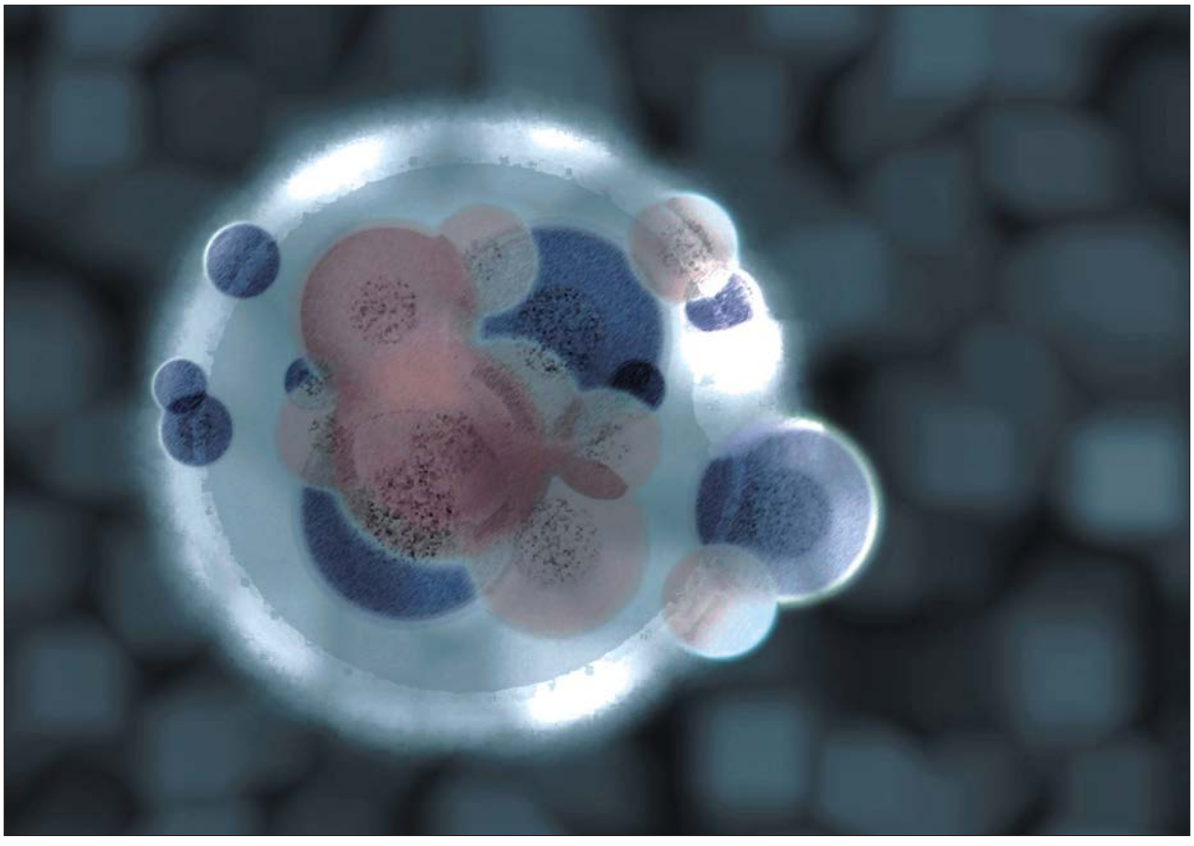

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Synergic Interactions Between Selected Botanical Extracts and Tetracycline Against Gram Positive and Gram Negative Bacteria

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Abstract: The potential use of plants as antimicrobial agents in medicine has not been fully exploited. The present study was carried out to investigate the synergic interaction of dichloromethane/methanol extracts from *Punica granatum* (PG), *Thymus vulgaris* (TV), *Commiphora molmol* (CM) and *Achillea fragrantissima* (AF) with tetracycline against Gram positive (*Staphylococcus aureus* ATCC 25923 and *Bacillus megaterium* ATCC 14591) and Gram negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumonia* ATCC 700603) bacterial strains. All extracts had a significant antibacterial activity against all tested bacteria within concentrations range of (0.1-50 mg/disk). The lowest Minimum Inhibitory Concentration (MIC) recorded was 1 mg mL^{-1}. The recorded MICs values of PG and TV against Gram negative bacteria were higher than those against Gram positive strains. Combination of PG, TV, or CM with tetracycline achieved synergetic interaction against tested bacteria as indicated from fractional inhibitory concentration and confirmed by time kill assays. CM, PG and TV significantly increased the Post Antibiotic Effect (PAE) of tetracycline from 1 h to 3, 4 and 5 h, respectively. The results suggested the use of these plants in extending the lifetime of fading antibiotics.

