary to prevent antibiotics becoming obsolete, and where appropriate, alternatives to antibiotics ought to be considered.

Acknowledgement

We are grateful to the Director, Central Drug Research Laboratory, Lucknow (UP) for providing GC-MS facilities.

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