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Full Length Research Paper

Study on combined antimicrobial activity of some biologically active constituents from wild *Moringa peregrina* Forssk.

M. A. Abdel-Rahman Tahany[1]*, A. K. Hegazy[1], A. Mohsen Sayed[1], H. F. Kabiel[1], T. El-Alfy[2] and S. M. El-Komy[2]

[1]Cairo University, Faculty of Science, Cairo, Egypt.
[2]Cairo University, Faculty of Pharmacy, Cairo, Egypt.

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Three plants were used in this study: *Moringa pregrina, Achillea fragrantissima* and *Coleome droserifolia*. Six active constituents were separated from n-hexane and ethyl acetate fractions of *Moringa pregrina*. These active constituents were lupeol acetate, α-amyrin, β-amyrin, β-sitosterol, β-sitosterol-3-O- β-D-glucoside and apignin were assayed individually and in combination against pathogenic bacteria and fungi. All constituents were proved to be more antibacterial than antifungal agents. *Aspergillus flavus* and *Fusarium solani* were completely resistant to all constituents. α-amyrin was the most effective antibacterial compound. The least relative activity was achieved by β-sitosterol against *Bacillus subtilis* compared to ampicillin. Reasonable antifungal activity was recorded in case of lupeol acetate, α-amyrin and β-amyrin, while β-sitosterol and β-sitosterol-3-O- β-D-glucoside, revealed no antifungal activity. Apignin missed both antifungal and antibacterial activities. Low MICs were detected by α-amyrin, β-amyrin and β-sitosterol-3-O-β-D-glucoside against all tested bacteria. Concerning fungi, β-sitosterol and β-sitosterol-3-O-β-D-glucoside showed no antimycotic activity. Lupeol acetate, α-amyrin and β-amyrin, however, have slightly high MICs for all tested dermatophytic fungi compared to that of fluconazole. Among ninety nine assayed combination mixtures, thirty seven synergistic combination mixtures were detected which exerts 37 synergisms against different pathogens with FICI less than 0.5, which indicates high efficacy of combination mixtures over monotherapy treatments.

Key words: Combined antimicrobial therapy, active constituents, *Moringa peregrina, Achillea fragrantissima*, *Coleome droserifolia*.

INTRODUCTION

In the field of ethno medicine it has been recorded that therapeutic efficacy was more pronounced when active compound was left in a particular combination with other principles (Obute, 2005). Combination therapy has many advantages and disadvantages. The advantages are additive or synergistic effect, increased spectrum of activity and decreased resistance, while the disadvantages are antagonistic effects, increased risk of drug interactions, increased toxicity and increased cost (Baddely and Pappass, 2005). Although the use of combination therapy is an appealing alternative to monotherapy to improve the treatment outcome of invasive microbes, it remain controversial, this controversy is based on the specific mode of action of the agent used. When the combined agents act on the same molecule differently, therefore theoretically, using these two agents may lead to antagonism (Sanati et al., 1997). There are several arguments that justify the strategy of combining antifungal drugs to optimize therapy such as the *in vitro* data showing a potential for a synergistic effect, broader spectrum of activity and decreased risk of emergence of resistant strains and absence of a negative or harmful effects of monotherapy (Kontoyian-nis and Lewis, 2004 and Marr, 2004; Ramesh Putheti, Okigbo, R. N 2008; Chandraseker et al., 2004; Steimbach, 2005; Baddley

*Corresponding author. E-mail: mohsenab2005@yahoo.com. Tel: +202 0105191713.

and Pappas, 2005;).

The terminology of combination therapy used to place results in interpretive categories is often the subject of debate and confusion. Synergism and antagonism have clear and intuitive meanings. The terms (additive) and (summation) may refer to positive interaction and lead to misinterpreted. Mathematically the terms (indifferent) and "no interaction" can be used to describe results with precise interpretation (Johnson et al., 2004).

This work was conducted to evaluate the antimicrobial activity of active constituents of *Moringa pregrina, Achillea fragrantissima* and *Coleome droserifolia* in different combinations on some pathogenic bacteria and fungi.

MATERIALS AND METHODS

Study species

***Moringa peregrine* Forssk. Fiori (Family Moringaceae):** The plant grows on steep slopes of the mountains and in gravel or coarse sand sides of the wadi in Egypt. It becomes threatened by habitat distribution, over collection as fuel and seed collection. Traditionally it is used to treat slimness, constipation, headache, fever, burns, back and muscle pains. Seeds are used as coagulants in developing countries.

***Achillea fragrantissima* Forssk. Sch. Bip. (Family: Compositae):** The plant grows in the limestone wadis in Egypt and occupies various desert soil types. It becomes threatened by drought conditions and over collection for herbal medicine. Traditionally, plant is used for hyperglycemic treatments, cough and cold flu.

***Coleome droserifolia* Forssk. Delile (Family: Cleomaceae):** The species distribution in Egypt covers a wide range of phytogeographical regions. It becomes threatened by over-collection for herbal medicine. It is used traditionally for wound healing, treatment of hyperglycemia, diabetes mellitus, skin allergy and dermatitis (Abd El Ghani, 1988; Boulos, 1995 and 1999)

Extraction

UV spectra were measured using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer, Mass spectra were measured using Finningan Mat SSQ 7000, 70 eV. NMR spectra were recorded at 300 (^{1}H) and 75 MHz (^{13}C) on a Varian Mercury-300 instrument. NMR spectra were recorded in DMSO-d_6, and chemical shifts were given in δ (ppm) relative to TMS as internal standard. Electrothermal 9100 for determination of melting points (uncorrected) (U.K.)**.**For column chromatography, Sephadex LH-20 (pharmacia, Uppsala, Sweden), Silica gel: precoated plates G_{60} F_{254} for thin layer chromatography (TLC) and for column chromatography (Merck).

Plant material

The aerial parts of *Moringa peregrina* (Forssk.) Fiori (Family Moringaceae) were collected and identified by Prof. Hegazy, (Department of Botany, Faculty of Science, Cairo University). The collected material was air-dried reduced to powder and kept for extraction.

