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Synergistic interactions between plant extracts, some antibiotics and/or their impact upon antibiotic-resistant bacterial isolates

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In this study, the antibacterial activities of medicinal plant extracts [of *Rehum palmatum* (R), *Cassia angustifolia* (C), *Glycyrrhiza glabra* (G), *Chichorium intybus* (Ch), and *Matricaria chamomilla* (M)], on antibiotic- resistant isolates (*Staphylococcus aureus* and *Alcaligenes xylosoxidans*) collected from clinical samples, pharmaceutical products, and different hospital water drains was detected (single, combined extract). This investigation shows that the extracts of *G. glabra*, *R. palmatum* and *C. angustifolia* and their combination with the selected antibiotic, variously inhibited the growth of the bacterial isolates. The methanol extraction ingredients recorded the maximum Inhibition Zone Diameter (mm IZD); 18.8/R, 12.6/C and 12.8/G plants. Prominent synergism occurred between plants extract mixture and Gentamycin, Ceftasidine, Tobramycin, Cefoperazone and Spictinomycin (GD) antibiotics. *Rehum* plant extract was the most potent antibacterial agent against *S. aureus* and *A. xylosoxidans*, especially when extracted with methanol solvent.

Key words: Synergism, antagonism, *Glycyrrhiza glabra*, *Rehum palmatum*, *Cassia angustifolia*, antibiotic-resistant bacteria, *Staphylococcus aureus, Alcaligenes xylosoxidans*, hospitals drain, clinical samples, volatile oils, total flavonoids.

INTRODUCTION

Plants have formed the basis of sophisticated traditional medicine and their natural products led for new drug development (Newman et al., 2000). Approximately, 80% of the world inhabitants rely on traditional medicine for their primary health care and medicinal plants play important roles on the remaining 20% population (Cragg et al., 1999). Medicinal plants differ greatly in the quality and quantity of the active ingredients where *Glycyrrhiza glabra* contains antibacterial active compounds as flavones, flavanones, isoflavones, isoflavans, chalcones, pterocarpans and antioxidant flavonoid compound activities (Sungeun et al., 2001; Taro et al., 2002; Hassan et al., 2005; Ajay et al., 2010). *Rehum palmatum* contains chrysophanol glycosides, along with di-O, C-glucosides of the monomeric reduced forms (rheinosides A to D) and dimeric reduced forms (sennosides A to F). It also contains quercetin-3-O-glucoside, quercetin-3-O-galactoside, quercetin-3-O-rutinoside, quercetin-3-O- arabinoside

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Abbreviation: R, *Rehum palmatum*; **C,** *Cassia angustifolia*; **G,** *Glycyrrhiza glabra*; **C, S1, S2, S3 and S4,** *Staphylococcus aureus* isolates; **A,** *Alcaligenes xylosoxidans* isolate.

and quercetin 3-O- [6″-(3-hydroxy-3- methylglutaroyl) - glucoside (WHO mongraphs, 1999; Tsukasa, 2004) while *Cassia senna* contains flavonoids and glucosides (Ghanadi et al., 2000; El-Sawi and Sleem, 2010; Periyasamy, 2010).

The chemical composition and antibacterial activities of some common consumed herb's as *Matricaria chamomilla*, *Origanum vulgare*, *Thymus vulgaris* was detected (Sokovic et al., 2010) and found that linolool, limonene, α-pinene and carvacol, β - pinene have the broadest activities but carvacol had the highest antibacterial action against pathogenic *Bacillus subtilis, Enterobacter cloacae, Escherichia coli*, *Micrococcus flavus*, *proteus mirabilis*, *Pseudomonas aeruginosa*, *salmonella enteritidis*, *S. epidermidis*, *S. typhimurlum* and *Staphylococcus aureus*. Also, Okwu (2011) isolated bioactive cannabinoid alkaloid (4-butylamine 10-methyl-6-hydroxy cannabinoid dornabinol) from the seeds of *Cassia alata*, and showed that the isolated compound successfully inhibited *P. aeruginosa*, *Klebsiella pneumonia*, *E. coli*, *S. aureus*, *Candida albicans* and *Aspergillus niger.* The virucidal effect of hot glycerin extract of *Cassia angustifolia*, *Rheum afficinale* was tested against herbs simplex virus type 1. The active components in these plants were identified as anthraquinones (Sydiskis et al., 1991). Locoricidin exhibited the highest activity against the upper respiratory tract bacteria such as *Streptococcus pyogenes*, *Haemophilus influenza* and *Moraxella catarrhalis* (Tanaka et al., 2001).

Severe microbial pollution of environment and pharmaceuticals; impact of pathogenic bacteria on human beings; the support, promotion and facilities are offered by the World Health Organization (WHO) for the effective use of herbal medicine in developing countries. Furthermore, the microbial mutations and appearance of new recombinant pathogenic microorganisms necessitate the continuous assessment of new antimicrobial activities of different medicinal plants.

The aim of this investigation was to assess and detects the antimicrobial agents against nosocomial and drain water contents of infecting microorganisms such as *Staphylococci* and *Alcaligenes* species.

MATERIAL AND METHODS

Screening experiment

Medicinal plants and extraction solvents

The active ingredient of different medicinal plants of *R. palmatum* (R), *C. angustifolia* (C), *G. glabra* (G), *Chichorium intybus* (Ch) and *M. chamomilla* (M) were extracted by different solvents viz: chloroform (Chl), ethyl acetate (EA), ethanol (E), methanol (M), hot water (HW) and cold water (CW).

Organisms used, sampling sites, their antibiotic sensitivity

The different bacterial isolates, sampling sites and their antibiotic response are given in Table 1.

Media used and bacterial identification

Two media were used for bacterial sampling in addition to selective media: Peptone: Tween: water (1 g: 10 ml: 1000 ml deionized water used for collection of samples from pharmaceutical drugs), and peptone water (1 g: 1000 ml deionized water) were used for waste drain water samples. Muller Hinton Agar (MHA) was used for the determination of antibiotic resistant bacteria. Selective media were: lactose broth (LB) and Selenite – cystine tetra-thionate broth (SCTB).

