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RESEARCH ARTICLE

THE ANTIFUNGAL PROPERTIES OF SOME MEDICINAL PLANTS EXTRACTS

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ABSTRACT

The antifungal activity of Alcoholic Medical Extracts (AMPE) of Leaf Thyme, *Thymus mastichina L.* Lamiaceae (AMPE-1); Leaf Rosemary, *Rosmarinus officinalis* L., Lamiaceae (AMPE-2); Cloves, *Eugenia caryophyllus* (AMPE-3); Bark, *Cinnamomum zeylanicum* (AMPE-4) and Roots Ginger, *Zingiber officinale* L. Zingiberaceae (AMPE-5), on fungal species suchas *Aspergillus flavus, A.niger and A.ochraceus*were evaluated by a standard disc diffusion method after determining the activated agents in each medical plants. Besides, the effects of each AMPE on the ability of each fungal on mycotoxins producing were estimated. The results showed that AMPE contained the most activated agentssuch as Flavonoids, Polyphenols, phenolic glycosides, tannins, alkaloids, saponins and cyanogenic glycosides compounds that's assessed. The minimum inhibition concentrations for each of AMPE against *Aspergillus flavus, A.niger* and *A.ochraceus* showed an inhibitory activation at 25 mg/ml in some AMPE .The inhibition activity was found to increase with the higher concentrations of other serial concentrations. The values of 25, 50 and 100 mg/ml from each AMPE antifungals activity revealed by the standard disc diffusion method showed that the inhibition activity were significantly ($p<0.05$) increased with the increased of each AMPE concentration. The concentration of 100 mg from each AMPE types was found to have an inhibitory activation at a range between 12 to 19 mm for the fungal strains. The results of the AMPE type's effects on mycotoxins produced from the fungal strains revealed that AMPE-1 and AMPE-2 at a concentration of 100 mg/ml increased significantly the Aflatoxin B1 and ochratoxin A production while other concentrations of AMPE types decreased significantly mycotoxins productions.

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INTRODUCTION

The medicinal plants extracts have found multi potential applications in traditional medicine, phyto-preparations, cosmetic, perfume industries, and foodtechnology (Gali-Muhtasib *et al.,* 2004; El Astal *et al*., 2005; Abu-Dar wish, and Al-Ramamneh, 2012).An increased number of new antibiotics from the pharmacological industries were produced during last four decades. Likewise the resistance to these drugs by microorganisms has also been increased (Cohen, 1992).The microbial resistance to antibiotics coupled with side effects of existing drugs urged the need for new drugs that are structurally and functionally different from currently used ones.

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The current trend is turning to" nature"to offer appropriate and efficient antimicrobial drugs to the patient (Stahl-Biskup, and Saez, 2002;Nascimento, *et al*., 2000). Examination of indigenous plants material have been reported from all around the world (Ahmad and Beg, 2001; Penna *et al*., 2001). Most of their propertiesare due to the active materials that able to control microorganisms related to food spoilage by their antimicrobial effects (Elgayyar *et al*., 2001; Canillac and Mourey, 2001). These properties are due to many active phytochemicals, including flavonoids, terpenoids, carotenoids, coumarinsand curcumins (Tepe *et al*., 2005). They are usually obtained by steam or hydro-distillation (Nasir, *et al*., 2015). Aspergillusis a genus consisting of several hundred mold species found in various climates worldwide known to cause health problems in humans *(*Samson *et al*., 2001; Steinbach and Stevens, 2003).The incidence of mediated *Aspergillus* infections, that can sometimes be fatal, has dramatically increased with the increase in patients with compromised