Key words: Minimum inhibitory concentration, synergy, fractional inhibitory concentration, Post-antibiotic effect

INTRODUCTION

The failure of antibiotics therapy for many pathogens has revived the search for antimicrobial compounds from natural sources. Plant extracts have played an important role in the potential discovery of new antimicrobial agents for treatment of infectious diseases (Darwish *et al*., 2002; Konning *et al*., 2004; Karim *et al*., 2011; Sohail *et al*., 2011). Hundreds of plants have been used worldwide in traditional medicine for controlling bacterial infections (Martin and Ernst, 2003). Several studies have proposed that natural compounds in combination with antibiotics could offer a new strategy for developing therapies against bacterial infections through potentiating the activity of antibiotics (Braga *et al*., 2005).

Pomegranate (*Punica granatum*), myrrh (*Commiphora molmo*), thyme (*Thymus vulgaris*) and gaysum (*Achillea* sp.) are used in folk medicine for decades. Fruits, peels and roots of pomegranate have been commonly used in herbal remedies in many countries. The antimicrobial activity of peels has been demonstrated against pathogenic bacteria including *S. aureus*, *Salmonella paratyphi*, *Shigella dysenteriae*, *E. coli*, *P. aeroginosa*, *K. pneumonia*, *Streptococcus pneumonia* and *Listeria monocytogenes* and *Bacillus subtilis* (Braga *et al*., 2005; Reddy *et al*., 2007; Al-Zoreky, 2009; Hayouni *et al*., 2011). In addition, *P. granatum* was effective at inhibiting the growth of multiresistant strains of *S. aureus* which acquired resistance to all conventional antibiotics among hospitalized patients (Machado *et al*., 2003; Melendez and Capriles, 2006). *C. molmol* is used as an antiseptic and anti-inflammatory for the topical treatment of mouth and throat infections (Kumari *et al*., 2011). Different extracts of *Commiphora* sp. showed antimicrobial potency against Gram negative and Gram positive bacteria (Suleiman *et al*., 2010). Also, the essential oils of the plant exerted antimicrobial activity especially against Gram positive bacteria (Mothana *et al*., 2010). *T. vulgaris* is a well known medicinal plant frequently used as a tea or as an additive in commercial spice mixtures of many foods for aroma and flavor (Sokovic *et al*., 2010). There are numerous reports on

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antibacterial activities of the essential oils from various *Thymus* species against human and food pathogenic bacteria (Couladis *et al*., 2004; Kotan *et al*., 2010). The antibacterial activities of the essential oils of some *Achillea* species against various bacteria have been previously reported (Kotan *et al*., 2010). In addition, the methanolic extract of some species of *Achillea* plant enhanced the inhibitory effects of different antibiotics including, chloramphenicol, neomycin, doxycycline, cephalexin and nalidixic acid against the growth of sensitive and resistant strains of *E. coli* (Darwish and Aburjai, 2010).

The present study aimed to evaluate the antimicrobial effect of dichloromethane / methanol extract of *Punica granatum*, *Thymus vulgaris*, *Commiphora molmol* and *Achillea fragrantissima* against Gram positive and Gram negative bacteria. The Minimum Inhibitory Concentration (MIC) of each plant extract was determined. To examine the potentiating activity of these botanical extracts on the effect of antibiotic to combat bacterial infections, synergistic interaction between the examined plant extracts and tetracycline was carried out and the Fractional Inhibitory Concentration (FIC) was calculated. The results of synergistic interaction assays were confirmed by growth kill assays. The effect of plant extracts on the Post Antibiotic Effect (PAE) of tetracycline was assessed. Post Antibiotic Effect (PAE) is the term used to describe suppression of bacterial growth that persists after brief exposure of organisms to antimicrobials. The increased PAE could offer an alternative way for the extension of the useful lifetime of antibiotics (Spangler *et al*., 1998).

MATERIALS AND METHODS

Chemicals: All reagents were purchased from Sigma (St. Louis, MO) except where indicated in the specified methods.

Plant materials: The oleo-resin of *C. molmol*, leaves of *T. vulgaris*, barks of *A. fragrantissima* and peel of *P. granatum* were purchased from a local market (Alhasa, KSA) and identified with the help of a specialized taxonomist.

Extraction of the plant material: Extraction of the plants was performed using continuous technique in which the specific parts of the plants were dried and milled. The powder of each plant was extracted with dichloromethane: methanol (1:1 v/v) at room temperature for 3 days and filtered. The solvent was then evaporated under vacuum.