Bacterial identification: Bacterial isolates were routinely cultivated on LB and SCTB media for identification tests to select Gram-positive *Staphylococci* and *Alcaligenes* strain producing colonies on LB and SCTB. Following morphological and biochemical schemes derived from Bergey's manual of systematic bacteriology; (Holt, 1986). The isolates were assigned to be *S. aureus* C, S_1, S_2, S_3 and S_4 and *Alcaligenes xylosoxidans* A.

Sensitivity tests

Effect of medicinal plant extracts on the activities of bacterial isolates: It was detected on different bacterial isolates *S. aureus* (C, S_1, S_2, S_3, and S_4) and *A. xylosoxidans* (A) (NCCLS, 1993). The plant (R, C, G) extracts were single, double (RG, RC, GC) and triple mixtures RCG.

Medicinal plant extract- antibiotics combination impact experiment

Synergistic effect of medicinal plant extract and some selected antibiotics upon different bacterial isolates were detected by potentiating the isolate-unspecific antibiotic (Table 1) with plant extract and carrying out antibiosis test (NCCLS, 1993).

Chemical analysis and measurements

Extraction of volatile oils by hydro-distillation

The essential oil was prepared from the powdered plant according to Egyptian Pharmacopoeia (1984) method.

Investigation of volatile constituents using GC/MS: This method was carried out using GC/MS-5989B Gas Chromatography mass with the following conditions: Searched library: Wiley 275. LIB. Column: DBI, 30 m, 0.53 mm ID, 1.5 µm film; carrier gas: helium (flow rate 1 ml / min) (Nermin and Gouda, 2011; Mohammed et al., 2010; Moussa et al., 2005); ionization mode: EL (70 ev. Temperature program: 40°C (static for 2 min) then gradually increased (160°C at a rate of 2°C/ min) up to 250°C (static for 7.5 min); detector temperature of 250°C and injection temperature of 250°C. After stabilization of the chromatographic condition, the samples were injected and the mass spectrum for each peak was determined and from the G1035Awiley PBM Library (probability based matching) of gas chromatography mass, the equivalent compound name, molecular weight and structure were concluded.

Estimation of the total flavonoids

Calibration curve: Replicate aliquots of the methanolic solution of rutin equivalent to 20, 40, 60, 80, 100, 120, 140, 160 and 180 µg were separately introduced into test tubes, evaporated till dryness on a hot water bath (40 to 60°C); and then 5 ml of 0.1 M aluminum

Table 1. Different bacterial isolates, sampling sites and their antibiotic response.

Code number	Site and origin of samples antibiotic used In antibiosis test	Clinical samples (urine)	Hospital waste water, Cairo, Egypt			Pharmaceuticals company product, Cairo, Egypt	
		S. aureus (C)	*S. aureus* (S_1)	*S. aureus* (S_2)	*S. aureus* (S_3)	*S.aureus* (S_4)	*Alcaligenes xylosoxidans* (A)
1	Gentamycin (10 µg)	-	-	-	-	-	-
2	Ceftazidine (30 µg)	-	-	-	-	-	-
3	Ceftriaxone (30 µg)	-	-	-	-	-	-
4	Cefotaxime (30 µg)	-	-	-	-	-	-
5	Tobramycin (10 µg)	-	-	-	-	-	-
6	Cefoperazone (75 µg)	-	-	-	-	-	-
7	Imipenem (10 µg)	-	$16.0^{B} \pm 0.6$	-	-	-	-
8	Cotrimoxazole(25 µg)	-	-	-	-	-	-
9	Vancomycin(30 µg)	16.4 ± 0.3	$16.0^{B} \pm 0.6$	$16.0^{B} \pm 0.6$	$17.0^{B} \pm 0.6$	-	-
10	Amikacin (30 µg)	-	$17.0^{B} \pm 0.6$	-	-	-	-
11	Ampicillin sulbactam(20 µg)	-	$17.0^{B} \pm 0.6$	-	-	-	-
12	Cefepime(30 µg)	-	-	-	-	-	-
13	Ciprofloxacin(5 µg)	-	-	-	-	$16.0^{B} \pm 0.6$	$16.0^{B} \pm 0.6$
14	Spictinomycin GD (30 µg)	-	-	-	-	-	-
15	Azithromycin dehydrate (25 µg)	-	-	-	-	-	$18.0^{AB} \pm 0.7$
16	Kanamycin acid sulphate (25 µg)	-	-	-	-	$18.0^{AB} \pm 0.7$	$19.0^{A} \pm 0.8$
17	Oxytetracycline 30 µg)	-	-	-	-	$18.0^{AB} \pm 0.7$	$18.0^{AB} \pm 0.7$
18	Colistin sulphate (30 µg)	-	-	-	-	$19.0^{A} \pm 0.6$	$19.0^{A} \pm 0.6$
19	Spictinomycin HCl (25 µg)	-	-	-	-	-	-
20	Amoxycillin trihydrate GE (25 µg)	-	-	-	-	-	-
21	Ciprofloxacin HCl	-	-	-	-	-	$20.0^{A} \pm 0.8$

The data with the same letter, at the same column, are insignificant. -, Negative result.

chloride reagent was added (Karawy and Aboutable, 1982). The intensity of the developed yellow color was measured at λ_{max} = 415 nm (wavelength of maximum absorbency) against a blank prepared in the same way but replacing rutin solution by methanol, using the spectrophotometer. Determinations were carried out and absorbance was plotted versus concentration.

Method: 1 g of defatted air-dried powdered plants was accurately weighed and extracted with methanol (till exhaustion). The methanolic extract was transferred to a measuring flask of 100 ml and the volume was completed with methanol; from the methanolic extract, 0.5 ml was transferred into a test tube and evaporated to dryness. To the residue, 5 ml of aluminum chloride were added and the procedure continued as mentioned earlier. The flavonoid content of powder plant calculated as rutin was deduced from the established standard calibration curve.

$$\% \text{ of flavonoid calculated as rutin in the powders} = \frac{X \times 100}{5000}$$

Where, X is the amount of rutin in µg obtained from standard curve for the sample.