Extraction and Isolation

The air-dried aerial parts (650 g) were powdered then extracted by percolation with 95% ethanol (4 x 7 L) to yield (250 g) ethanolic extract residue. The residue (200 g) was suspended in distilled water and partitioned between n-hexane, chloroform, ethyl acetate and *n*-butanol (saturated with water). The solvents were separately evaporated under reduced pressure to yield 6, 3, 4.7 and 5 g respectively.

n-Hexane fraction (HF)

4 g was chromatographed over a vacuum liquid chromatography column (VLC) (Si gel H, 30 g, 5 x 3 cm). Gradient elution was carried out using n-hexane-chloroform mixtures, and chloroform-ethyl acetate mixtures. Fractions 100 ml each were collected to yield four main fractions (A-D). Fraction A (15 - 20% chloroform/n-hexane, 0.5 g) was rechromatographed over a Si gel 60 column (25 x 2 cm, 50 g), using *n*-hexane as an eluent to give 18 mg of compound 1 [R_f 0.51 in system (*n*-hexane-ethyl acetate (9.5 : 0.5 v/v)] it gives a reddish purple colour with *p*-anisaldehyde-sulphuric acid spray reagent. Fraction B (25 - 30% chloroform/n-hexane, 0.6 g) was rechromatographed over a Si gel 60 column (25 x 2 cm, 50 g), using *n*-hexane-ethyl acetate (9.9: 0.1 v/v) as an eluent to give 15 mg of white needle crystals of compound 2 [R_f 0.56 in system *n*-hexane-ethyl acetate (9 : 1 v/v)]. It gives a purple colour with *p*-anisaldehyde-sulphuric acid spray reagent. Fraction C (40% chloroform/n-hexane, 1.2 g) was purified by passing several times over sephadex LH-20 columns (40 × 2 cm) using chloroform-methanol (1:1 v/v) as an eluent. The purified fraction was rechromatographed over a Si gel 60 column (25 x 2 cm, 50 g), using *n*-hexane-ethyl acetate (9.5: 0.5 v/v) as an eluent to yield compound 3 (20 mg) and compound 4 (33 mg) [R_f 0.4 and 0.25, respectively, in system *n*-hexane-ethyl acetate (9:1 v/v)] they give a purple and violet colours respectively, with *p*-anisaldehyde-sulphuric acid spray reagent. Fraction D (100% ethyl acetate, 0.9 g) was rechromatographed over a Si gel 60 column (25 x 2 cm, 50 g), using chloroform-methanol (9.6: 0.4 v/v) as an eluent to give white powder of compound 5 [0.37 in system Chloroform-methanol (9.5 : 0.5 v/v), it gives a violet colour with *p*-anisaldehyde-sulphuric acid spray reagent].

Chloroform fraction (CF)

2 g was chromatographed over VLC column as mentioned under the n-hexane extract. Similar fractions were pooled together to yield two main fractions. Purification of these fractions over several silica columns as under fraction C yielded compounds 4 and 5.

Ethyl acetate fraction (EF)

2 g was fractionated over a sephadex LH-20 column (25 x 3 cm) using 20, 40, 60 and 80% methanol in water mixtures as an eluent. Fractions 200 ml were collected to yield two main fractions (E and F). These fractions were purified by passing several times over sephadex LH-20 columns, using methanol as an eluent to yield compounds 6 (11 mg) and 7 (10 mg) [R_f 0.45 and 0.42, respectively, in system Chloroform-methanol (9:1 v/v)].

n-Butanol fraction (BF)

4 g) was fractionated over a sephadex LH-20 column (25 x 3 cm) using 20, 40, 60 and 80% methanol in water mixtures as an eluent. Fractions 200 ml were collected to yield three main fractions (G-I).

Fractions G, H and I were purified by passing several times over sephadex LH-20 columns, using methanol and methanol-water mixtures (1:1 v/v) as an eluent to yield compounds 8 (20 mg), 9 (45mg) and 10 (30 mg), respectively [Rf 0.23, 0.34 and 0.5 respectively, in system ethyl acetate-methanol-water-formic acid (100: 16: 12:1: 0.1 v/v/v/v).

Compound 1: White microcrystalline powder, m.p. 222 - 224°C, EIMS (70 ev, rel. int.), m/z at 468 $[M]^+$ (7.9 %), 408 (40 %) $[M-CH_3COO]^+$, 218 (56%), 203 (77%), 189 (100%).

Compound 2: White needle crystals from *n*-hexane, m.p. 195 - 197°C, EIMS (70 ev, rel. int.), m/z at 426 $[M]^+$ (10%), 218 (100%), 203 (79%) and 189 (60%).

Compound 3: White needle crystals from *n*-hexane, m.p. 185 - 186°C. EIMS (70 ev, rel. int.), m/z at 426 $[M]^+$ (12.3 %), 218 (100%), 203 (38.46%) and 189 (34.61%).

Compound 4: White needle crystals from *n*-hexane, m.p. 140-141°C, **EIMS**: (70 ev, rel. int.), m/z at 414 $[M]^+$ (100%), 396 (51%), 329 (42%), 303 (44%), 273 (60 %) and 255 (80%).

Compound 5: White microcrystalline powder, m.p. 290°C, ^{1}H-NMR: δ (300 MHz, DMSO) 0.66 (3 H, d, J = 5.5 Hz, Me-21), 0.78 (3H, t, J = 6.3,Me-29), 0.83 (3H, d, J = 6.2 Hz, Me-26), 0.90 (3 H, d, J = 6.3 Hz, Me-27), 0.92 (3 H, s, Me-18), 0.96 (3H, s, Me-19), 3.03 (1 H, m, H-3), 4.21 (1H, d, J = 7.5, H-1`), 5.33 (H, br.s, H-6) ppm.

Compound 6: Yellow microcrystalline powder, soluble in methanol, m.p. 348-350°C. UV spectral data (Table 1).

Compound 7: Yellow microcrystalline powder, soluble in methanol, m.p.294-296°C. UV spectral data (Table 1).

