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The Synergetic Efficacy of the Combination of Amphotericin B and Certain Essential Oils against Selected Fungal Clinical Isolates

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ABSTRACT

The synergistic effect of five potential essential oils from each of the following plant species: *Thymus vulgaris*, Lamiaceae, *Cinnamomum zeylanicum*, Lauraceae, *Eugenia aromatica*, Myrtaceae, *Eucalyptus globulus*, Myrtaceae and *Zingiber officinalis*, Zingiberaceae was assessed in combination with the antifungal agent amphotericin B. The oils were analysed and their main components identified using GC/FID and GC/MS. The antifungal activities of the oils were investigated against two clinical isolates of the fungal species; *Aspergillus niger* and *Candida albicans*. The minimum inhibitory concentrations of the individual oils were evaluated using the agar disc diffusion assay while the synergetic effects between each of the oils and amphotericin B were evaluated using the agar dilution checkerboard micro titer test. The results indicated significant synergetic effects between thyme oil and amphotericin B against *C. albicans* and *A. niger* with FIC indices of 0.25 and 0.13 respectively. Moreover, cinnamon oil acted synergistically with amphotericin B against *C. albicans* with an FICI value of 0.28, however when tested against *A. niger*, an additive effect was observed (FICI = 1). The combination of thyme oil and to a less extent, cinnamon oil with amphotericin B is suggested for the treatment of infections caused by *C. albicans* and *A. niger*.

INTRODUCTION

Fungal infections are one of the major causes of diseases and in some cases death worldwide. Some *Aspergillus* species are responsible for diseases caused by food contamination as well as opportunistic infections of humans (Baker, 2006). *Candida albicans* causes severe infections and colonizes mucosal surfaces of the oral and vaginal cavities as well as in the digestive tract causing a variety of illnesses (Terlecka *et al.*, 2007). Recently, much focus has turned to these opportunistic fungi responsible for causing infections in vulnerable patients, such as patients treated with immunosuppressors, or other acquired immunodeficiency conditions and chemotherapy patients (Pappas, 2010). Due to the rapid development of bacterial and fungal resistance, many antimicrobial medications can turn out to be ineffective. Hence, researchers continue to search for new antimicrobials from natural products. Plant oils remain a promising source for the production of antimicrobial drugs despite the fact that their antibacterial and antifungal activity was proven less than that of commercial synthetic drugs (Giamperi *et al.*, 2002) but considering the increasing pathogen resistance, the implementation of essential oils and their components have gained new attention.

Amphotericin B has been widely used to treat fungal infections since the 1950s but it can cause many adverse effects (chills, fever, headache, loss of appetite, muscle or joint pain, nausea, stomach pain and weight loss) as well as fatal syndromes of hepato and nephrotoxicity (Dupont *et al.*, 1996).

The synergetic or additive effect produced by the combination between plant essential oils and antimicrobial drugs was referred as a strategy for combating microbial development (Wagner and Ulrich-Merzenich, 2009).

Hence, many studies are conducted towards reducing the amphotericin B dose by combining it with new products that have antifungal action in attempt to produce an effective synergetic action as well as reduce the adverse effects of amphotericin B (Bidgoli, 2010; Giordani *et al.*, 2006; Mahboubi and Saad *et al.*, 2010; Rosato *et al.*, 2008; Silva *et al.*, 2011; Yongmoon, 2007).

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In this study, the antifungal activity of five essential oils; thyme oil (*Thymus vulgaris* L, Lamiaceae), cinnamon oil (*Cinnamomum zeylanicum* Blume, Lauraceae), clove oil (*Eugenia aromatica* Thunberg, Myrtaceae), eucalyptus oil (*Eucalyptus globulus* L., Myrtaceae) and ginger oil (*Zingiber officinalis* Roscoe, Zingiberaceae) were investigated against two fungal species; clinical isolates of *Aspergillus niger* and *Candida albicans*. The oils under investigation were chosen for their prominent antimicrobial action that has been revealed throughout years of literature and evidently contributed to a number of oxygenated terpenoid and phenolic compounds which, in pure from, have shown to exhibit antimicrobial activity (Ahmad *et al.,* 2010; Ali *et al.,* 2008; Bakkali *et al.,* 2008; Braga *et al.,* 2007; Burt, 2004; Gilles *et al.,* 2010; Tajkarimi *et al.,* 2010).