Statistical analysis

The experiment followed complete randomized design; the obtained data were subjected to analysis of variance (ANOVA) (Snedcor and Cochran, 1980) using Mastate programme. The least significant differences were used to compare means of treatments or 5% probability (Walter and Duncan, 1969).

RESULTS

Screening experiment

As shown in Table 1, the collected samples (isolates) of *S. aureus* (C, S_1, S_2, S_3 and S_4) and *A. xylosoxidans* (A) varies greatly in their resistance to different antibiotics. They were all resistant to antibiotics coded (1 to 6, 14, 19 and 20); but showed variable sensitivity to the remaining antibiotics. The response of the isolates was insignificant toward the individual antibiotic tested, while that of clinical samples to antibiotics was similar to the response of S_2 and S_3 isolate. The data (Table 2 and Figure 1) showed that, the different concentrations (10 and 30%) of the individual plants (R, C and G) extract inhibited different bacterial isolates growth. The maximum inhibition zone diameter (26 mm) was undertaken by 30% methanol extract of R plant / A isolate while the minimum inhibition zone diameter (IZD) was achieved by 10% of C and G plants (8 mm IZD) on S_4 isolate (*S. xylo*soxidans). Other plants (Ch and M) had no inhibitory effect. Thoroughly, examination of the results (Table 2) further showed that, the mean interaction (effect) between different isolates and plant extract, resulted in a higher potent effect (14.3 mm IZD)/ 30% concentration of R plant extract upon A (*A. xylosoxidans*) isolates while the least potent effect was done by C plant extract upon S_4 isolate (*S. aureus*); 2.0 mm IZD/10% concentration. Further screening of the mean effect of the results had showed that methanol extract ingredients (Figure 2) recorded the maximum inhibitory effect against all isolates (18.8/R, 12.6/C and 12.8/C plants) so it can be used as the sole extraction solvent in further studies. The data (Table 2) revealed that the triple mixture had the maximum significant inhibitory effect of 29.00 mm IZD/A isolate and a significant minimum of 15.0 mm IZD/S_4 isolate; these results led to the conclusion that the R plant extract was the most potent, methanol solvent was the best extraction one; *A. xylosoxidans* (A) isolates were the most sensitive and *S. aureus* S_4 isolate was the most resistant to single triple plant extract mixture.

Combined plant extract-antibiotic impact experiment

The results (Table 3) show that, potentiating the isolate-uninhibitory antibiotics with medicinal plant triple extract mixtures resulted in a synergistic effect upon antibiotics where the inhibition zone diameters (33.0 mm IZD) increased than that from under the effect of mixtures alone. The maximum insignificant synergism of plant extract upon antibiotic activity was achieved on gentamycin (10 µg) antibiotic where it inhibited all the tested isolates, the least synergism occurred with cefoparazone (75 µg) which inhibited S_3 isolate only while intermediate synergism occurred on ceftazidine (30 µg), spictinomycin GD (30 µg) that inhibited 3 (of 5) isolates and Tobramycin (10 µg) inhibited one isolate only. The data further showed that, the antibacterial actions of plant extract mixture (R × C × G) was effective against all bacterial isolates but significantly lower than their combination with the tested antibiotic. The GC/MS analysis of medicinal plant volatile oils have showed that, the total volatile content of *G. glabra* equal 0.1%, *R. palmatum* equal 0.2% and *C. angustifolia* equal 0.25% (Table 4).

Furthermore, the GC/mass chormatograph (Table 4) revealed the following: *G. glabra* contains α-pinene, octanol, γ-terpinene, stragole, Iso fenchon, β-caryophyllene, citronellyl acetate, caryophyllene oxide and geranyl linalool. It is obvious that geranyl linalool represented the highest percent (35%) while β-pinene was the lowest one (2%). *R. palmatum* contains α-thugen, Octanol, γ – Stragole, trans-ocimene, trans-anethole, eugenol, α-copaene, α-farnesene, δ-cadinene and palmitic acid. Eugenol represented the highest percent (25%) while α-thugen was the lowest one (4%). *C. angustifolia* contains α-pinene, β-pinene, octanol, γ-terpinolene, stragole, cis-limonene oxide, trans-ocimene, trans-anethole, β-caryophyllene, caryophyllene oxide, geranyl linalool and palmitic acid. Cis-limonene oxide represented the highest percent (25%) while α-pinene was the lowest one (1%).

Flavonoids content of medicinal plants

The data (Table 5) have showed that, the total flavonoids content of *G. glabra* equaled 1.6%, *R. palmatum* 2.88% and *C. angustifolia* was 2.68%.

DISCUSSION

This study was done aiming at finding some antibacterial agents that may be mixed with pharmaceuticals or combined with some antibiotics to inhibit the growth of the virulent pathogenic microorganism. In this connection, Yang et al. (2010) stated that, in recent years, human pathogenic microorganisms have developed multiple drug resistance and caused nosocmial infections. Moreover, it was suggested that, plant extract from *Llicium verum* can be further developed into antibiotic medicine due to their proven antibacterial activity. In the present study and on testing the sensitivity of different bacterial isolates to antibiotics, it was found that they greatly differs, which might be due to continuous genetic recombination of microorganisms (Saleh, 2000). There are two main factors that

Table 2. Antibacterial effect of different concentrations (10 and 30%) of individual and combined medicinal plant extracts (mm inhibition zone diameter, IZD).