Bacterial strains: The antibacterial assay was performed against standard strains of Gram positive (*S. aureus* ATCC 25923 and *B. megaterium* ATCC 14591) and Gram negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *K. pneumoniae* ATCC 700603) bacterial strains. All strains were purchased from ATCC (Manassas, VA). The strains were cultured aerobically overnight on nutrient agar (Oxoid, UK) at 37°C and then frozen at -80°C in 30% glycerol solution (Pro-labs, UK) until used. In all assays, inocula were prepared using overnight cultures on nutrient agar that were then suspended in nutrient broth to a turbidity equivalent to 0.5 McFarland containing 1.5×10^8 cell forming unit mL^{-1} (CFU mL^{-1}).

Antimicrobial activity of plant extracts by disk diffusion method Overnight growing cultures were adjusted to match 0.5 McFarland standard and then diluted 1:10 using Mueller-Hinton broth (Difco Laboratories, Detroit, MI) and used for inoculation of agar media. The seeded agar media were immediately mixed and poured in Petri plates and allowed to solidify. Filter paper disks (Whatman No.1, 6 mm in diameter) were loaded with different concentrations (0.1, 0.5, 1, 10, 50 mg/disk) of different plant extracts. The loaded disks were placed over agar surface and left in refrigerator for 2-3 h to allow diffusion of the extracts. All plates were inverted and incubated at 37 °C for 24 h. Following incubation, the mean diameter of inhibition zones around the disks was measured to the nearest mm. All experiments were carried out in triplicates.

Minimum Inhibitory Concentration (MIC) of the tested botanical extracts: The minimum inhibitory concentration of plant extracts was determined by broth microdilution method according to National Committee for Clinical Laboratory Standards (1999). All strains were cultured on Muller Hinton agar (Oxoid, UK) and incubated at 37°C for 24 h prior to MIC determination. An inoculum density of 10^5 CFU mL^{-1} of each of the test organisms was prepared in Muller Hinton Broth (MHB). MHB was dispensed into wells of a 96- well microtitre plate. A stock solution of each plant extract was prepared and diluted in DMSO. A DMSO control was included in all assays. Plant extracts were serially diluted in the wells followed by the addition of the bacterial inocula. Control samples were performed simultaneously by growing different bacterial strains without the plant extracts. The microtitre plates were incubated at 37°C for 18 h. The plates were subjected to reading at 600 nm using plate reader. The MIC value was recorded as the lowest concentration at which no growth was observed. All MIC values were determined on three independent experiments.

Synergic interaction between plant extracts and tetracycline against tested bacterial strains: To estimate the effect of the tested botanical extracts in combination with antibiotics on the growth of used bacterial strains, increasing concentrations of tetracycline (0.5 to 128 μg mL^{-1}) were prepared. The prepared concentrations of tetracycline were added to MHB inoculated with 10^5 CFU mL^{-1} of each test strain. The botanical extracts were prepared at 0.25×MIC and added to each plate. The assay was carried out for tetracycline alone and each plant extract as control. The plates were incubated at 37°C for 24 h, the lowest antibiotic concentration in combination with plant extract that prevented the development of turbidity was regarded as the MIC*.

Determination of the fractional inhibitory concentration (FIC): The activity of antibiotic and each plant extract in combination was determined by calculating the Fractional Inhibitory Concentration (FIC) index as described by Mackay *et al*. (2000) as follows: FIC of drug A = (MIC* drug A+Plant extract) / MIC drug A alone, FIC ≤0.5 indicating synergy, FIC>0.5 to 4 means indifference and FIC>4 indicating antagonism.

Effect of selected botanical extracts and tetracycline on the growth of *Escherichia coli* ATCC 25922: Growth curves for *E. coli* ATCC 25922 were performed in MHB in the presence of either plant extract (0.25×MIC), tetracycline (0.5×MIC), or both. A standard inoculum of approximately 10^5 CFU mL^{-1} was used and then the tubes were incubated at 37°C on a shaking-platform incubator for 24 h. At various time intervals (0, 4, 8, 12, 16 and 24 h) during the incubation period, samples were subjected to a 10-fold serial dilutions and 0.1 mL aliquots of these dilutions were spread onto nutrient agar and incubated at 37°C for 24 h for viable cell count or CFU determination.

