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Antibacterial Activities of *THYMUS VULGARIS* and Rifmpicin on Methicillin Resistant *STAPHYLOCOCCUS AUREUS* (MRSA)

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Abstract: Mixtures of thyme and rifampicin were tested for inhibitory activity against 45 *Staphylococcus aureus* isolates alone and in combination. The results indicate that MICs of thyme extract mixed with rifampicin for all tested strains of *S. aureus* ranged between 0.2g/ml of rifampicin plus 5 mg/ml of Thyme ethyl-acetate extract or Thyme methanol extract, and 16 g/ml of rifampicin plus 6 mg/ml of Thyme ethyl-acetate extract or Thyme methanol extract. Time-killing curve of rifampicin in combination with Thyme ethyl-acetate extract and with thyme methanol extract exhibit zero CFU after 8hrs, 10hrs respectively incubation period at 37°C that was compared with time-killing curve of rifampicin, time-killing curve of Thyme ethyl-acetate extract and Thyme Methanol extract

Keywords: : Methicillin Resistant *Staphylococcus aureus*, rifampicin, and thyme

1 Introduction

The genus *Thymus* comprises 215 species with *Thymus vulgaris* being one of the most important and thoroughly investigated aromatic plant. Chemistry, processing and application of *Thymus* species were previously investigated. *Thymus* species as well as many other aromatic plants biosynthesize remarkable amount of volatile compounds referred as the essential oil; therefore chemical classification of such plants is based on the main essential oil components. Chemical polymorphism is characteristic to the species of *Thymus*. Essential oils of thyme containing high amount of thymol and carvacrol were reported to possess the highest antioxidant activity [1]. The leaf of thyme (*Thymus vulgaris*) is a popular culinary herb that has a history of use in traditional herbal medicine, particularly in the treatment of cough and branchial catarrh. Both Germany's Commission E and a wide assortment of herbal texts support thyme's traditional use as an expectorant [4]. Thyme showed broad antibacterial activity by inhibiting the growth of both gram-positive and gram-negative bacteria. However, gram positive bacteria *Clostridium botulinum* and *Clostridium perfringens* appeared to be more sensitive than the gram-negative organisms [18].

In an *in vitro* antibacterial study, thyme showed greatest inhibition against *A. hydrophila* compare to other psycrotrophic food-borne bacteria such as *Aeromonas hydrophila*, *Listeria monocytogenes* and *Yersinia enterocolitica*. Inhibition of growth was tested by using the paper disc agar diffusion method, while the MIC was determined by the broth microdilution method [8].
Rifampicin is a semi synthetic derivative of rifamycin antibiotics which are produced by the fermentation of a strain of *Streptomyces medterranei* a species which was first isolated in Italy in 1957 from a soil sample collected in France. The fermentation produces Rifamycin B. Rifampicin is a semisynthetic derivative of rifamycin B, one of five rifamycins isolated from *Streptomyces mediterranei* [17].

2 Material and Methods

2.1 Bacterial strains

Forty five strains of MRSA (Coagulas positive) isolated from clinical specimen, sputum, septic wound, urine and blood samples, were obtained from faculty of medicine, Assiut University, Egypt. The bacterial strains were

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maintained on agar slants. Each culture was activated by transferring a loopful from brain heart infusion BHI slants into nutrient broth (10 ml) followed by incubation at 37°C for 24 and subcultures were freshly prepared before use. Bacterial inocula were made in 5 ml of BHI media and grown for 24 hrs at 37°C.

2.2 Rifampicin

Rifampicin (3-4-methylpiperazinyliminomethyl rifamycin sv), Approx 95% (HPC) (13292-46-1) Ec no 236-312-0, Sigma-Aldrich Co. p.o Box.

2.3 Determination of minimal inhibitory concentration (MIC)

The MICs of rifampicins on MRSA were determined using an agar dilution method [16]. Petri plates of Brain Heart Infusion (BHI) agar containing various concentrations of rifampicin were inoculated with 24 hrs culture of bacterial strain in triplicates [12], was individually spread on the surface of the solid wed agar plates. Test and control plates were then incubated at 35°C. Plates were evaluated for the presence or absence of colonies after 24 hrs of incubation period. For each treatment, the absence of colonies on all tested plates was considered as an inhibitory effect. The lowest concentration of rifampicin required to completely inhibit the growth of the tested microorganism was designated as the MIC.