Medicinal plants	Extraction solvent	Plant extract concentration (%)	Inhibition zone diameter (mm) IZD							
			Staphylococcus aureus					*Alcaligenes xylosoxidans* (A)	Mean effect of different solvent	Main effect Control
			C	S1	S2	S3	S4			
R *palmatum* (R)	Cold water (CW)	10	$10^{B} \pm 0.4$	$11^{B} \pm 0.5$	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	-	$6.5^{D} \pm 0.3$	-
		30	$13^{B} \pm 0.4$	$14^{B} \pm 0.5$	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	-	-	$8^{D} \pm 0.3$	-
	Hot water (HW)	10	$10^{B} \pm 0.4$	$10^{B} \pm 0.6$	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	-	$6^{D} \pm 0.2$	-
		30	$13^{B} \pm 0.4$	$13^{B} \pm 0.5$	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	-	-	$7.8^{D} \pm 0.3$	-
	Ethanol (96%), (E)	10	-	-	-	-	$11^{B} \pm 0.4$	$17^{B} \pm 0.6$	$5.6^{D} \pm 0.2$	-
		30	-	-	-	-	$13^{B} \pm 0.4$	$20^{B} \pm 0.6$	$5.4^{D} \pm 0.2$	-
	Methanol (M)	10	$14^{B} \pm 0.5$	$15^{B} \pm 0.6$	$14^{B} \pm 0.5$	$14^{B} \pm 0.5$	$12^{B} \pm 0.5$	$23^{B} \pm 0.8$	$5.6^{D} \pm 0.6$	-
		30	$18^{AB} \pm 0.7$	$19^{AB} \pm 0.6$	$18^{AB} \pm 0.7$	$18^{AB} \pm 0.6$	$14^{B} \pm 0.4$	$26^{A} \pm 0.7$	$18.8^{B} \pm 0.6$	-
	Ethylacetate (EA)	10	$12^{B} \pm 0.5$	$14^{B} \pm 0.6$	$12^{B} \pm 0.5$	$12^{B} \pm 0.5$	$11^{B} \pm 0.4$	$17^{B} \pm 0.6$	$13.2^{C} \pm 0.5$	-
		30	$16^{AB} \pm 0.5$	$18^{AB} \pm 0.6$	$16^{AB} \pm 0.5$	$16^{AB} \pm 0.5$	$1^{B} \pm 0.4$	$20^{B} \pm 0.6$	$16.4^{B} \pm 0.6$	-
	Chloroform (Chl)	10	$10^{B} \pm 0.4$	$10^{B} \pm 0.5$	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	$11^{B} \pm 0.4$	$19^{B} \pm 0.3$	$10.2^{C} \pm 0.3$	-
		30	$13^{B} \pm 0.4$	$13^{B} \pm 0.5$	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	$20^{B} \pm 0.6$	$14.2^{C} \pm 0.4$	-
Mean effect (interaction) of plant extract /isolate		10	$9.3^{C} \pm 0.3$	$9.3^{C} \pm 0.4$	$9.3^{C} \pm 0.4$	$9.3^{C} \pm 0.3$	$7.5^{C} \pm 0.3$	$11.16^{C} \pm 0.5$		$9.41^{C} \pm 0.3$
		30	$12.16^{A} \pm 0.5$	$12.2^{B} \pm 0.5$	$12.16^{B} \pm 0.5$	$12.16^{A} \pm 0.5$	$8.8^{C} \pm 0.3$	$14.3^{C} \pm 0.5$		$11.93^{B} \pm 0.3$
C angustifolia (C)	Cold water (CW)	10	$10^{B} \pm 0.4$	$10^{B} \pm 0.5$	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	-	$5.8^{D} \pm 0.1$	-
		30	$12^{B} \pm 0.4$	$13^{B} \pm 0.5$	$12^{B} \pm 0.4$	$12^{B} \pm 0.4$	-	-	$7.2^{D} \pm 0.2$	-
	Hot water (HW)	10	-	$10^{B} \pm 0.5$	-	-	-	$10^{C} \pm 0.3$	$5.8^{D} \pm 0.1$	-
		30	-	$12^{B} \pm 0.5$	-	-	-	$12^{C} \pm 0.4$	$7^{D} \pm 0.2$	-
	Ethanol (96%), (E)	10	$10^{B} \pm 0.4$	-	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	-	$3.8^{E} \pm 0.1$	-
		30	$12^{B} \pm 0.4$	-	$12^{B} \pm 0.4$	$12^{B} \pm 0.4$	-	-	$4.6^{E} \pm 0.1$	-
	Methanol (M)	10	$11^{B} \pm 0.4$	$10^{B} \pm 0.5$	$11^{B} \pm 0.4$	$11^{B} \pm 0.4$	$8^{C} \pm 0.2$	$12^{C} \pm 0.3$	$10.2^{C} \pm 0.2$	-
		30	$14^{B} \pm 0.4$	$13^{B} \pm 0.5$	$14^{B} \pm 0.4$	$14^{B} \pm 0.4$	$10^{B} \pm 0.3$	$14^{C} \pm 0.4$	$12.6^{C} \pm 0.3$	-

Table 2. Contd.