Post antibiotic effect (PAE): *E. coli* ATCC 25922 strain was grown overnight at 37°C and then diluted with the MHB and further incubated at 37°C for 2-3 h to enter the logarithmic growth phase. The actively growing culture was subsequently diluted with MHB to achieve turbidity matching that of a 0.5 McFarland standard. The inocula equivalent to 10^4 CFU mL^{-1} were incubated at 37°C for 30 min with or without tetracycline (1×MIC), the plant extract alone (0.25×MIC), or a combination of antibiotic and the plant extract. After 30 min, bacteria were diluted 1000 fold with MHB to eradicate the antibiotic and/or plant extracts. Control experiments were performed simultaneously for cells unexposed to either antibiotic or plant extracts. A 100 μL of each treatment was obtained at time 0 and every hour thereafter until turbidity developed and spread onto nutrient agar medium for total viable cell count determination. The number of CFU mL^{-1} was determined after incubation at 37°C for 24 h. PAE was calculated as described by Craig and Gudmundsson (1996): PAE = T-C, where T is the time required for the viable cell counts of the exposed bacteria to increase by a factor of $\log_{10}$ from the initial time immediately after washing and C is the corresponding time for cells unexposed to either antibiotic or plant extract. Each assay was carried out in triplicates and results were expressed as the means of PAE values. To avoid the overgrowth of control culture, a 100-fold dilution was prepared using MHB at the same time points as scheduled for antibiotic exposures during the study.

RESULTS

Antimicrobial activity of plant extracts by disk diffusion method: In the present study, the antimicrobial activity of dichloromethane/methanol extracts from PG, TV, CM and AF was tested against Gram positive and Gram negative bacterial strains (Table 1). The results showed that PG extract exhibited a potent antibacterial activity against all tested strains within the used range of concentrations (0.5-50 mg/disk), producing 10-32 mm inhibition zone in a dose-dependent manner. TV extract inhibited the growth of *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at concentrations of 10 and 50 mg/disk. In addition, it also inhibited the growth of *B. megaterium* ATCC 14591 and *P. aeruginosa* ATCC 27853 at concentrations ranging from 1-50 mg/disk but had no inhibitory effect on the growth of *K. pneumonia* ATCC 700603 at any of the concentrations investigated. CM and AF extracts had no effect on *E. coli* ATCC 25922 and were effective against *S. aureus* ATCC 25923 at 10-50 and 50 mg/disk, respectively. Similarly, the inhibition in the growth of *B. megaterium* ATCC 14591 was achieved at concentrations of 10-50 and 50 mg/disk of CM and AF, respectively. CM had better efficiency against the growth of *P. aeruginosa* ATCC 27853 and *K. pneumonia* ATCC 700603 at low extract concentrations (0.5-50 mg/disk). AF was unique among all extracts under investigation in inhibiting the growth of *P. aeruginosa* ATCC 27853 at 0.1 mg/disk. It also inhibited the growth of *K. pneumonia* ATCC 700603 at the highest two concentrations studied; 10 and 50 mg/disk (Table 1). AF was also unique being the extract with the least antimicrobial potency.

Minimum Inhibitory Concentration (MIC): All botanical extracts were assessed for MIC determination using broth microdilution method using Muller Hinton broth medium. The MIC values of the tested extracts varied with the

Table 1: Antimicrobial activity of tested plant extracts by disk-diffusion method.

Treatment (mg/ Disk)	Mean diameter of inhibition zone (mm)				
	Staphylococcus aureus ATCC 25923	*Bacillus megaterium* ATCC 14591	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumonia* ATCC 700603	*Escherichia coli* ATCC 25922
PG*					
50	22	26	27	32	23
10	18	20	21	29	21
1.0	12	14	12	17	13
0.5	10	-	10	11	10
0.1	-	-	-	-	-
TV*					
50	19	32	18	-	16
10	9	22	13	-	9
1.0	-	10	9	-	-
0.5	-	-	-	-	-
0.1	-	-	-	-	-
CM*					
50	14	16	25	26	-
10	9	9	17	21	-
1.0	-	-	13	16	-
0.5	-	-	11	12	-
0.1	-	-	-	-	-
AF*					
50	12	13	28	14	-
10	-	-	21	10	-
1.0	-	-	18		-
0.5	-	-	14	-	-
0.1	-	-	13	-	-

*PG: *Punica granatum*, TV: *Thymus vulgaris,* CM: *Commiphora molmol,* AF: *Achillea fragrantissima* –; no inhibition. The data are expressed as the means of three independent experiments

Table 2: Minimum inhibitory concentration (MIC) of the tested botanical extracts against different bacterial strains

Microorganism	MIC+ Plant extract (mg mL^{-1})			
	Punica granatum	*Thymus vulgaris*	*Commiphora molmol*	*Achillea fragrantissima*
Staphylococcus aureus	8	16	8	128
Bacillus megaterium	1	4	16	128
Escherichia coli	64	32	16	256
Pseudomonas aeruginosa	32	32	16	64
Klebsiella pneumonia	16	16	1	128

+The data are expressed as the means of three independent experiments

Table 3: Synergic interaction between the tested botanical extracts and tetracycline against the tested bacterial strains