Although the antifungal action of the essential oils under investigation have been studied thoroughly, no comparative study has been run under the same experimental conditions in order to target those that exhibit the highest probability of potentiation of antifungal drugs exemplified in amphotericin B. The present study aims at verifying the possible synergetic effect between the selected essential oils and the antifungal compound amphotericin B in an effort to find alternatives for the use of high doses of amphotericin B. The combination of amphotericin B with any of the potential essential oils is likely to reduce its minimum effective dose thus minimizing its side effects and slow down the onset of resistance produced by the fungal species as that reported by *Candida* strains (Yongmoon, 2007).

MATERIALS AND METHODS

Plant material and essential oil extraction

Fresh plant material (leaves of *Thymus vulgaris* L, Lamiaceae; barks of *Cinnamomum zeylanicum* Blume, Lauraceae; flower buds of *Eugenia aromatica* Thunberg, Myrtaceae; leaves of *Eucalyptus globulus* L., Myrtaceae and rhizomes of *Zingiber officinalis* Roscoe, Zingiberaceae) were collected from herbal stores in Cairo, Egypt. The identity of the plant was ascertained morphologically by Prof. Abdel Rahman Al-Newaihi, Department of Botany, Faculty of Science, Ain Shams University. Voucher specimens (SA522-526) were kept at the Department of Pharmacognosy, Faculty of Pharmacy, Ain Shams University. The corresponding oil for each plant was prepared using hydrodistillation for 4 hours by means of a Clevenger type apparatus, dehydrated over anhydrous sodium sulfate and stored at 4 °C.

Gas chromatography analyses

GC-FID analysis

The GC analyses were carried out on a Focus GC® (Thermo Fisher Scientific®, Milan, Italy) equipped with TR5-MS fused bonded column (30 m x 0.25 mm x 0.25 µm) (Thermo Fisher Scientific ®, Florida, USA) and FID detector; carrier gas was nitrogen (1.5 ml/min); the operating conditions were: initial temperature 40°C, 1 min. isothermal followed by linear temperature increase till 230°C at a rate of 4°C/min. 230°C, then 5 min. isothermal. Detector and injector temperatures were 300 and 220°C, respectively.

The split ratio was 1: 20. Chrom-card® chromatography data system ver. 2.3.3 (Thermo Electron Corp.®, Florida, USA) was used for recording and integrating of the chromatograms. Average areas under the peaks of three independent chromatographic runs were used for calculation the percentage composition of each component.

GC-MS analysis

The analyses were carried out on Focus GC® (Thermo Fisher Scientific®, Milan, Italy) equipped with the same column and conditions mentioned in the GC/FID. The capillary column was directly coupled to a quadrupole mass spectrometer Polaris Q, (Thermo Electron Corp.®, Milan, Italy). The injector temperature was 220°C. Helium carrier gas flow rate was 1.5 ml/ min. All the mass spectra were recorded with the following condition: filament emission current, 100 mA; electron energy, 70 eV; ion source, 250°C; diluted samples were injected with split mode (split ratio, 1: 15).

Compound identification and quantitation

The identification of the oil constituents was based on a comparison of their linear retention indices (determined by the Kovats method) relative to (C8–C22) homologous series of *n*-alkanes with those of literature. Further identification was made by comparing their spectral data and retention indices with Wiley Registry of Mass Spectral Data 8th edition, NIST Mass Spectral Library (December 2005), and the literature (Adams, 2004).

Antifungal activity

Fungal isolates

The fungal isolates included clinical isolates of *Candida albicans* (RCMB 005009) and *Aspergillus niger* (RCMB 002007) obtained from The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt. The isolates were cultured in Sabouraud dextrose agar for 48 h at 25°C.

Disc diffusion assay

MIC values of the individual samples were determined by the disc diffusion assay method. The oils were serially diluted two-fold over a range of 200 to 0.39 µg/mL with 10% DMSO. Amphotericin B was used as the reference antifungal control. Two-fold serial dilutions of amphotericin B ranging from 50 to 1.56 µg/mL were used. Filter paper discs containing 10µL of different concentrations of each oil were applied to the surface of the inoculated plates and left for 30 min at room temperature to allow the diffusion of the oils. The plated were then incubated at 25°C for 48 h. The MIC (Minimum Inhibitory Concentration) was defined as the lowest concentration that did not result in any visible growth of the microorganism compared with the growth in the control plate (Rosato *et al.,* 2007).