Medicinal plants	Extraction solvent	Plant extracts concentration (%)	Inhibition zone diameter (mm) IZD							
			Staphylococcus aureus					*Alcaligenes xylosoxidans* (A)	Mean effect of different solvent	Main effect Control
			C	S1	S2	S3	S4			
C angustifolia (C)	Ethylacetate (EA)	10	$10^{B} \pm 0.4$	-	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	$10^{C} \pm 0.3$	$4^{E} \pm 0.1$	-
		30	$13^{B} \pm 0.4$	-	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	-	$12^{C} \pm 0.4$	$5^{E} \pm 0.1$	-
	Chloroform (Chl)	10	$10^{B} \pm 0.4$	$9^{C} \pm 0.3$	$10^{B} \pm 0.4$	$10^{B} \pm 0.4$	-	$11^{C} \pm 0.3$	$7.8^{D} \pm 0.2$	-
		30	$12^{B} \pm 0.4$	$12^{B} \pm 0.6$	$12^{B} \pm 0.4$	$12^{B} \pm 0.4$	-	$13^{C} \pm 0.4$	$9.6^{D} \pm 0.2$	-
Mean effect (interaction) of plant extract /isolate		10	$8.5^{C} \pm 0.2$	$8.5^{C} \pm 0.2$	$8.5^{C} \pm 0.2$	$8.5^{C} \pm 0.2$	$2^{D} \pm 0.06$	$7.47^{D} \pm 0.3$		$6.91^{C} \pm 0.3$
		30	$10.3^{B} \pm 0.3$	$10.3^{B} \pm 0.3$	$10.3^{B} \pm 0.3$	$10.5^{B} \pm 0.3$	$17^{AB} \pm 0.01$	$8.5^{D} \pm 0.4$		$10.88^{C} \pm 0.3$
	Cold water (CW)	10	-	-	-	-	-	-	-	-
		30	-	-	-	-	-	-	-	-
	Hot water (HW)	10	-	-	-	-	-	-	-	-
		30	-	-	-	-	-	-	-	-
G glubra (G)	Ethanol (96%), (E)	10	$9^{C} \pm 0.3$	$15^{B} \pm 0.6$	$10^{B} \pm 0.4$	$9^{C} \pm 0.3$	$8^{C} \pm 0.3$	$18^{B} \pm 0.6$	$12^{C} \pm 0.3$	-
		30	$11^{B} \pm 0.4$	$19^{AB} \pm 0.6$	$13^{B} \pm 0.4$	$11^{B} \pm 0.4$	$10^{B} \pm 0.3$	$21^{B} \pm 0.6$	$12.8^{C} \pm 0.3$	-
	Methanol (M)	10	$11^{B} \pm 0.4$	$14^{B} \pm 0.6$	$10^{B} \pm 0.4$	$11^{B} \pm 0.4$	$9^{C} \pm 0.3$	$17^{B} \pm 0.6$	$10.2^{C} \pm 0.2$	-
		30	$13^{B} \pm 0.4$	$18^{AB} \pm 0.6$	$13^{B} \pm 0.4$	$13^{B} \pm 0.4$	$11^{B} \pm 0.3$	$20^{B} \pm 0.6$	$15^{B} \pm 0.3$	-
	Ethylacetate (EA)	10	$10^{B} \pm 0.3$	$15^{B} \pm 0.5$	$10^{B} \pm 0.4$	$10^{B} \pm 0.3$	-	-	$7^{D} \pm 0.2$	-
		30	$12^{B} \pm 0.4$	$19^{AB} \pm 0.6$	$13^{B} \pm 0.4$	$12^{B} \pm 0.4$	-	-	$8.8^{D} \pm 0.2$	-
	Chloroform (Chl)	10	$11^{B} \pm 0.3$	-	-	$11^{B} \pm 0.3$	-	-	$2.2^{E} \pm 0.02$	-
		30	$13^{B} \pm 0.4$	-	-	$13^{B} \pm 0.4$	-	-	$2.6^{E} \pm 0.02$	-
Mean effect (interaction) of plant extract /isolate		10	$6.83^{C} \pm 0.2$	$6.83^{C} \pm 0.2$	$5^{C} \pm 0.1$	$6.83^{C} \pm 0.2$	$1.3^{E} \pm 0.06$	$5.8^{D} \pm 0.2$		$5.5^{C} \pm 0.2$
		30	$8.2^{C} \pm 0.3$	$8.2^{C} \pm 0.3$	$6.5^{C} \pm 0.1$	$8.2^{C} \pm 0.3$	$1.7^{E} \pm 0.1$	$8.2^{D} \pm 0.2$		$7.0^{C} \pm 0.2$
R × C (mixture)	Methanol	10	$17^{AB} \pm 0.5$	$18^{AB} \pm 0.6$	$18^{AB} \pm 0.6$	$17^{AB} \pm 0.5$	$13^{B} \pm 0.4$	$22^{B} \pm 0.7$	$18^{B} \pm 0.6$	-
		30	$21^{A} \pm 0.7$	$22^{A} \pm 0.7$	$22^{A} \pm 0.7$	$21^{A} \pm 0.7$	$17^{AB} \pm 0.6$	$27^{A} \pm 0.8$	$21.9^{A} \pm 0.7$	-

Table 2. Contd.

Medicinal plants	Extraction solvent	Plant extract concentration (%)	Inhibition zone diameter (mm) IZD							
			Staphylococcus aureus					*Alcaligenes xylosoxidans* (A)	Mean effect of different solvent	Main effect Control
			C	S1	S2	S3	S4			
R × G (mixture)	Methanol	10	$12^{B} \pm 0.3$	$19^{AB} \pm 0.6$	$19^{AB} \pm 0.6$	$12^{B} \pm 0.3$	$10^{B} \pm 0.3$	$20^{B} \pm 0.7$	$17^{A} \pm 0.6$	-
		30	$16^{AB} \pm 0.5$	$23^{A} \pm 0.7$	$23^{A} \pm 0.7$	$16^{AB} \pm 0.5$	$13^{B} \pm 0.4$	$23^{B} \pm 0.7$	$19.6^{A} \pm 0.7$	-
C × G (mixture)	Methanol	10	$10^{B} \pm 0.3$	$18^{AB} \pm 0.6$	$13^{B} \pm 0.5$	$10^{B} \pm 0.3$	$9^{C} \pm 0.3$	$19^{B} \pm 0.6$	$14^{B} \pm 0.5$	-
		30	$14^{B} \pm 0.5$	$22^{A} \pm 0.7$	$17^{AB} \pm 0.6$	$14^{B} \pm 0.5$	$12^{B} \pm 0.4$	$23^{B} \pm 0.7$	$17.6^{B} \pm 0.6$	-
R × C × G (mixture)	Methanol	10	$18^{AB} \pm 0.6$	$22^{A} \pm 0.7$	$21^{A} \pm 0.7$	$18^{AB} \pm 0.6$	$17^{AB} \pm 0.5$	$23^{B} \pm 0.7$	$21^{A} \pm 0.7$	-
		30	$23^{A} \pm 0.7$	$26^{A} \pm 0.8$	$26^{A} \pm 0.8$	$23^{A} \pm 0.7$	$21^{A} \pm 0.7$	$29^{A} \pm 0.8$	$25^{A} \pm 0.8$	-
Mean effect (interaction) of plant extract (RCG) /isolate		10	$14.25^{B} \pm 0.5$	$14.25^{B} \pm 0.5$	$17.75^{AB} \pm 0.6$	$14.25^{B} \pm 0.5$	$12.25^{B} \pm 0.5$	$21^{B} \pm 0.5$		$16.53^{B} \pm 0.5$
		30	$18.5^{AB} \pm 0.7$	$18.5^{AB} \pm 0.7$	$22^{A} \pm 0.7$	$18.5^{AB} \pm 0.7$	$15.75^{AB} \pm 0.6$	$25.5^{A} \pm 0.7$		$20.58^{A} \pm 0.7$

-The data with the same letter, at the same column, are insignificant.
Negative inhibition zones in case of *C. intybus* and *M. camomila.*
Antibiotic disc diameter: mm.