immune systems.(Crampri *et al.,* 1998a). In addition, this mold is the leading cause of ABPA (allergic bronchopulmonary (Aspergillosis) and is one of the major risk factors leading to allergic asthma (Crampri *et al.,* 1998b). The toxic effects of mycotoxins such as aflatoxins, ochratoxins and trichothesises, etc. are mostly known from veterinary practice (AL-Malikey, 2009). Mycotoxicosis, which can occur in both industrialized and developing countries, arise when environmental, social and economic conditions combine with meteorological conditions as humidity and temperature which work in favor of molds growth (Steyn, 1998). Involvement of mycotoxins in disease causation should be considered when a disease appears in several persons, with no obvious connection to a known etiological agents, such as microorganisms (Peraica, *et al*., 1999). Therefore, this study was undertaken to investigate the five medical plants composition as *Thymus mastichina* L.; *Rosmarinus officinalis* L.; *Eugenia caryophyllus*;*Cinnamomum zeylanicum* and *Zingiber officinale* L. for the active materials contents and to find out the inhibitory effects of its extracts on the growth of the *Aspergillus flavus, A. niger* and *A. ochraceus* and on the ability to AFB1(AFB1) and OCA(OC-A) production from *Aspergillus flavus* and *A. ochraceus*, respectively.

MATERIALS AND METHODS

Medicinal plants samples collections: A total of five samples from medical plants at ten replicates for each one were collected randomly from Turkey markets in Istanbul at one kilogram distance from each other .1. Leaf Thyme (*Thymus mastichina L*. Lamiaceae); 2. Leaf Rosemary (*Rosmarinus officinalis* L., Lamiaceae); 3. Cloves (*Eugenia caryophyllus*); 4. Bark (*Cinnamomum zeylanicum*) and 5. Roots Ginger (*Zingiber officinale* L. Zingiberaceae) were situated in polymers packages. The samples then cleaned by removal the foreign materials when needed (Roberts and Greenwood, 2003).

Medicinal plants extraction: The plant pieces collected were gently plucked, washed and finely cut into pieces. The plant materials were then subjected to the hydro distillation process for a period of 3 h using a Clevenger type apparatus (Sokovi´c and Van Griensven, 2006). Hydro-alcoholic extraction for each medicalplantswas conducted according to method adopted byTajkarimi, (2010), with some modification. Samples of 200-250g from the dried and powdered medical plant materials were treated using 80% methanol. The filtration process was performed through What man filter paper No.1. The solvent was evaporated using a Rotary evaporator and the powder extracts were kept in a stoppered sample vial at 4º C until used in following tests.

Chemical constituents assay: part of each of the extracts was used in screenings for the major secondary metabolites such as polyphenols, cyanogenic glycosides, saponins, phenolic glycosides, flavonoids, tannins, and alkaloids according to (Debella, 2002).

Screening for antifungal potential of the Medicinal plant extracts

The medical plants extracts antifungal screening was conducted through cultivation of each fungalspecies on Saboroued dextrose agar (SDA) medium at 28 °C for 5 days. From each fungal speciesa preparationof theconidial suspension were made available at a concentration of 1×10^6 conidia/mL with adding a 3-4 drops of Tween 40 (Martinez *et al*., 1996; Dabur *et al*., 2004). The extracts antifungal activity concentrations ranges were determined by the Minimal Inhibitory Concentration (MIC) for each fungal species *A.flavus, A.niger and A.ochraceus*. 100 µl of 10^6 conidial/mL from each fungal species was inoculated in tubes with yeast extract sucrose broth medium (YES) supplemented with different concentrations (10 – 1000 mg/ml) of the medical extracts, respectively. After cultivation for 72 h at 28 °C, the MIC of each extracts samples were determined by measuring the optical density in the spectrophotometer at 620 nm, comparing the samples readout with the non-inoculated (YES) broth. MICs were determined at the lowest concentration that inhibited visible fungal growth. Nystatin was used as positive control.

The standard disc diffusion method (Kirby-Bauer Disc method), which described by Goldman and Green, (2008) with some modification was used to determine the medical extracts antifungals activity that followed by an inoculation of each fungal species on the surface of SDA plates. Subsequently, filter paper discs (6 mm in diameter) saturated with 50 µl from either medical extract of active concentration which determined in MIC test and placed on surface of each inoculated plate. Evaluation the efficiency of the methodology, each extract was inserted simultaneously in a well at 50 µl from the sample concentrations in new plates. The plates were incubated at 28 °C for 72 h. After this period, it was possible to observe an inhibition zone. Generally, fungal cultured with halos equal to or greater than 7 mm were considered susceptible to either the medical extract tested. DMSO and Tween 40 to 2% were used to dissolve the extracts in the culture media when necessary. The controls were the solvents used for each extract and they showed no inhibitions in preliminary studies. For the positive control, Nystatin corresponding wells were used.