Bacterial strains	Tetracycline ($\mu g\ mL^{-1}$)	PG+		TV+		CM+	
		MIC*	FIC	MIC*	FIC	MIC*	FIC
Staphylococcus aureus	8	4	0.5	4	0.5	0.5	0.063
Bacillus megaterium	4	0.5	0.125	0.25	0.063	1	0.25
Escherichia coli	8	0.5	0.063	1	0.125	8	1
Pseudomonas aeruginosa	8	32	4	1	0.125	2	0.25
Klebsiella pneumonia	16	8	0.5	0.5	0.031	2	0.125

+PG: *Punica granatum*, TV: *Thymus vulgaris,* CM: *Commiphora molmol* MIC: Minimum inhibitory concentration of tetracycline. MIC*: Minimum inhibitory concentration of tetracycline (0.5 X MIC) in combination of plant extract (0.25×MIC). FIC: Fractional inhibitory concentration (MIC*/ MIC of tetracycline). FIC≤0.5 indicating synergy, FIC>0.5 to 4 means indifference and FIC > 4 indicating antagonism. The data are expressed as the means of three independent experiments

bacterial strain tested, ranging from 1- 256 mg mL^{-1} (Table 2). PG was most effective against *B. megaterium* with MIC value of 1mg mL^{-1} and least effective against *E. coli* with MIC value of 64 mg mL^{-1}. The recorded MICs of PG against the growth of S. *aureus*, *K. pneumonia* and *P. aeruginosa* were 8, 16 and 32, respectively. CM was most effective against *K. pneumonia* and *S. aureus* with MIC values of 1 and 8 mg mL^{-1}, respectively. *E. coli*, *P. aeruginosa* and *B. megaterium* required higher concentration of CM to be inhibited and the recorded MIC was 16 mg mL^{-1}. TV exerted higher inhibitory activity against Gram positive bacteria with MIC values of 4 and 16 mg mL^{-1} against the growth of *B. megaterium* and *S. aureus*, respectively. The reported MIC values of TV for *K. pneumonia*, *E. coli* and *P. aeruginosa* were 16, 32 and 32, respectively. AF extract showed the least antibacterial activity with high MICs ranging from 64 to 256 mg mL^{-1} against the bacterial strains under investigation (Table 2). Overall, CM was the most effective extract with low MIC values against most bacterial strains.

Synergic interaction between botanical extracts and tetracycline: The interaction of PG, TV, or CM with tetracycline was investigated against the growth of the bacterial strains under investigation (Table 3). The reported MIC values for tetracycline were 4 $\mu g\ mL^{-1}$ for *B. megaterium,* 8 $\mu g\ mL^{-1}$ for *S. aureus, E. coli*

and *P. aeruginosa* and 16 μg mL^{-1} for *K. pneumonia*. The effect of combination of each plant extract at a concentration of 0.25×MIC with 0.5×MIC of tetracycline was examined. The lowest antibiotic concentration in combination with the plant extract that prevented the development of turbidity was regarded as the MIC* and the Fractional Inhibitory Concentration (FIC) index was calculated. Indifference (FIC = 4) was shown with PG extract against *P. aeruginosa* ATCC 27853. Combination of PG extract with tetracycline achieved synergistic activity against the other tested strains with FIC ranging from 0.063 to 0.5. Co-treatment of all tested bacterial strains with TV and tetracycline increased the antibacterial activity of tetracycline as indicated from FIC values (0.031-0.5). CM had no effect on the activity of tetracycline against *E. coli* (FIC = 1). However, it showed a significant synergy in inhibiting the growth of all other bacterial strains tested with FIC 0.063-0.25 (Table 3).

Effect of selected plant extracts and tetracycline on the growth of *Escherichia coli* ATCC 25922: For time-kill assays, growth curves for *E. coli* ATCC 25922 were performed in the presence of either plant extract (0.25×MIC), tetracycline (0.5×MIC), or both. The total viable cell count in relation to time was determined. Treatment of bacteria with either tetracycline, PG or TV were similar to the control in starting the exponential phase of growth after 4 h, while the combination of PG or TV and tetracycline extended the lag phase and prevented the growth for the following 24 h (Fig.1 and 2). Using CM extract with tetracycline (Fig. 3) had no significant effect on tetracycline activity which in agreement with the synergistic study.