Compound 8: Yellowish-white amorphous powder, soluble in methanol, UV spectral data (Table 1). ^{1}H-NMR δ ppm [300 MHz, DMSO]: 1.59 (1H,dd,J = 15,4,H-6 ax), 1.78 (2 H,m, H-2 ax,eq), 1.94 (1 H,dd,J = 13,9,H-6 eq), 3.79 (1 H,br.s, H-4), 3.94(1 H,br.s, H-5), 5.14(1 H,m,H-3), 6.18(1 H,d,J = 15.9 Hz, H-8`), 6.73 (1 H,d,J = 6.6Hz, H-5`), 6.94 (1H,dd,J = 8.1,2Hz, H-6`), 7.04 (1 H,br.s, H-2`), 7.40 (1 H,d,J = 15.9Hz , H-7`).

Compound 9: Yellow amorphous powder, soluble in methanol, UV spectral data (Table 1). ^{1}H-NMR δ ppm [300 MHz, DMSO]: 0.97 (3 H,d,J = 5.1, CH_3-6```), 3.84(3 H,s,OCH_3), 4.39(1 H, d,J = 2.1,H-1```),5.42(1 H,d,J = 7.2,H-1``), 6.18 (1 H, d, J = 2.4 Hz, H-6), 6.37 (1 H, d, J = 2.1 Hz, H-8), 6.90 (1 H,d, J = 8.7 Hz, H-5`), 7.50 (1 H,dd, J = 1.2, 6.6 Hz, H-6`), 7.53(1 H,s,H-2`). $^{13}$C-NMR: δ [75MHz, DMSO] 66.84 (C-6``), 70.09(C-4``), 74.27(C-2``),75.91(C-5``),76.39(C-3``), 101.22 (C-1``), 17.68(C-6```), 68.26(C-5```) 70.29(C-2```), 70.58(C-3```), 71.79 (C-4```), 100.86(C-1```), 93.60(C-8), 98.78 (C-6), 103.88(C-10), 115.07(C-2`), 116.22(C-5`), 121.03(C-6`), 122.26 (C-1`), 133.00(C-3), 144.73(C-3`), 146.87 (C-2), 148.43(C-4`), 156.37 (C-9), 161.14(C-5), 164.44 (C-7), 178.07(C-4).

Compound 10: Yellow amorphous powder, soluble in methanol, UV spectral data (Table 1). ^{1}H-NMR δ ppm [300 MHz, DMSO]: 3.70 (3H,s,OCH_3), 3.74 (3 H,s,OCH_3), 4.66 (1 H,d,J = 9.6,H-1``), 6.75 (1 H, s, H-3), 6.89 (2 H,d,J = 8.2Hz, H-3`,5`), 7.98 (2 H,d,J = 8.2Hz, H-2`,6`).$^{13}$C-NMR : δ [75 MHz, DMSO] 61.96 (C-6``), 70.85(C-4``), 70.86 (C-2``), 73.45(C-1``), 78.83(C-3``),82.01(C-5``), 56.61(4`-$OCH_3$), 61.7(6-$OCH_3$),102.41(C-3), 103.96(C-8), 104.60(C-10), 116.14(C-3`,5`), 121.95 (C-1`), 129.36 (C-2`,6`), 133.07 (C-6), 156.05 (C-9), 160.43(C-5,4`), 162.88 (C-2), 164.11 (C-7), 181.99(C-4).

Active constituents

Five compounds were isolated from the n-hexane fraction and were identified as lupeol acetate (1), β-amyrin (2), α-amyrin (3), β-sitosterol (4) and β-sitosterol -3-O- β-D-glucoside (5). One compound was isolated from the ethyl acetate was identified as apigenin (6) Table 1 (El-Alfy et al., 2009).

Antimicrobial activity

The antimicrobial activity was assayed against three Gram +ve: *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* (ATCC 12600) and *Streptococcus faecalis* (ATCC 19433) and three Gram--ve: *Escherichia coli* (ATCC 11775), *Neisseria gonorrhoeae* (ATCC 19424) and *Pseudomonas aerugmosa* (ATCC 10145), and seven fungal species: two filamentous moulds: *Aspergillus flavus* L. and *Fusarium oxysporum* L. and four dermatophytic fungi: *Candida albicans* (ATCC 26555), *C. krusei* (ATCC 6258), *C. parapsilosis* (ATCC 22019), *C. tropicalis* (ATCC 750) and *Saccharomyces cerevisiae* (2180-1A).

Bacteria and dematophytic fungi were grown and maintained on nutrient agar slants and Sabouraud dextrose agar slants, respectively. They were then stored under aerobic conditions. The dermatophytic fungi were cultured overnight, while moulds for 3 days at 30°C in Sabouraud dextrose broth and bacteria were cultured overnight at 35°C in nutrient broth. The minimum inhibitory concentration (M1C) was determined according to National Committee for Clinical laboratory Standards (NCCLS) M38-A microdilution method (Ibrahim et al., 2009), using (12 x 8 wells) microtitre plates. Aliquots (50 µ1) of the sample (single or combined 1:1 v/v) stock solution and 200 µl of the inoculum were pipetted to the well labeled as (A). Only 100 µl of each inoculum were added to the wells labeled (B-H). The inoculum and sample in the well (A) were mixed thoroughly before transferring 100 µl of the resultant mixture to well B. the same procedure was repeated for inoculum mixture in well (B) to (C) and repeated from wells (C-H) thus creating a serial dilution of the test materials.

Ampicillin was used as a standard reference antibiotic for comparison with the antibacterial activities, while fluconazol was used as the standard in the antifungal activity test. After an incubation period at 30°C for 24 h. turbidity was taken as indication of growth. Thus the lowest was taken as the minimum inhibitory concentration (MIC). The MIC was recorded as the mean of triplicates.

Quantitative mathematical analysis of combination therapy

The fractional inhibitory concentration index (FICI), calculated by use of checkerboard method, has long been the most commonly used way to characterize the activity of antimicrobial combinations in the laboratory (Mukherjee et al., 2005). FICI is determined by dividing the MIC of each drug when used in combination by the MIC of each drug when used alone.

FICI = MIC_a in combination / MIC_a tested alone + MIC_b in combination / MIC_b tested alone, Where MIC_a and MIC_b are the MICs of drugs a, b. FICI > 4 defines antagonism FICI 0.5-4 defines no interaction (indifference), FICI < 0.5 defines synergism

Statistical analysis

Chi-square test and one way ANOVA test were used. All results are expressed as mean ± St. Error. P values < 0.05 were considered significant (Lewis, 1984).