Table 3. Antibacterial effect of medicinal plant mixture, potentiated with some antibiotic, on growth of different bacterial isolates.

30% RCG mixture + antibiotic	Inhibition Zone diameter (mm IZD) *Staphylococcus aureus Alcaligenes xylosoxidans*					
	C	S_1	S_2	S_3	S_4	A
1- Gentamycin 10µg)	$32.0^{A} \pm 1.4$	$33.0^{A} \pm 1.4$	$33.0^{A} \pm 1.4$	$32.0^{A} \pm 1.4$	$31.0^{A} \pm 1.2$	$34.0^{A} \pm 1.4$
2- Ceftazidine(30 µg)	-	$33.0^{A} \pm 1.3$	$33.0^{A} \pm 1.3$	-	$33.0^{A} \pm 1.3$	-
3- Ceftriaxone(30 µg))	-	-	-	-	-	-
4- Tobramycin (10 µg)	-	$33.0^{A} \pm 1.3$	$32.0^{A} \pm 1.2$	-	-	-
5- Cefoperazone(75 µg)	$32.0^{A} \pm 1.2$	-	-	$32.0^{A} \pm 1.2$	-	-
6- Cefotaxime (30 µg)	-	32.0^{A}	-	-	-	-
7-Spictinomycin (30 µg) (GD)	$32.0^{A} \pm 1.2$	-	-	$32.0^{A} \pm 1.2$	$33.0^{A} \pm 1.2$	$33.0^{A} \pm 1.2$
8- Spictinomycin HCl (25 µg)	-	-	-	-	-	-
9-Amoxycillin in hydrate GE (25 µg)	-	-	-	-	-	-
10- Plant extract mixture alone (R×C×G)	$18.5^{B} \pm 0.7$	$23.25^{B} \pm 0.6$	$22.0^{B} \pm 0.7$	$18.5^{B} \pm 0.7$	$15.75^{B} \pm 0.6$	$25.5^{B} \pm 0.7$

The data with the same letter, at the same column, are insignificant.

Table 4. GC/ MS analysis of volatile oils (% dry weight) of the studied medicinal plants.

Compound name	Retention time	G (%)	R (%)	C (%)
α-Thugen	4.0^{C}		4^{C}	
α-Pinene	4.13^{C}	4^{C}		1^{C}
β-Pinene	4.3^{C}	2^{C}		2^{C}
Octanol	6.11^{B}	5^{C}	10^{B}	7^{BC}
γ-Terpinene	7.23^{B}	12^{B}		
γ-Terpinolene	7.6^{B}			9^{B}
Stragole	7.8^{B}	10^{B}	9^{B}	8^{B}
Trans-Ocimene	8.1^{B}		12^{B}	
Cis-Limonene oxide	8.92^{B}			25^{A}
Trans-Anethole	9.1^{B}		8^{B}	10.5^{B}
Iso-Fenchon	9.6^{B}	15^{B}		
Eugenol	11.11^{A}		25^{A}	
α-Copaene	11.4^{A}		8^{B}	
α-Farnesene	13.22^{A}		6^{B}	
δ-Cadinene	13.5^{A}		6^{B}	
β-Caryophyllene	13.55^{A}	8^{C}		4.5^{C}
Citronellyl acetate	14.03^{A}	4^{C}		
Caryophyllene oxide	14.33^{A}	5^{C}		15^{B}
Geranyl linalool	14.88^{A}	35^{A}		10^{B}
palmitic acid	15^{A}		12^{AB}	8^{B}
Total percentage	--	0.10^{C}	0.20^{B}	0.25^{A}

The data with the same letter, at the same column, are insignificant. Small letters (a, b and c) represent the total percentage of volatile oils.

Table 5. Assay of total flavonoids of medicinal plants.

Medicinal plant	Total flavonoid content (%)
Glycyrrhiza glabra	1.6^{B}
Rehum palmatum	2.88^{A}
Cassia angustifolial	2.68^{A}

The data with the same letter, at the same column, are insignificant.

greatly affect the bacterial sensitivity to medicinal plant extracts; firstly, the active ingredients content of flavornoids and essential oils, second, is the efficiency of the extraction solvents. These flavonoids amounted to 1.6/G, 2.88/R and 2.68/C plants (Table 5). Concerning solvent effect, Al-Fatimi et al. (2010) concluded that both methanol and dichloromethane solvent extract showed strong activities against most human pathogenic fungi. In contrast, methanol extract showed broader and stronger antibacterial activities than the dichloromethane. On the contrary, water extract did not show any biological activity. In this concern Dawoud and Ewesis (2006) reported that, the least inhibitory effect of medicinal plants was caused by the aqueous extract (25.0 g/100 ml) of *Salvia officinalis* on mushroom pathogenic bacteria *Pseudomonas stutzeri* and that of *Eucalyptus globules* on *Pseudomonas tolassii* representing 2.59 and 5.78% control dry mass inhibition.