Anti-mycotoxins potential of the Medical extracts: The effects of 25, 50 and 100 mg of each alcoholic medical extracts(AMPE) on AFB1 and OCA production from each *Aspergillusflavus and A.ochraceus*. species on cultures initiated from conidia were studied according the procedure in Martinez, *et al*., (1996) with some modification using 50-ml Erlenmeyer flasks containing 15ml of yeast extract sucrose (YES) medium. Medical extracts was added by diluting a stock solution of each extracts in distilled water, and transferring 1.0 ml to each flask to yield the desired final concentrations. Each flask then received 0.4 ml of each Aspergillus species conidial suspension to produce an inoculum of approximately 1.0 x 10^6 conidia/ml. All flasks were incubated at 28°C for 7 days, then autoclaved at 121°C for 15 mint. and stored at 4 °C in order to be analyzed for AFB1 or OCA production.

Aflatoxins analysis: After autoclaving the fungal growth medium, each flask components was extracted thrice with 20 ml portions of CHCI3.Then the toxin samples extracts were more purified by using the chromatographic column and eluted aflatoxins by hexane (AOAC, 2002). The extracts were

concentrated on a rotary evaporator (Heidolph, Germany), and stored in a small screw glass container at 4 c until used in the analysis. The quantification of aflatoxin B1 (AFB1) contents was determined using high performance liquid chromatography (HPLC, LC-10-Shimadzu-Japan system) according to Clara, *et al*., (2002), with some modifications as follow: each extraction samples were dissolved in 1 ml of chloroform. AFB1 were determined in extracts in HPLC system which used pump 4015 and fluorescence detector at 366 nm, flow rate at 0.8 ml/ mint. 20 µl of the samples was used to be injected. All unknowns toxin in samples were compared with the standard solution of AFB1 provided by Sigma Company (USA).

Ochratoxin A assay: each autoclaving fungal growth flasks were homogenized well after mixing with 60% of acetonitrile. The extraction of the OCA was performed through elution with hexane in the chromatographic column that prepared according the (AOAC, 2002). The eluted then concentrated using the rotary evaporator and re-dissolved with 1 ml of methanol in small glass screw containers and stored at 4 c until used in OCA analysis. Methanol samples were analysis to OCA content according the procedure mentioned in Pena *et al*., (2006), by injection in the pump of HPLC system (LC-10-Shimadzu-Japan) which used pump 4015 and fluorescence detector at 340 nm, flow rate 0.5 ml/ mint. 50 µl for the samples was used for injection. All unknowns toxin in samples was compared with the standard solution of OCA provided by Sigma Company (USA).

Statistical analysis: Data were analyzed by the ANOVA analysis, using the general linear model of the Statistical Analysis System (SAS Institute, 2001). Significant treatment differences were evaluated using Duncan's multiple-range test (Duncan, 1955). All statements of significance are based on the 0.5 level of probability.

RESULTS AND DISCUSSION

Phytochemical composition of AMPE: The results presented in Table (1) revealed that the active ingredients which have been found in each medical plant were inconsistent.

Table 1. The activated materials in alcohol Medical extracts (AMPE) types

Medical types	Types of some activated agents in Medical contents						
	Flavonoids	Polyph-enols	Phenolic glycosides	Tannins	Alkaloids	Saponins	Cyanogenic glycosides
AMPE-1	+	+	+	+	+	+	-
AMPE-2	+	+	+	-	-	+	+
AMPE-3	+	+	+	+	+	-	+
AMPE-4	+	+	+	+	+	+	+
AMPE-5	+	+	+	+	+	+	+

(-) not found, (+) found.AMPE-1.*Thymus mastichina L*.AMPE-2.*Rosmarinus officinalis* L. AMPE-3.*Eugenia caryophyllus*AMPE-4.*Cinnamomum zeylanicum*AMPE-5.*Zingiber officinale* L.