Table (1). FICI of the synergistic combination mixtures to the tested microorganisms.

Synergistic combinations	*B. subtilis*	*E. coli*	*N. gonorrhoroea*	*P. aeruginosa*	*S. aecalis*	*C. lbicans*	*C. krusei*	*C. arapsilesis*	*C. tropicalis*	*S. cereviseae*	*S. aureus*	Total no. of synergy
	FICI	FICI	FICI	FICI	FICI	FICI	FICI	FICI	FICI	FICI	FICI	
Lupeol acetate + β-amyrin			0.37									1
Lupeol acetate + β-sitosterol		0.49										1
Lupeol acetate + β-sitosterol -3-O- β-D-glucoside		0.35									0.39	2
β-amyrin + α-amyrin			0.47									1
β-amyrin + β-sitosterol	0.35			0.32								2
α-amyrin + β-sitosterol -3-O- β-D-glucoside			0.41		0.46							2
β-sitosterol + β-sitosterol -3-O- β-D-glucoside				0.40								1
Lupeol acetate + A. vol. oil				0.48		0.41	0.28	0.39	0.35			5
Lupeol acetate + C. vol. oil							0.38					1
α-amyrin + A. vol. oil						0.30	0.40					2
α-amyrin + C. vol. oil			0.40			0.30	0.40					3
β-amyrin + A. vol. oil						0.40		0.40				2
β-amyrin + C. vol. oil			0.40			0.30						2
β-sitosterol + A. vol. oil		0.40	0.40									2
β-sitosterol + C. vol. oil	0.40		0.30									2
Lupeol acetate + Flaconazole						0.27	0.30					2
α-amyrin + Fluconazole							0.30			0.40		2
β-amyrin + Fluconazole						0.30	0.40					2
β-amyrin + ampicillin	0.35											1
β-sitosterol -3-O- β-D-glucoside + ampicillin				0.40								1
Total no. of synergy	3	3	7	4	1	7	7	2	1	1	1	37

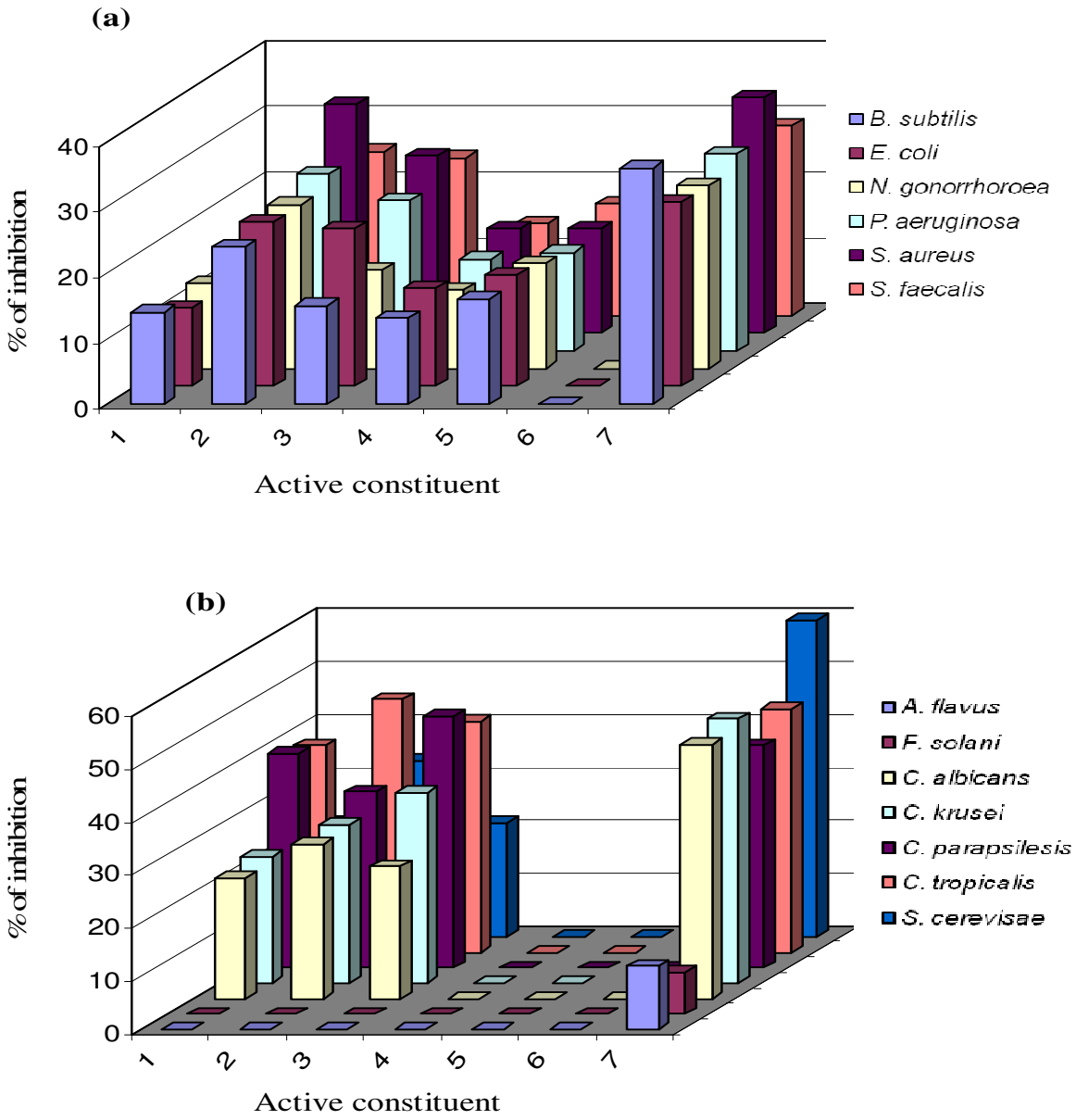

Figure 1. Antimicrobial susceptibility of the active constituents separated from *Moringa Peregrina* for (a) Bacteria: P < 0.01, F = 0.5 and (b) Fungi P < 0.01, F = 0.6)). 1: Lupeol acetate, 2: β-amyrin, 3: α-amyrin, 4: β-sitosterol, 5: β-sitosterol-3-O-glucoside, 6: apigenin, 7: ampicillin or fluconazol (standard drugs).