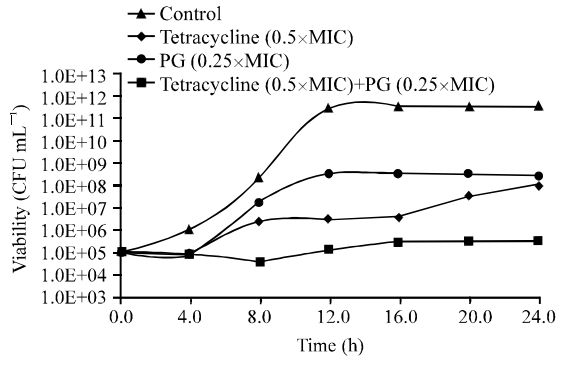

Fig. 1: Growth curves of *Escherichia coli* ATCC 25922 (control), in the presence of *Punica granatum* (PG) (0.25 MIC), tetracycline (0.5 MIC), or a combination of the plant extract (0.25 MIC) and tetracycline (0.5 MIC). Data are expressed as the mean of three independent assays

Post Antibiotic Effect (PAE): The post antibiotic effect induced by the investigated botanical extracts was carried out using *E. coli* ATCC 25922. The tested bacteria was exposed to tetracycline (1×MIC), each of the plant extract (0.25×MIC) and a combination of each plant extract with tetracycline for 30 min. Control experiments were performed simultaneously for cells unexposed to tetracycline or plant extracts. The change in total viable cell count over time under the exposure conditions was represented in (Fig. 4-6). The estimated PAEs for tetracycline, PG, TV, or CM were 1, 1, 3 and 0 h, respectively. The combination of tetracycline and PG, or TV resulted in a 4-fold increase in PAE (from 1 to 4 h) for PG and a 5-fold

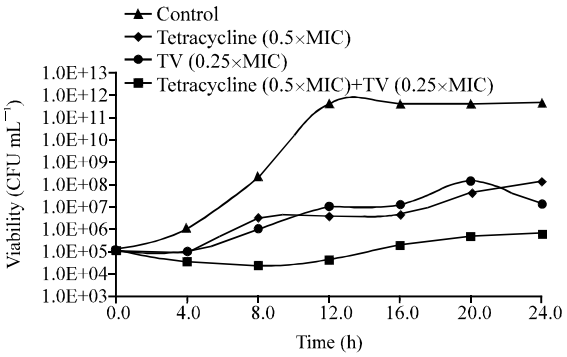

Fig. 2: Growth curves of *Escherichia coli* ATCC 25922 (control), in the presence of *Thymus vulgaris* (TV) (0.25 MIC), tetracycline (0.5 MIC), or a combination of the plant extract (0.25 MIC) and tetracycline (0.5 MIC). Data are expressed as the mean of three independent assays

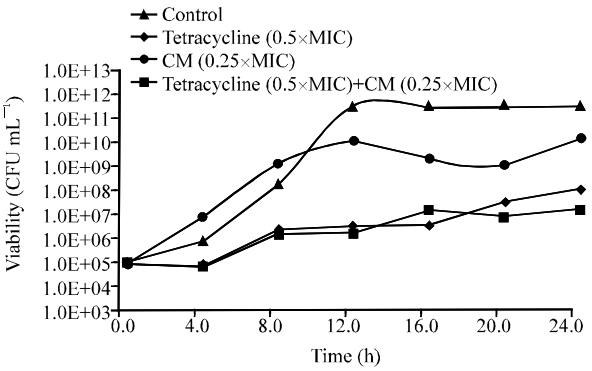

Fig. 3: Growth curves of *Escherichia coli* ATCC 25922 (control), in the presence of *Commiphora molmol* (CM) (0.25 MIC), tetracycline (0.5 MIC), or a combination of the plant extract (0.25 MIC)and tetracycline (0.5 MIC). Data are expressed as the mean of three independent assays

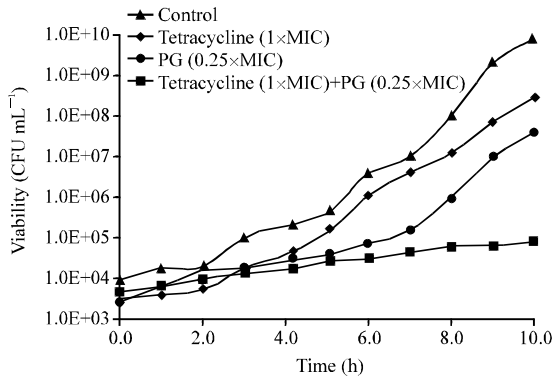

Fig. 4: The post-antibiotic effect (PAE) of *Punica granatum* (PG) (0.25 MIC), tetracycline (1 MIC), or a combination of the plant extract and tetracycline on *Escherichia coli* ATCC 25922. Control curve is representing the growth of bacteria unexposed to either the plant extract or tetracycline. Data are expressed as the mean of three independent assays