The Leaf Thyme (*Thymus mastichina L*., Lamiaceae) constituents were Flavonoids, Polyphenols, Phenolic Glycosides, Tannins, Alkaloids and Saponins. It's apparent that there no cyanogenic glycosides compounds in leaf thyme. Our findings were found to be in agreement with the results reported in (Fullas, 2003), they found that the medical plants extracts studied containing an essential oils such as Thymol, Arvacrol, Flavonoids, Polyphenols, Phenolic glycosides, Tannins, Alkaloids, and Saponins but not the Cyanogenic glycosides (Bisset, 1994). Leaf of the Rosemary (*Rosmarinus officinalis* L., Lamiaceae) was found to contain an active ingredients of the following: Flavonoids, Phenolic acids (Caffeic, Chorogenic and Rosmarinic), essential oils (Camphor and Cineole) and diterpenes (Carnosol). Furthermore, no ingredients of Tannins and Alkaloids were recorded. The finding of our investigation were in agreement with (Newall, *et al*., 1996) whom they found that the alcoholic Rosemary extract contain the sample active ingredient as that mentions in this study. Cloves (*Eugenia caryophyllus*) medical plant extracts was found to contain all the active ingredients mentioned so far excluding saponins compounds. Bark (*Cinnamomum zeylanicum*) was contains all the active ingredients found in other medical extracts of this study. Besides, Ginger roots (*Zingiber officinale* L. Zingiberaceae) extracts also contained the sample active ingredients of other medical extracts recorded in this study.The phytochemical analysis for MAPE revealed that the components were markedly different in composition. The variations in the type's components have been attributed to some or more of intrinsic factors including pieces, cultivar, clone, ecotype and ecological factors and technological factors (Russo et al., 2012).

AMPE Antifungal activities

All Aspergillus spp. strains were screened for their sensitively to the fifth AMPE types effects. Table (2) presents the results of the MIC test for strains sensitivity to the fifth AMPE. It's evident that all strains appeared to be resistant to 10 mg from each AMPE in the medium. Besides, the sensitivity of strain *A. flavus* to 25 mg was evident with AMPE-1 and 5 whereas, that of *A.ochraceus* was sensitive to AMPE-2 and 4. Increasing the AMPE concentration up to 50 mg resulted in an initial inhibition for all fungal strain, with the exception of *A.niger* which was found to be resistant to AMPE-2 and 5. Furthermore, increased the concentration up to 100 mg caused an inhibition to all fungal strains at middle range with the exception of *A.niger* which showed a sensitivity to AMPE 2 and 5 at lower ranges.

All fungal strains was found to be sensitive for all AMPE at middle ranges for 250 mg and at high ranges for 500 and 1000 mg of AMPE in medium. The standard disc diffusion method (Kirby-Bauer Disc method), that determine the antifungals activity of 25, 50 and 100 mg/ml concentration for each AMPE (Table 3).

Table 2. The Minimal Inhibitory Concentration (MIC) for alcohol Medical extracts (AMPE) types against some fungal species

Medical types No.	Fungal species	AMPE concentrations (mg/ml) of media							
		0	10	25	50	100	250	500	1000
AMPE-1	*A.flavus*	-	-	+	+	++	++	+++	+++
	A. niger	-	-	-	+	++	++	+++	+++
	A.ochraceus	-	-	-	+	++	++	+++	+++
AMPE-2	*A.flavus*	-	-	-	+	++	++	+++	+++
	A. niger	-	-	-	-	+	++	++	++
	A.ochraceus	-	-	+	+	++	++	+++	+++
AMPE-3	*A.flavus*	-	-	-	+	++	++	+++	+++
	A. niger	-	-	-	+	++	++	+++	+++
	A.ochraceus	-	-	-	+	++	++	+++	+++
AMPE-4	*A.flavus*	-	-	-	+	++	++	+++	+++
	A. niger	-	-	-	+	+	++	+++	+++
	A.ochraceus	-	-	+	+	++	++	+++	+++
AMPE-5	*A.flavus*	-	-	+	+	++	++	+++	+++
	A. niger	-	-	-	-	+	++	+++	+++
	A.ochraceus	-	-	-	+	++	++	+++	+++