RESULTS AND DISCUSSION

The data presented in Figure (1) showed that all compounds separated from n-hexane and ethyl acetate fractions of *M. peregrina* proved to be more antibacterial than antifungal agents. The filamentous moulds *A. flavus* and *F. solani* were completely resistant to all compounds. β-amyrin was the most effective antibacterial compound and achieved high relative activity with the reference antibiotic apmicillin reaching 97.2% in case of *S. aureus*. Least relative activity (36.1%) was achieved by β-sitosterol against *B. subtilis.*

Reasonable antifungal activity was recorded in case of lupeol acetate, α-amyrin and β-amyrin with relative activity ranging from 21.6 - 47.6% compared to fluconazole activity. B-sitosterol and B-sitosterol-3-o-glucoside however, revealed no antifungal activity. On the other hand, Apignin missed both antifungal and antibacterial activities, so it was excluded from the next experiments. Relatively low MICs were detected by α-amyrin, β-amyrin and B-sitosterol-3-o-glucoside against all tested bacterial species while lupeol acetate recorded higher MIC (Figure 2). Concerning fungi, β-sitosterol and β-sitosterol-3-o-glucoside showed no antimycotic activity, lupeol acetate,

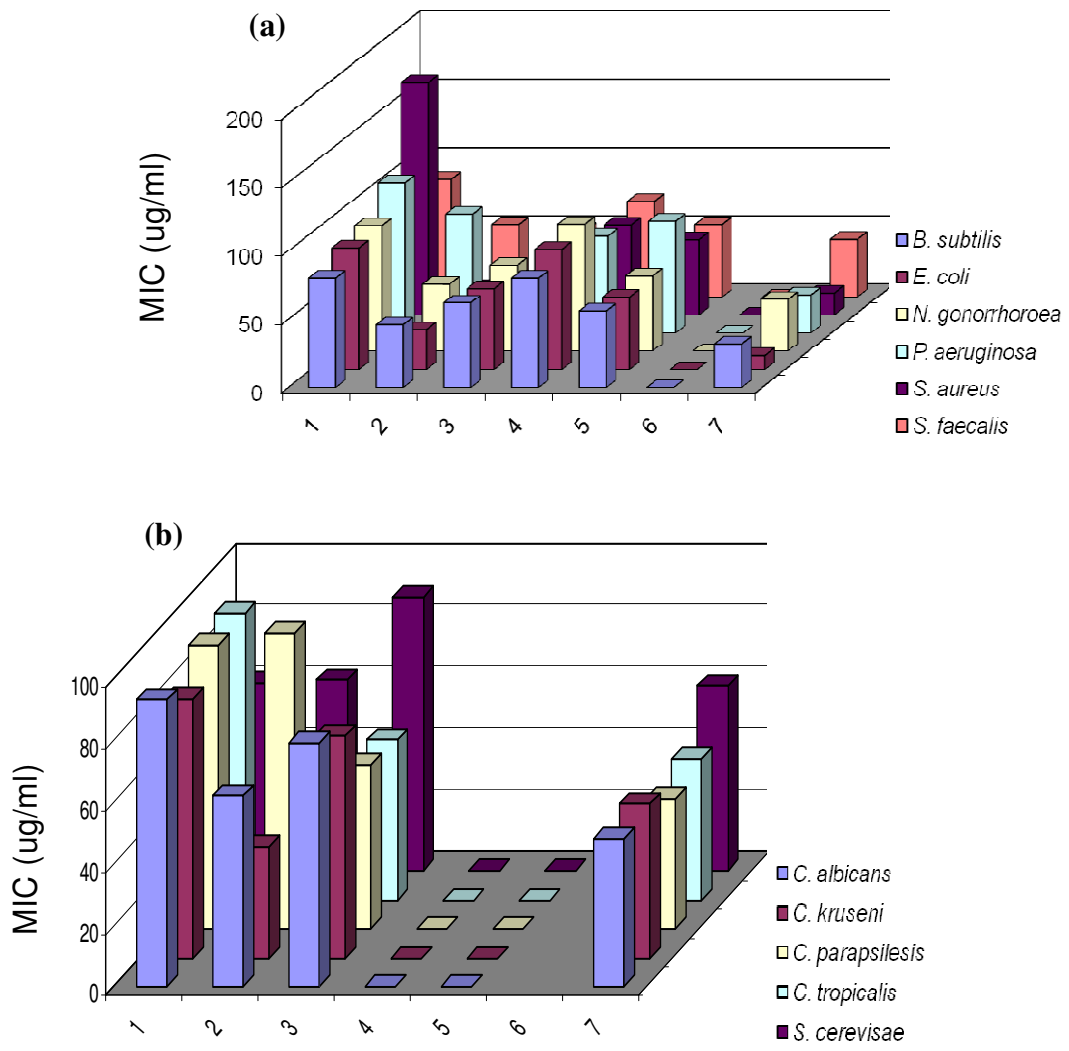

Figure 2. MIC for the active constituents separated from *Moringa Peregrina* for (a) Bacteria ($P < 0.02$, $F = 2.1$) and (b) Fungi ($P < 0.03$, $F = 1.2$)). 1: Lupeol acetate, 2: β-amyrin, 3: α-amyrin, 4: β-sitosterol, 5: β-sitosterol-3-O-glucoside, 6: apigenin, 7: ampicillin or fluconazol (standard drugs).

β-amyrin and α-amyrin, however, have slightly high MICs for all tested dermatophytic fungi compared to that of fluconazole.

Previous surveys demonstrated the wide occurrence of active antimicrobial substances in higher plants. For centuries plant and herbs have been used in various parts of the world for the treatment of certain diseases. Yet a scientific study of plants to determine their content of antimicrobial materials is comparatively new. Our finding that *M. peregrina* compounds have wide spectrum antimicrobial activity was also recorded by Eilert et al. (1981) who screened the antimicrobial activity of *M. peregrina* against six Gram +ve and seven Gram –ve bacteria and found that water extract, ethanol and petroleum ether extracts possess high antimicrobial activity. *Moringa oleifera* provides a rich and rare combination of zeatin, quercetin, β-sitsterol, caffeoylquinic acid and kaempferol which have antifungal and antibacterial activities (Anwar et al., 2006; Ramesh Putheti, Okigbo RN, 2008).