2.4 Plant samples

The plant samples were collected from local markets at Assiut governorate, Egypt. The Thyme cultivated in Egypt.

2.5 Preparation of extracts

The air-dried plant materials were ground into fine powder in grinder and extracted with hexane, ethyl-acetate, methyl alcohol 80% and distilled water respectively by maceration. A 100g sample of each ground plant was soaked in 500 ml solvent ($3 * 48h$) [5]. The extract was filtered through a Buchner funnel with Whatman filter paper number1, after filtration of total extracts; the extracts were evaporated under reducing pressure to dryness at 45°C on a rota-evaporator (Bchi R114), all extracts were soluble in dimethylformaamid (DMF) with exception of the aqueous extracts dissolved in distilled water.

2.6 Antibacterial assay

An agar dilution method [16], used to determine the antibacterial activity and minimal inhibitory concentration (MIC) for all different extracts of all test medicinal plants.
Serial concentrations of all test plants extracts were achieved (% v/v) in plates containing BHI agar medium, as follows: 0.02, 0.04, 0.08, 0.2, 2.5, 0. 4, 0.8, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,12 and13 mg/ml.
Petri plates of BHI agar containing various concentrations of extracts were inoculated with 24 h culture of bacterial strain in triplicates were individually spread on the surface of the solid wed agar plates.
Each antibacterial test also included plates containing the culture medium plus solvent (DMF) in order to obtain a control of the solvent antimicrobial effect. After inoculation procedures, using triplicates, test and control plates were then incubated at 35°C. Plates were evaluated for the presence or absence of visible growth of each strain on the agar plate after 24 hrs of incubation. The absence of colonies on all plates tested was considered as an inhibitory effect. The lowest concentration of extracts required to completely inhibit the growth of the tested microorganism was designated as the MIC.

2.7 Synergism assays

In vitro synergism assays of rifampicin in combination with different extracts of all tested medicinal plants were carried out after evaluating the MIC of all test different extracts on BHI agar medium, by agar dilution method.

2.8 Killing curve

2.8.1 Assessment of killing curve [10]

Selected strains of MRSA isolates were grown overnight in BHI broth medium. 0.2 ml of inoculums was added to 20 ml Nutrient Broth test tubes containing antibacterial agent MIC for rifampicin, for natural extracts, and (1/2MIC of natural extract plus 1/2MIC of rifampicin) for combination. Test tubes were then incubated at 35°C without shaking. After 2 hrs the flasks were strongly agitated and a 0.1ml sample was diluted and immediately plated; the test tubes were immediately returned to incubation.

3 Results

3.1 Determination of antibacterial activities of Rifampicin μg/ml against 45 isolates of MRSA isolates.

According to the present results MIC for two strains of MRSA exhibit 0.4 μg/ml, whereas MIC_{2s} was 0.8 μg/ml,

MIC_{4s} was 2 µg/ml, MIC_{3s} was 4 µg/ml, MIC_{9s} was 8 µg/ml, MIC_{22s} was 16 µg/ml and MIC_{3s} was 32 µg/ml of rifampicin. Mean of time-growth rate of MRSA exhibit 4.9 $\log_{10}$ CFU/ 0.1 ml of nutrient broth medium after 24 hrs of incubation period at 37°C, time-killing curve of selected strains studied with rifampicin exhibit 2.8 $\log_{10}$ CFU/0.1ml at 24 hrs of incubation period while Time-growth curve of MRSA exhibit 4.8 $\log_{10}$ at 24 hrs of incubation period.

3.2 Determination of antibacterial activities of Thymus vulgaris (thyme) extracts on methicillin-resistant Staphylococcus aureus (MRSA).