Checkerboard titer test

To assess the synergism between amphotericin B and each of the oils, the checkerboard titer test was used. Twelve serial two-fold dilution of the oils and 8 two-fold dilutions of amphotericin B were prepared. From each dilution, 50 µl of each oil was added to the wells of 96 well plates in vertical orientation and 50 µL of amphotericin B dilution was added in horizontal orientation. In this way, all amphotericin B dilutions were mixed with the appropriate oil concentrations, thus obtaining a series of different concentrations. The analyses of the combination of the oils and amphotericin B were performed by calculating the fractional inhibitory concentration (FIC). The results were interpreted as a synergetic effect when the value of the FICI was ≤ 0.5, as indifferent when it was > 0.5 - 2.0, and as antagonistic effect when it was > 2.0 (Davidson and Parish, 1989). The synergic effect is shown graphically by applying the Isobole method (Wagner and Ulrich-Merzenich, 2009).

Statistical analysis

All assays were duplicated and all the data were statistically evaluated using student's *t*-test (GraphPad Prism® 5.01, GraphPad Software, Inc., CA, USA) followed by Dunn's post-hoc multiple comparison test when the significance value is < 0.05 using the same significance level. The criterion for statistical significance was taken as $P < 0.05$.

RESULTS AND DISCUSSION

GC-FID and GC-MS analyses

Analysis and compound identification of the investigated oils revealed the following main components (≥ 4%) observed in Table 1: the main components of thyme oil included thymol (46.2%) and p-cymene (34.6%). Cinnamon oil was comprised mainly of cinnamaldehyde (90.2%) while clove oil constituted mainly of eugenol (47.1%) and *β*- caryophyllene (30.4%). Cineole was the main component of eucalyptus oil (93.7%) while ginger oil constituted mainly of zingiberene (31.3%) and geranial (10.9%). These results are in accordance with previously published data (Chaieb *et al.,* 2007; Chizzola *et al.,* 2008; Gilles *et al.,* 2010; Giordani *et al.,* 2006; Singh *et al.,* 2008). The studied essential oils constituted a high percentage of components with prominent antimicrobial activity including phenolic terpenoids as thymol in thyme oil and eugenol in clove oil as well as oxygenated terpenoids as cineole in eucalyptus oil and geranial in ginger oil (Bakkali *et al.,* 2008; Burt, 2004).

Antifungal activity

The antifungal activities of the five investigated oils are shown in Table 2. Amongst the five oils, thyme oil showed the most effective growth inhibition against *C. albicans* and *A. niger* with MIC values of 25 and 50 ug/mL respectively followed by cinnamon oil with MIC values of 50 ug/mL against both species. Both clove oil and eucalyptus oil showed moderate activity with MIC values of 100 ug/mL with a slightly higher inhibiting activity for clove oil against *C. albicans* at an MIC value 50 ug/mL. Ginger oil showed no apparent activity against either species.

Synergetic effect of oils and amphotericin B

To explore the possibility of developing a more potent combination therapy of the tested antifungal oils with amphotericin B, the checkerboard micro titer test was performed. Results of the combinations of the oils with the reference antifungal agent, amphotericin B were emphasized using the FIC values as shown in Table 2 and the Isobolograms revealed in Figure 1. Ginger oil was disregarded from this study for its inactivity against both species in the previous results. A highly synergetic effect was shown when using thyme oil against *C. albicans* as well as *A. niger* significantly potentiating the effect of amphotericin B from an MIC of 8.0 and 16.0 ug/mL to 2.0 ug/mL with FICI values of 0.25 and 0.13 respectively. Another significant synergetic effect was shown using cinnamon oil against *C. albicans* where the MIC of amphotericin B was lowered from 8.0 to 2.0 ug/mL with an FICI value of 0.28, however when tested against *A. niger*, an additive effect was observed (FICI = 1). The significant synergetic results are also depicted in Figure 1(a) for thyme oil and Figure 1(b) for cinnamon oil by the distinctly left-deviating curves (Davidson and Parish, 1989). Even though the MIC values of Amphotericin B were lowered as a result of all the oil combinations but results revealed that the effect of both clove oil and eucalyptus oil against both species was indifferent at FICI values between 0.5 and 2.