The high mortality of microbial infections and the relatively limited efficacy of current agents have produced significant interest in combination therapy for these agents to treat infections so in the present study, ten combination mixtures of dual active constituents were investigated for MIC and FICI determination against bacteria and fungi.

The fractional inhibitory concentration index (FICI) recorded "no interaction" (FICI 0.5-4) in relatively large number of combinations between the active constituents of *M. peregrina* reaching 100% in combination of α-amyrin plus β-sitosterol (mixture 8) (Figure 3). Antagonism, however, rarely occurred as it recorded once in case of lupeol acetate plus β-amyrin (mixture 1) against

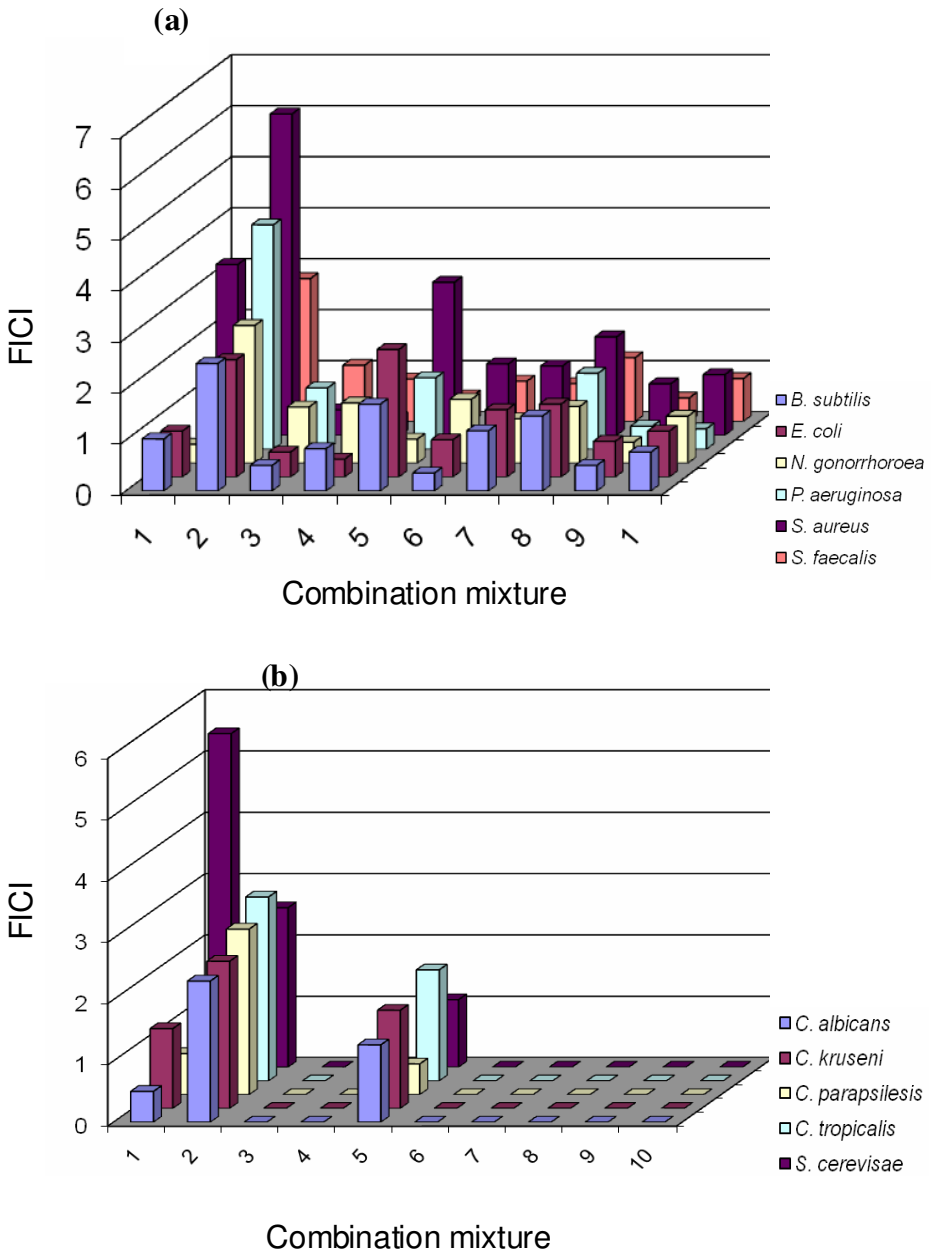

Figure 3. Combination antimicrobial of dual active constituents separated from of *M. peregrine* (Bacteria ($P < 0.03$, $F = 2.1$), Fungi ($P < 0.03$, $F = 1.9$)). Combination mixtures: 1: Lupeol acetate + β-amyrin, 2: Lupeol acetate + α-amyrin, 3: Lupeol acetate + β-sitosterol, 4: Lupeol acetate + β-sitosterol-3-O-glucoside, 5: β-amyrin + α-amyrin, 6: β-amyrin + β-sitosterol, 7: β-amyrin + β-sitosterol-3-O-glucoside, 8: α-amyrin + β-sitosterol, 9: α-amyrin + β-sitosterol-3-O-glucoside, 10: β-sitosterol + β-sitosterol-3-O-glucoside

S. cerevisiae (FICI, 5.44) and twice in case of lupeol acetate plus α-amyrin (mixture 2) against *P. aeruginosa* and *S. aureus* (FICI, 4.4 and 6.3 respectively). It was worthy noting that eleven cases of synergy between *M. peregrina* active constituents against all tested bacterial species (Table 1). Synergy means that the combined therapy is more effective in killing pathogen than mono-therapy.