Flavonoids and essential oils greatly affect the pathogenic microorganism where, Chandrashekar and Kulkarni (2011) stated that, the ethanolic extract of *Annona squamosa* leaves was flavones type compounds that inhibited *P. aeruginosa*, *S. aureus*, *B. subtilis* and *Aspergillus flavus* showing high inhibition zone. The antimicrobial studies show that these isolated compounds successfully inhibited *P. aeurgenisa*, *K. pneumonia*, *S. aureus, Alcaligenes*, *E. coli*, *C. albicanus* and *A. niger.* In the present study, the data (Table 5) further show that the highly potent mean and main effect of *R. palmatum* plant might be due to its high percentage of flavonoids (2.88%) compared with the least amount found in *G. glabra* (1.6%). In addition, *R. palmatum* plant contains a moderate amount of essential oil (2%) compared with 1% in case of *G. glabra* which potentiate its antibacterial activities. In this

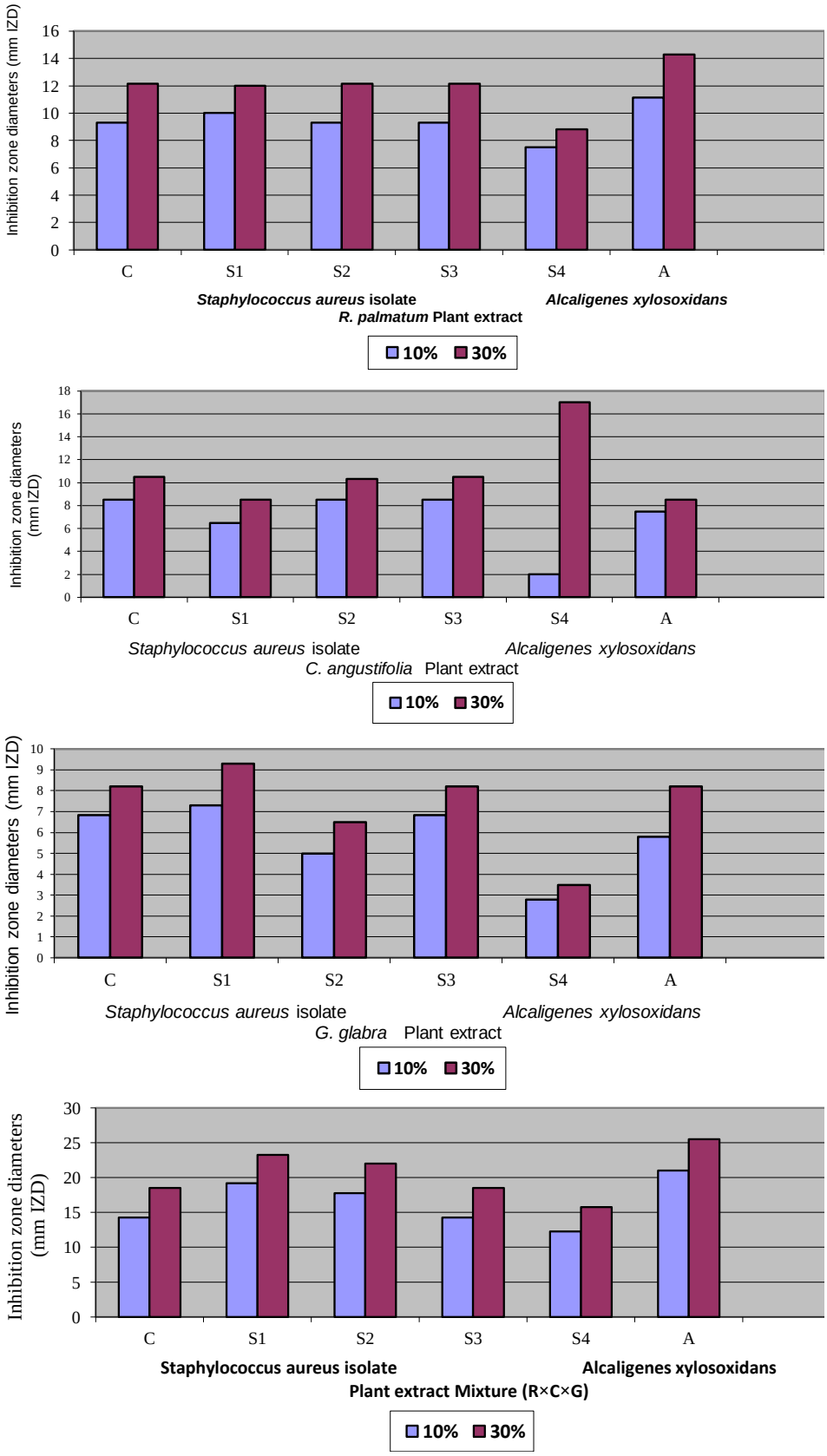

Figure 1. Antibacterial effect of different concentrations (10 and 30%) of individual and combined medicinal plant extracts (mm inhibition zone diameter, IZD).