The significant activity of thyme oil is reported widely in literature attributed to its thymol content (Ahmad *et al.,* 2010; Saad *et al.,* 2010). The activity of cinnamon oil is mainly attributed to the high percentage (91.2%) of the aromatic aldehyde, cinnamaldehyde reported to be the main antimicrobial component in this oil (Giordani *et al.,* 2006). These results are in complete coherence with that obtained from the synergetic study of thyme and cinnamon oil revealing the highest potentiation of amphotericin B antifungal activity by the former oil against *C. albicans* and *A. niger*. Many studies on the antifungal activity on the essential oils of *Eucalyptus* species are reported in literature; the oils are regarded as potent antibacterial than antifungal agents and occasionally show a higher inhibition on *Candida* species over *Aspergillus* species (Tyagi and Malik, 2011). The activity of eucalyptus oil is usually attributed to the whole oil rather than its main component cineole (Vilela, 2009). The phenolic components; eugenol and thymol were shown to possess potent antifungal activity against *Candida albicans.* This activity was attributed in a recent study to the Inhibition of H^+-ATPase leading to intracellular acidification and cell death which was more prominent in case of thymol (Ahmad *et al.,* 2010). This may provide an explanation for the potency of thyme oil over clove oil in the presented study. Clove oil rich in eugenol that may reach 90% in some clove oils was also reported to inhibit the growth of *Aspergillus niger* (Chaieb *et al.,* 2007; Pawar and Thaker, 2006) which is consistent with the presented results showing moderate activity of the studied clove oil constituting 48.1% of eugenol. The antimicrobial

properties of these compounds are partly associated with their lipophilic character, leading to their accumulation in membranes and to subsequent membrane-associated events such as energy depletion. Phenolic components of essential oils sensitize the phospholipids bi-layer of the cell membrane, causing an increase of permeability and leakage of vital intracellular constituents or impairment of microbial enzyme systems (Gilles *et al.*, 2010). Ginger oil showed no activity against either species, this may be rationalized by the absence of phenolic components (eugenol, shogaols, zingerone, gingerdiols, gingerols, etc.) that attribute to the antifungal activity of ginger oil as mentioned in literature (Ali *et al.*, 2008; Singh *et al.*, 2008).

Table. 1: Main components and concentrations of oils.

Thyme oil		**Cinnamon oil**		**Clove oil**		**Eucalyptus oil**		**Ginger oil**	
Compound	**Conc. %**	**Compound**	**Conc. %**	**Compound**	**Conc. %**	**Compound**	**Conc. %**	**Compound**	**Conc. %**
Thymol	46.2	cinnamaldehyde	90.2	eugenol	47.1	cineole	93.7	zingiberene	31.3
p-cymene	34.6	benzaldehyde	4.6	*β*- caryophyllene	30.4			geranial	10.9
γ-terpinene	6.6	cinnamyl acetate	4.1	eugenyl acetate	19.4				
sabinene	4.5								
carvacrol	4.2								

Table. 2: Antifungal activity of selected oils alone and in combination with amphotericin B against *C. albicans* and *A. niger* showing minimum inhibitory concentrations (MICs), fractional inhibiting concentrations (FICs) and FIC indices (FICIs).

	Candida albicans				***Aspergillus niger***			
	MIC_a	MIC_c	FIC	FICI	MIC_a	MIC_c	FIC	FICI
Thyme oil – Amp B								
Thyme oil (ug/mL)	25.0	0.024	0.001		50.0	0.39	0.008	0.133
Amp B (ug/mL)	8.0	2.0	0.25	0.25	16.0	2.0	0.125	
Cinnamon oil – Amp B								
Cinnamon oil (ug/mL)	50	1.56	0.031		50.0	25.0	0.5	1.0
Amp B (ug/mL)	8.0	2.0	0.25	0.28	16.0	8.0	0.5	
Clove oil – Amp B								
Clove oil (ug/mL)	50.0	6.25	0.125		100.0	50.0	0.5	1.0
Amp B (ug/mL)	8.0	4.0	0.5	0.625	16.0	8.0	0.5	
Eucalyptus oil – Amp B								
Eucalyptus oil (ug/mL)	100	50	0.5		100.0	25.0	0.25	0.75
Amp B (ug/mL)	8.0	4.0	0.5	1.0	16.0	8.0	0.5	
Ginger Oil	No Activity							