High synergistic mixtures were detected for fungi, but no interaction mixtures were detected for fungi and no antagonistic interactions were recorded except mixture 2 was antagonistic to *S. cerevisiae*. As the focus of this study is on the efficacy of combination antimicrobial drugs especially pathogens, another combination experiment

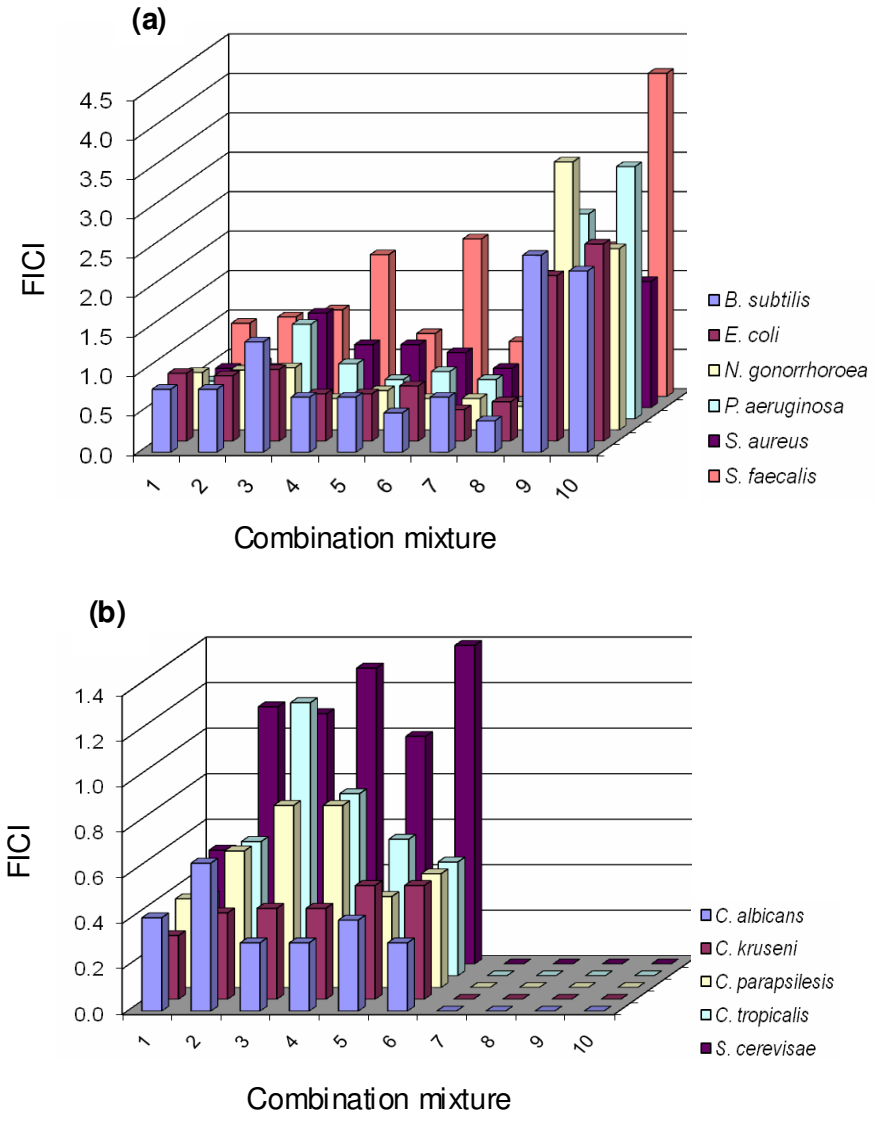

Figure 4. Combination antimicrobial therapy of the active constituents separated from *M. peregrine* plus volatile oils extracted from *Achillea fragrantissima* and *Coleome droserifolia* (Bacteria ($P < 0.03$, $F = 1.2$), Fungi ($P < 0.01$, $F = 0.8$)). Combination mixtures: 1: Lupeol acetate + A. vol. oil, 2: Lupeol acetate + C. vol. oil, 3: α-amyrin + A. vol. oil, 4: α-amyrin + C. vol. oil, 5: β-amyrin + A. vol. oil, 6: β-amyrin + C. vol. oil, 7: β-sitosterol + A. vol. oil, 8: β-sitosterol + C. vol. oil, 9: β-sitosterol-3-O-glucoside + A. vol. oil, 10: β-sitosterol-3-O-glucoside + C. vol. oil.

using *M. peregrina* active constituents plus volatile oils from *Achillea fragrantissima* or *Cleome droserifolia* was carried out.

Data in (Figure 4) revealed "no interaction" against bacteria in almost all combinations (FICI 0.5-4) except seven cases of synergism and one case of antagonism. The antagonistic interaction was between the B-sitosterol-3-o-glucoside and volatile oil of *C. droserifolia* (mixture 10) against *S. faecalis*. Four of the seven synergistic mixtures acted against the serious pathogen *N. gonorrhoeae* one acted against each of *P. aerugnosa*, *E. coli* and *B. subtilis* (Table 1).

Regarding fungi, high synergy was recorded in the combination between volatile oil of *A. fragrantissima* plus lupeol acetate covering 4 of the 5 tested dermatophytes (table 1) which represent promising finding in antimycosis therapy. Five of 6 mixtures acted synergistically against *C. albicans* while 4 of 6 mixtures were synergistic against *C. krusei* where the rate of killing increased with reduction of MIC (Table 1). Mixtures from 7 to 10 have lost the antifungal activity totally. The antimicrobial activity of *A. fragiantissima* and *C. viscose* was also reported by Barel