The minimal inhibitory concentrations (MIC) of thyme ethyl-acetate extract (TEAE) mg/ml on MRSA ranged between 10 mg/ml and 12 mg/ml and MIC of thyme methanol extract (TME) mg/ml on MRSA ranged between 10 mg/ml and 12 mg/ml. Aqueous and hexane extract of leaves of thyme hasn't antibacterial activities against all tested MRSA strains. Aqueous and hexane extract of leaves of thyme hasn't antibacterial activities against all tested MRSA strains.
Time-killing curve of selected strains of MRSA affected with TEAE exhibit zero CFU after 8 hrs of incubation period at 37°C and for TME exhibit zero CFU after 12 hrs of incubation periods at 37°C.

3.3 Determination of antibacterial activities of Rifampicin plus Thyme extracts on methicillin-resistant Staphylococcus aureus (MRSA).

According to these results minimal inhibitory concentration (MIC) of rifampicin µg/ml plus thyme ethyl-acetate extract (TEAE) mg/ml on MRSA ranged between 0.2 µg/ml of rifampicin plus 5 mg/ml of TEAE and 16 µg/ml of rifampicin plus 6 mg/ml of TEAE and MIC of rifampicin µg/ml plus thyme methanol extract (TME) mg/ml on MRSA ranged between 0.2 g/ml of rifampicin plus 5 mg/ml of TME and 16 µg/ml of rifampicin plus 6 mg/ml of TME.
Ethyl-acetate extract and methanol extract of thyme produce synergetic effect with rifampicin on all tested MRSA strains. Moreover, MIC of rifampicin was decreased for half.
The time-killing curve of selected strains of MRSA affected with combination of TEAE plus rifampicin exhibit zero CFU after 8 hrs of incubation period at 37°C and for combination of TME plus rifampicin on MRSA exhibit zero CFU after 12 hrs of incubation period at 37°C.
There is no synergy observed in time-killing curve of selected strains of MRSA affected by combination or TEAE or TME alone.

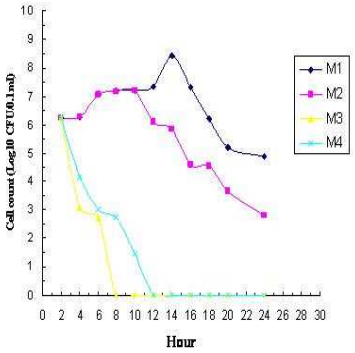

Fig. 1: Time-killing curve ($\log_{10}$ CFU/0.1ml) of MRSA affected with different ethyl-acetate and methanol extract of *Thymus vulgaris* (thyme) mg/ml.

M1= mean population ($\log_{10}$ CFU/0.1ml) of MRSA without any antibacterial agent (control).
M2= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with rifampicin.
M3= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with thyme ethyl-acetate extract (TEAE).
M4= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with thyme methanol extraction (TME).
** = Highly significant at 0.1 level of probability.
L.S.D. = Least significant difference.

4 Discussion

Methicillin-Resistant *Staphylococcus aureus* (MRSA) it's initial isolation in the UK in 1961 and has continued to be a major pathogen causing infections in hospitals and in the community and are increasingly isolate in hospitals worldwide [19].
Studies the Clinical uses and control of Rifampicin and Clindamycin in they examined 243 selected strains, 34 sensitive to Rifampicin, Clindamycin, lincomycin, and Erythromycin, 43 resistant to penicillin only, 29 resistant to penicillin, streptomycin, and tetracycline, and 137 resistant to erythromycin (that is all the erythromycin-resistant staphylococci isolated in this laboratory in the past five years). Among the more resistant strains, 25 were also resistant to Methicillin. Rifampicin is the most active drug: all strains are inhibited by 0.03µg/ml or less, except two resistant strains with MICs of 8 µg/ml and 32 µg/mI [15].
Frequencies at which mutants resistant to rifampicin arose in vitro were determined in *Staphylococcus aureus* strains including methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), rifampicin (16

Table 1: minimal inhibitory concentration (MIC) of rifampicin on MRSA.