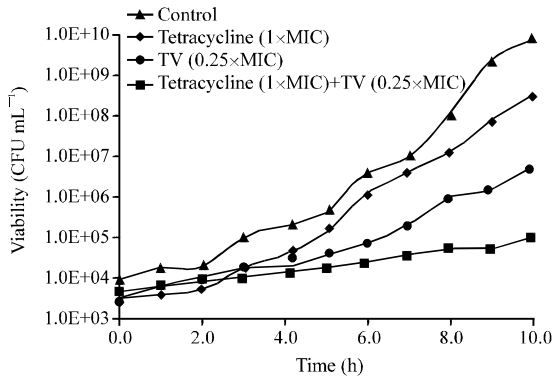

Fig. 5: The post-antibiotic effect (PAE) of *Thymus vulgaris* (TV) (0.25 MIC), tetracycline (1MIC), or a combination of the plant extract and tetracycline on *Escherichia coli* ATCC 25922. Control curve is representing the growth of bacteria unexposed to either the plant extract or tetracycline. Data are expressed as the mean of three independent assays

increase in PAE (from 1 to 5 h) for TV (Fig. 4 and 5). Although CM alone was not as effective as the other two plant extracts and showed no difference in PAE as compared to unexposed cells, the combination of CM extract with tetracycline resulted in a 3-fold increase in the PAE (Fig. 6).

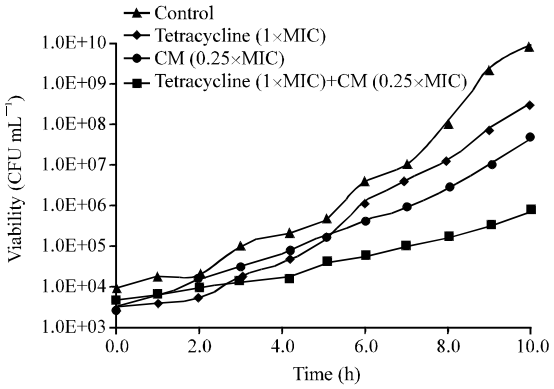

Fig. 6: The post-antibiotic effect (PAE) of *Commiphora molmol* (CM) (0.25 MIC), tetracycline (1MIC), or a combination of the plant extract and tetracycline on *Escherichia coli* ATCC 25922. Control curve is representing the growth of bacteria unexposed to either the plant extract or tetracycline. Data are expressed as the mean of three independent assays

DISCUSSION

The antimicrobial activities of dichloromethane/ methanol extracts of PG, TV, CM and AF were evaluated against Gram positive and Gram negative bacteria. The inhibitory activity of the tested extracts was initially determined by disk-diffusion method. Marked microbial inhibition was recorded with PG against all tested bacterial strains (Table 1) reflecting a broad spectrum activity of the plant extracts which in accordance with previous reports (Prashanth *et al*., 2001; Al-Zoreky, 2009). All tested strains were susceptible to TV extract except *K. pneumonia*. CM and AF were effective in inhibiting the growth of Gram positive bacteria (*B. megaterium* and *S. aureus*) but only at high concentrations 10 and 50 mg/disk. Also, CM and AF showed no activity against *Escherichia coli* (Table 1). In general *E. coli* was the least affected strain while *Pseudomonas aeruginosa* was affected by all extracts at most concentrations investigated. The antibacterial activity of CM may be attributed to the high terpenes content of the oleo-resin of the plant (Rahman *et al*., 2008).

The lowest concentration of the tested plant extract that prevented the growth of the tested bacteria was investigated using broth microdilution method and defined as MIC. The recorded MIC values of PG and TV against Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*) were higher than those for Gram positive strains (*Staphylococcus aureus* and *Bacillus megaterium)*

reflecting that tested Gram positive bacteria were more sensitive than Gram negative bacteria to both botanical extracts. This is in agreement with Holetz *et al*. (2002) who reported that PG showed significant activity against *S. aureus* and no activity against *E. coli* and *P. aeroginosa*. CM was the most effective botanical extract with high potency against the growth of all bacterial strains. The lowest MIC (1 mg mL^{-1}) was recorded with PG and CM extracts against *B. megaterium* ATCC 14591 and *K. pneumonia,* respectively (Table 2) while the highest MIC (64- 256 mg mL^{-1}) was recorded for AF with all tested bacterial strains. The low activity of different extracts from *Achillea* sp. was reported against *K. pneumonia, P. aeruginosa, S. aureus* and *E. coli* (Tuberoso *et al*., 2005; Konakchiev *et al*., 2011). The high values of MIC recorded with AF against the tested bacteria indicating low activity, suggested the elimination of this extract from further investigations.