MICa, MIC of one sample alone; MICc, MIC of one sample of the most effective combination
FIC of oil = MIC of oil in combination with Amp B / MIC of oil alone
FIC of Amp B = MIC of Amp B in combination with oil / MIC of Amp B alone
FICI = FIC of oil + FIC of Amp B

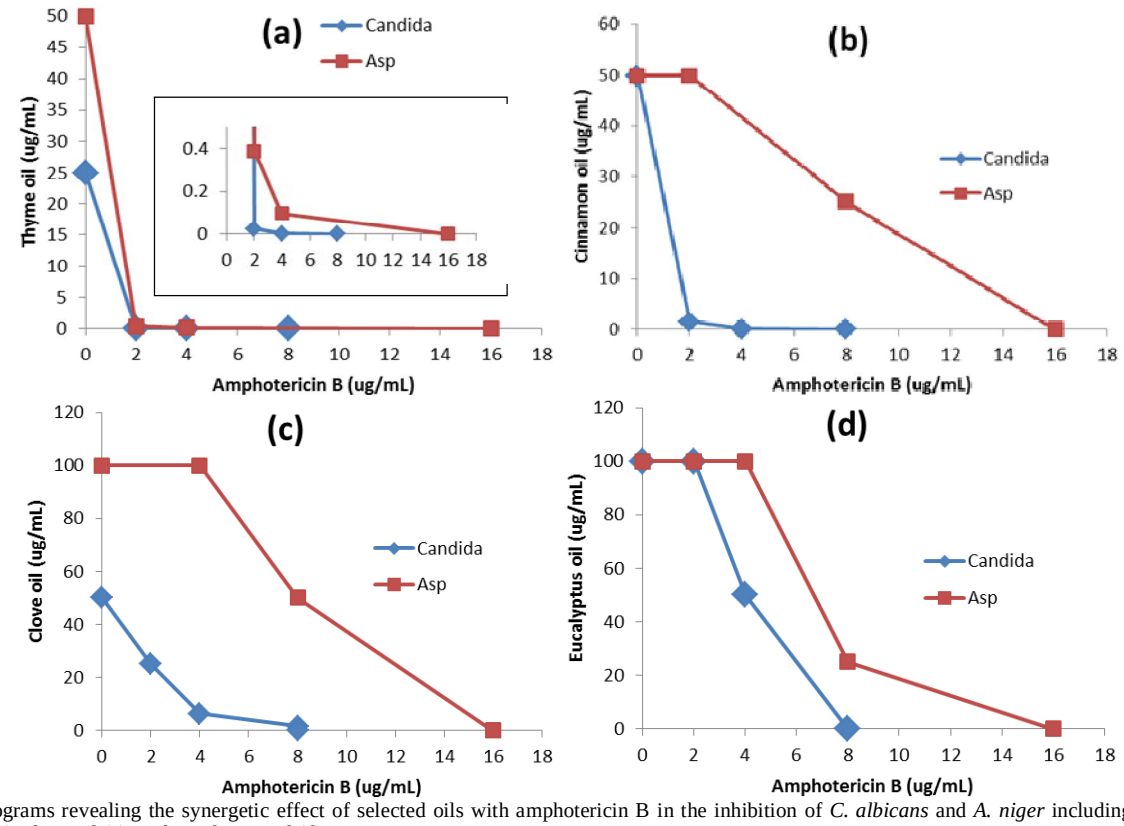

Fig. 1: Isobolograms revealing the synergetic effect of selected oils with amphotericin B in the inhibition of *C. albicans* and *A. niger* including thyme oil (a), cinnamon oil (b), clove oil (c), and eucalyptus oil (d).

CONCLUSION

The combination of thyme oil and amphotericin B is suggested for the treatment of infections caused by *C. albicans* and *A. niger*, and may reduce the efficacious dose of amphotericin B, thus minimizing its side effects. This field of synergistic studies is encouraged on a broader scope involving other pathogenic organisms. Also, *in vivo* experiments are necessary to assess the potential for therapeutic applications for these drug combinations.

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