Rifampicin	Concentration						
	0.4µg/ml	0.8 µg/ml	2 µg/m	4µg/ml	8 µg/ml	16 µg/m	32 µg/ml
No. of susceptible strain	2 MIC_{2s}	4 MIC_{2s}	8 MIC_{4s}	11 MIC_{3s}	20 MIC_{9s}	42 MIC_{22s}	45 MIC_{3s}
% No. 0f susceptible strain	4.4%	8.8%	17.7%	24.4%	44.4%	93.4%	100%

Table 2: Determination of minimal inhibitory concentration (MIC) of Thyme extracts on MRSA.

Thyme methanol extract (TME)	Concentration	
	10 mg/ml	12 mg/ml
No. of susceptible strain	10 MIC_{10s}	35 MIC_{35s}
(%) susceptible strain	22.2%	100%
Thyme ethyl-acetate extract (TEAE)	10 mg/ml	12 mg/ml
No. of susceptible strain	9 MIC_{9s}	45 MIC_{36s}
(%) susceptible strain	20%	100%

MIC = Minimal inhibitory concentration.
MIC_{ns} = Number of strains inhibited with minimal inhibitory concentration.

Table 3: Determination of minimal inhibitory concentration of rifampicin µg/ml in combination with thyme ethyl-acetate extract (TEAE) mg/ml and/or thyme methanol extract (TME) mg/ml on MRSA.

rifampicin Plus (TEAE)	**Minimal inhibitory concentration (MIC)**						
	0.2 µg/ml 5 mg/ml	0.4 µg/ml 5 mg/ml	1 µg/ml 5 mg/ml	2 µg/ml 5 mg/ml	4 µg/ml 5 mg/ml	8 µg/ml 5 mg/ml	16 µg/ml 6 mg/ml
No. of susceptible strains inhibited with MIC	2	2	4	3	9	22	3
rifampicin Plus(TME)	0.2 µg/ml5 mg/ml	0.4 µg/ml 5 mg/ml	1 µg/ml 5 mg/ml	2 µg/ml 5 mg/ml	4 µg/ml 5 mg/ml	8 µg/ml 5 mg/ml	16 µg/ml 6 mg/ml
No. of susceptible strains inhibited with MIC	2	2	4	3	9	22	3
(%) of susceptible strains inhibited with MIC	4.4%	4.4%	8.8%	6.6%	20%	48.8 %	6.6%

Table 4: Time-killing rate (log_{10}CFU/0.1ml) of MRSA affected with different extracts of *Thymus vulgaris* (thyme) mg/ml.

Hour	**M1**	**M2**	**M3**	**M4**	**F-test**	**L.S.D.**
2	6.22	6.22	4.26	4.34	**	0.027
4	6.27	6.27	3.08	4.15	**	0.022
6	7.06	7.06	2.73	3.01	**	0.040
8	7.22	7.16	0.00	2.74	**	0.89
10	7.22	7.21	0.00	1.46	**	0.157
12	7.33	6.10	0.00	0.00	**	0.049
14	8.44	5.84	0.00	0.00	**	0.051
16	7.32	4.58	0.00	0.00	**	0.116
18	6.20	4.55	0.00	0.00	**	0.54
20	5.19	3.67	0.00	0.00	**	0. 153
24	4.88	2.81	0.00	0.00	**	0.063

M1= mean population (log_{10} CFU/0.1ml) of MRSA without any antibacterial agent (control).
M2= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with rifampicin.
M3= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with thyme ethyl-acetate extract (TEAE).
M4= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with thyme methanol extraction (TME).
** = Highly significant at 0.1 level of probability.
L.S.D. = Least significant difference.

Table 5: Time-killing rate (Log10 CFU/0.1ml) of MRSA affected with rifampicin µg/ml in combination with different extracts of *Thymus vulgaris* (thyme) mg/ml.

Hour	**M1**	**M2**	**M5**	**M6**	**F-test**	**L.S.D.**
2	6.22	6.22	6.22	6.22	**	0.046
4	6.27	6.27	3.02	4.15	**	0.051
6	7.06	7.06	2.52	2.99	**	0.075
8	7.22	7.16	0.00	2.75	**	0.074
10	7.22	7.21	0.00	2.40	**	0.194
12	7.33	6.10	0.00	0.00	**	0.049
14	8.44	5.84	0.00	0.00	**	0.051
16	7.32	4.58	0.00	0.00	**	0.116
18	6.20	4.55	0.00	0.00	**	0.54
20	5.19	3.67	0.00	0.00	**	0. 153
24	4.88	2.81	0.00	0.00	**	0.063

M1= mean population (log_{10} CFU/0.1ml) of MRSA without any antibacterial agent (control).
M2= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with rifampicin.
M5= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with combination of rifampicin plus TEAE.
M6= mean population (log_{10} CFU/0.1ml) of MRSA inhibited with combination of rifampicin plus TME.