The interaction between the tested botanical extracts and tetracycline against the tested bacterial strains were evaluated. Tetracycline has been used since 1940s as a broad-spectrum antibiotic in the treatment of a wide variety of bacterial infections including the infections of respiratory tract, sinuses, middle ear, urinary tract and intestines. It is also used in the treatment of gonorrhea, especially in patients allergic to β-lactams and macrolides (Cunha and Garabedian-Ruffalo, 1990). The general usefulness of this antibiotic has been reduced with the onset of bacterial resistance (Roberts, 2005). To investigate the effect of the botanical extracts on the potency of tetracycline in inhibiting the bacterial growth, the interaction between tetracycline and the botanical extracts was evaluated. Initially, the MIC values of tetracycline were determined against the tested bacteria. The susceptibility testing was performed using growth microdilution method. In the combination assays, the used concentration of each plant extract was 0.25×MIC since higher concentration significantly reduced the total viable count of the tested bacteria (data not shown). In a similar way, the used concentration of tetracycline was 0.5×MIC. The interaction between tetracycline and the investigated botanical extracts was determined by calculating the fractional inhibitory concentration (Table 3). Synergy activity was recorded between PG extract and tetracycline against the tested bacteria (FIC: 0.063 to 0.5) except for *P. aeruginosa* ATCC 27853 with FIC = 4. PG extract had a significant effect on improving the activity of tetracycline which was previously reported by Braga *et al*. (2005). Treatment of bacteria with TV and tetracycline increased the antibacterial activity of tetracycline as indicated from FIC values (FIC 0.031- 0.5). Fujita *et al*. (2005) demonstrated that crude extract of *Thymus* leaves significantly increased the activity of tetracycline against MRSA clinical isolates. CM had no effect on the activity of tetracycline against *E. coli* (FIC = 1). However, it showed a significant synergy in inhibiting the growth of all other bacterial strains (FIC: 0.063-0.25). The displayed potentiation exerted by CM extract on tetracycline activity against *S. aureus* and *K. pneumonia* was reported by Rahman *et al*. (2008).

To confirm the possible use of synergistic antibiotic-herb combination for combating infections caused by a Gram negative pathogen , time kill assays for *Escherichia coli* ATCC 25922 in the presence of either plant extract (0.25×MIC), tetracycline (0.5×MIC), or both were performed. *Escherichia coli* was selected as one of the most causes of urinary tract infections (Bosch *et al*., 2011). Based on our findings, *Escherichia coli* was the least susceptible organism to the investigated plant extracts with high MIC values. Time kill assays were carried out to confirm the interaction between the botanical extracts and tetracycline. Treatment of bacteria with either tetracycline, PG or TV were similar to the control in starting the exponential phase of growth after 4 h, while the combination of PG or TV and tetracycline extended the lag phase and prevented the growth for the following 24 h (Fig. 1 and 2). Using CM extract with tetracycline (Fig. 3) had no significant effect on tetracycline activity which in agreement with synergistic study. Very few reports concerning the effect of medicinal plants in general and especially the plants under investigation concerning their effect on the synergism of antibiotics.

To date, many studies have been concerned with bactericidal or bacteriostatic activity of different plant extracts. However, it is important to consider the sustainability of tested antimicrobial extracts over a long period of time. The Post Antibiotic Effect (PAE) is the term used to describe the suppression of bacterial growth that persists after brief exposure of microorganism to antimicrobial agents. Drugs with no PAEs may require more frequent administration than those with PAEs (Spangler *et al*., 1998). The PAE of tetracycline, each of plant extract, or combination of both was tested. The estimated PAEs for tetracycline, PG, TV, or CM were 1, 1, 3 and 0 h, respectively. PG was as effective as tetracycline in delaying the growth of *E. coli*, while TV was more effective than the antibiotic resulting in a 3-fold increase in time required for *E. coli* to grow by $\log_{10}$. The combination of tetracycline and PG, or TV extended the

PAE to 4 and 5 h, respectively. CM alone was not as effective as PG or TV extracts and caused no difference in PAE as compared to unexposed cells. However, the combination of CM extract with tetracycline resulted in a 3- fold increase in the PAE. The increased PAE could help reducing the dosing regimens time and cost treatment and represent an alternative way for the extension of the useful lifetime of antibiotics. The availability of literature in this specific area was challenging, the search for post antibiotic effect and plant extracts on Pubmed.gov yielded only 9 abstracts. The broad spectrum antibacterial activity of the investigated botanical extracts and their role in potentiating antibiotic activity suggested the use of these plants in combating bacterial infections and in extending the lifetime of some antibiotics that lost efficiency or have fading antibacterial activity.

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