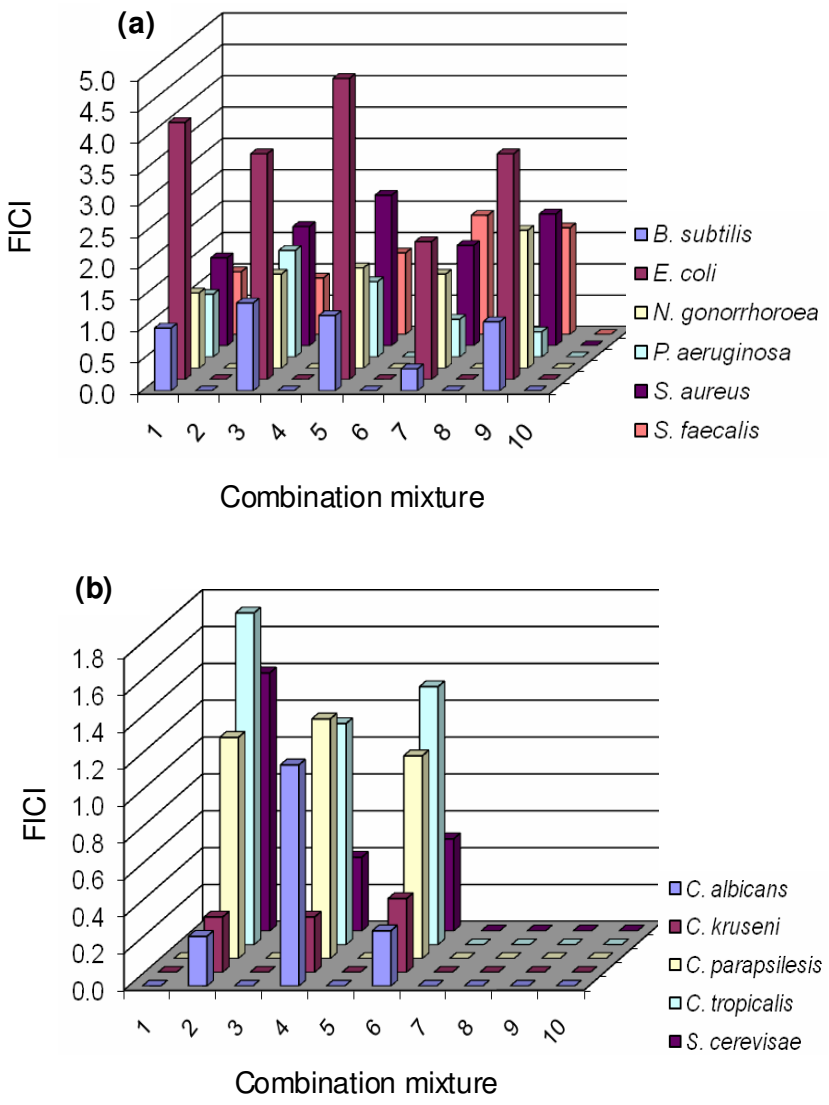

Figure 5. Combination antimicrobial therapy between active constituents separated from *M. peregrina* plus reference antibiotics from local market (Bacteria ($P < 0.01$, $F = 1.9$), Fungi ($P < 0.02$, $F = 0.9$)). Combination mixtures: 1: Lupeol acetate + ampicillin, 2: Lupeol acetate + flaconozote, 3: α-amyrin + ampicillin, 4: α-amyrin + fluconazole, 5: β-amyrin + ampicillin, 6: β-amyrin + fluconazole, 7: β-sitosterol-3-O-B-d glucoside + ampicillin, 8: β-sitosterol + fluconazole, 9: β-sitosterol + ampicillin, 10:6- β-sitosterol-3-O-glucoside + fluconazole

et al. (1991), Hashem and Wahba, (1999) and Sudhakar et al. (2005).

The efficacy of the active constituents separated from *M. peregrina* was finally assayed in combination with ampicillin and fluconazol from the local market (Figure 5). The data revealed "no interaction" in most combination for mixtures in inhibiting bacterial growth (FC1C 0.5-4). Antagonistic effects recorded in two were lupeol acetate plus ampicillin (mixt. 1) and □-amyrin plus ampicillin (mixture 5) against *E. coli* (FICI 4.1 and 4.8) where the antibacterial activity decreased in the individually tested compound. Synergy was recorded also in two are β-sitostrol plus ampicillin (mixture 1) against *S. aureus* and B-sitosterol-3-o-glucoside plus ampicillin (mixture 9) against *P. aeruginosa* (Table 1).

High increase in antifungal activity was observed in these combination mixtures. In case of fungi 6 of 15 synergistic mixtures were recorded against dermatophytes (Table 1) were mixture (1) against *C. albicans* and *C. krusei* mixture (4) against *C. krusei* and *S. cerevisiae* and mixture (6) against *C. albicans* and *S. cerevisiae*. This observation indicated the more efficacy of combined treatment for candidiasis than monotherapy. Polak (1989) reported that antifungal combination may increase the

magnitude and rate of microbial killing *in vitro*, shorten the total duration of therapy, prevent the emergence of drug resistance, expand the spectrum of activity and decrease the drug related toxicities by allowing the use of lower doses of antifungal. Sugar (1995) found that multi-compound therapies along the disease pathway may need to be manipulated simultaneously from an effective treatment. When one drug is used, the required high dosage for efficacy often produce bioavailability problems and unwanted side effects and drug resistance problems may also emerge (Zhang et al., 2007). Perhaps if we can focus on multiple targets in the microbial pathway through the use of co-drugs, high dosage of single drug will not be necessary (Cowen and Lindquist, 2005 and Heitman, 2005).

There are several mechanisms produced from synergy in combined antimicrobial therapy: (i) inhibition of different stages of some biochemical pathways represent one type of interaction; (ii) increased penetration of the antimicrobial agent as a result of cell wall or cell membrane antimicrobial activity from another agent which facilitate the 2nd agent to reach their target site; (iii) a transport interaction where one antimicrobial degrade cell wall allowing the other to remain at the site of its action within the wall; and (iv) Simultaneous inhibition of different microbial cell wall targets, such as cell wall and membrane targets. Antagonism among antimicrobial agents might occur in one of several ways including: (i) direct act at the same site which render the second agent inactive; (ii) adsorption to the surface by one agent inhibits binding of another antimicrobial agent; and (iii) modification of the pathogen upon exposure to one antimicrobial agent renders it less susceptible to the effect of the second agent (Johnson et al., 2004).

In conclusion twenty synergistic combined mixtures were detected in this *in vitro* study which is pathogen dependent. These mixtures need further *in vivo* studies to evaluate their actual effect. Table (1) summarizes these important conclusions. We want to pay attention to the higher efficacy of combined therapy against *Candida* species and some bacterial species than monotherapy. When one of the combined agents is of natural source, the concentration of chemical decreased, and the side effect and appearance of resistant pathogens delayed.

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