** = Highly significant at 0.1 level of probability.
L.S.D. = Least significant difference.

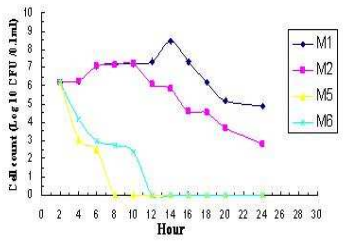

Fig. 2: Time-killing curve ($\log_{10}$ CFU/0.1ml) of MRSA affected with with rifampicin in combination with ethyl-acetate and methanol extract of *Thymus vulgaris* (thyme) mg/ml.

M1= mean population ($\log_{10}$ CFU/0.1ml) of MRSA without any antibacterial agent (control).
M2= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with rifampicin.
M5= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with combination of rifampicin plus TEAE.
M6= mean population ($\log_{10}$ CFU/0.1ml) of MRSA inhibited with combination of rifampicin plus TME.
** = Highly significant at 0.1 level of probability.
L.S.D. = Least significant difference.

and 1 mg/L) used for selection were equal to the expected maximum and minimum serum concentrations rifampicin [2].

The present results were almost agreed to some extent with the finding reported by [2, 15].

Carvacrol, a compound present in the essential oil fraction of oreganum and thyme showed a dose-related inhibition of growth of the pathogen *Bacillus cereus* [20].

The alcohol and ethanol extracts of thyme, thyme essential oil, thymol and carvacrol were found to have strong inhibition activity against *Bacillus subtilis, S. sonnei, E. coli* [9].

Thyme oil was tested for its antibacterial activity against *Campylobacter jejuni (C. jejuni), E. coli* O157:H7, *Listeria monocytogenes*, and *S. enterica* obtained from food and clinical sources and was found most effective against *E. coli, L monocytogenes S. enterica,and C. jejuni* [11].

Non-volatile antioxidants, such as flavonoids and vitamin E were also found in the extracts of *T. vulgaris* [6].

Water extract of leaves of thyme hasn't antibacterial activities against all test strains. This result is different with some authors [3, 7].

The water, ethanolic and methanol extractions of thyme showed various inhibitory effects on MRSA [3].

The different concentrations of aqueous extract of leaves of thyme collected from flora of Palestine, in general, had inhibitory action on *E. coli, Klebsiella pneumoniae, Enterobacter cloacae, Acinetobacter haemolyticus and Staphylococcus aureus*, but no effect on *Candida albicans, Proteus mirabilis and Salmonella typhi* [7].

Essential oils of *Thymus vulgaris* ; thymol 39.7%, p-cymene 30.0%, limonene 1.7%; 31.5% thymol exhibited the highest and broadest antibacterial activity against 14 strains of *staphylococcus aureus* (mithicillin-resistant) and 28 *staphylococcus coagulase negative* (S.C.N.) [13].

The studies the antibacterial activity of an essential oil Trans-thujanol of thyme which obtained from france against *Staphylococcus aureus* showed that, the MIC of Trans-thujanol were 0.4% (V /V)[14].

Aqueous and hexane extract of leaves of thyme hasn't antibacterial activities against all tested MRSA strains. Ethyl-acetate extract and methanol extract of thyme produce synergetic effect with rifampicin on all tested MRSA strains. Moreover, MIC of rifampicin was decreased for half. No synergetic effect was observed when different concentrations of ethanol extracts from lemon balm, clove and eugenol were combined with ampicillin to inhibit the growth of *K. pneumoniae* and *E. aerogenes*. Only the association of thyme (20 μg/mL) with ampicillin was able to cause such an effect [12].

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