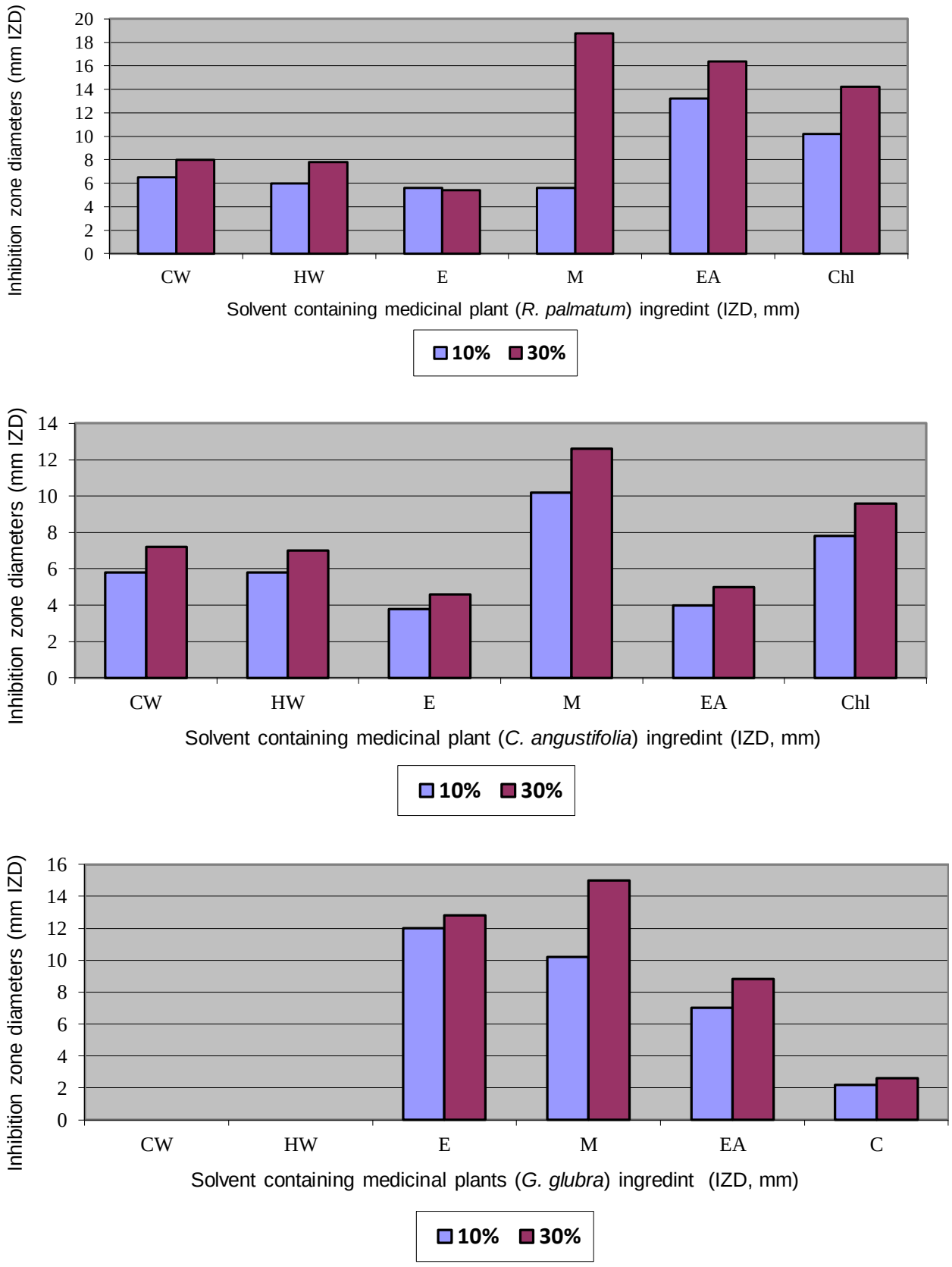

Figure 2. Extraction solvent gradient efficiency / different medicinal plant. CW, cold water; HW, Hot water; M, Methanol; E, Ethanol; EA, Ethyl acetate and Chl, chloroform solvents.

concern, Kovac and Kocsis (2011) found that the antibacterial activity of the essential oils (thyme, clove, cinnamon, thymol, carvacrol, engenol and cinnamic aldhyde) against methicillin-resistant *S. aureus* (MRSA) and methicillin – susceptible *S. aureus* (MSSA) could be related to their most abundant components but the effect of the minor component should also be taken into consideration.

Khalil and Li (2011) found that the essential oil (1.8-cineol, camphor, borneol, alpha- pinene, beta-pinene, camphene, beta-myrcene and caryophyllene) of *S. afficinalis* efficiently inhibited both *S. aureus* and *Streptococcus* group D after 10 min of contact at oil concentration of 20 µl /ml; but the essential oils showed temporary bacteriostatic effect on *E. coli*, *Salmonella typhi* as well as *P. aeruginosa*. In comparison with most known antibiotics, the efficiency of *S. officinalis* oil was much better, especially against antibiotic- resistant bacteria. On the other hand, *Lemon myrtle* essential oils have greater antimicrobial activity than Tea tree against *Alcaligenes faecalis*, *Mycobacterium phlei*, *S. aureus*, *Microsporum gypsium*, *Trichophyton mentrgrophytes* and *Trichophyton rabrum* (Wilkinson et al., 2003). Our data agree with these literature and verify the antibacterial activity of the plant extract obtained from *R. palmatum*, *C. acutifolia* and *G. glabra*; the mixture of plant extract contribute to the total quantity and quality of different compounds and finally to their increased antibacterial activity. Mixing plant extracts with different commercial antibiotics enhanced and synergized their antibacterial effect. In this regard, Liu et al. (2011) stated that many clinical isolates of *S. aureus* are resistant to numerous antimicrobials including fluroquinolones (FQs). Flavonoids such as biochanin A (BCA) are naturally present in fruits, vegetables and can be combined with FQs to produce a powerful antimicrobial agent. The possible synergistic action between the antimicrobial agents BCA and ciprofloxacin (CPFX) when used in combination, the inhibitory concentration index (FICI) data showed that there was synergy in all 12 of the 12 *S. aureus* tested.

Thoroughly, surveying the data and comparing between Tables 2, 3, to deduce the synergistic action of plant extract mixtures upon different groups of antibiotics, we can summarize that the aminoglycoside antibiotics (gentamycin, topramycin and vancomycin) were synergized. The mode of action of these glycoside antibiotics are the inhibition of amino acid polymerization (Aronson, 2010). Also, the β-lactam antibiotics (cefotaxime, spectinomycin GD) were synergized. The mode of action of these antibiotics is the binding to and inhibition of bacterial enzyme located on the cell membrane that is involved in bacterial cell wall synthesis (peptidoglycan), inhibition of cell growth, cell death and lysis. Ciprofloxacin (fluoroquinolone) antibiotics, tetracyclines (that inhibit ribosomal protein synthesis and vancomycin (inhibit bacterial cell wall synthesis) were also synergized. Further studies to elucidate the detailed and specific mode of action of these plant extracts upon pathogenic microorganisms will have to be done.

Conclusion

These data led us to approach the conclusion that, the plants extract mixture of *R. palmatum*, *C. angustifolia* and *G. glabra* may interfere with bacterial protein synthesis, enzyme activities and cell wall formation and can be used as a commercial antibiotic.

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