(-) resistant activity, (+) Low sensitive, (++) Middle sensitive, (+++) High sensitive AMPE-1.*Thymus mastichina L.*AMPE-2.*Rosmarinus officinalis* L. AMPE-3.*Eugenia caryophyllus*AMPE-4.*Cinnamomum zeylanicum*AMPE-5.*Zingiber officinale* L.

Table 3. Effect of alcohol Medical extracts (AMPE) types on fungal species inhibitions

Medical types No.	Conc. (mg/ml)	Fungal inhibition zone dimeter (mm)		
		A.flavus	*A. niger*	*A.ochraceus*
AMPE-1	25	^{f}9±0.62	^{g}5±0.70	^{e}6±0.47
	50	^{e}12±0.94	^{e}9±1.03	^{d}12±1.31
	100	^{c}16±0.76	^{c}13±0.81	^{c}15±2.63
AMPE-2	25	^{g}3±0.11	^{g}5±0.24	^{e}7±0.56
	50	^{d}14±0.73	^{d}11±0.92	^{d}12±0.95
	100	^{b}18±2.22	^{b}16±3.2	^{b}17±3.07
AMPE-3	25	^{g}3±0.21	^{g}4±0.33	^{g}3±0.22
	50	^{f}9±0.60	^{d}11±1.15	^{e}7±0.90
	100	^{e}13±1.54	^{d}12±0.92	^{d}12±0.85
AMPE-4	25	^{g}4±0.31	^{f}7±0.64	^{f}5±0.16
	50	^{d}15±0.83	^{d}12±0.85	^{c}15±2.11
	100	^{b}19±3.98	^{b}17±1.71	^{b}18±1.94
AMPE-5	25	^{f}9±0.70	^{g}5±0.72	^{e}6±0.44
	50	^{e}13±1.30	^{d}12±1.28	^{c}15±2.40
	100	^{c}17±2.71	^{c}14±0.97	^{b}18±3.17
Nystatin	5	a22±2.02	a19±1.85	a20±1.29

AMPE-1.*Thymus mastichina L.*AMPE-2.*Rosmarinus officinalis* L. AMPE-3.*Eugenia caryophyllus*AMPE-4.*Cinnamomum zeylanicum* AMPE-5.*Zingiber officinale* L.

Table 4. Effect of alcohol Medical extracts (AMPE) types on mycotoxins producing levels from fungal strains

Medical types No.	Conc. (mg/ml)	AFB1	OCA
		ηg/ml	
Control	0	213±7.11	104±4.71
AMPE-1	25	128±5.38	58±4.27
	50	133±6.06	65±3.72
	100	255±6.48	123±4.50
AMPE-2	25	205±6.22	95±7.36
	50	211±5.81	110±4.64
	100	220±7.44	119±3.82
AMPE-3	25	156±5.05	74±4.83
	50	93±6.38	41±3.22
	100	51±3.51	21±1.60
AMPE-4	25	201±7.60	100±2.83
	50	182±5.72	94±8.63
	100	215±8.44	91±4.49
AMPE-5	25	190±6.25	101±7.90
	50	184±5.72	105±5.43
	100	180±5.90	108±3.64
Nystatin	5	202±6.47	96±4.67

AMPE-1.*Thymus mastichina L.*AMPE-2.*Rosmarinus officinalis* L. AMPE-3.*Eugenia caryophyllus*AMPE-4.*Cinnamomum zeylanicum*AMPE-5.*Zingiber officinale* L.

It's apparent that the inhibition activity were found to be increased significantly ($p<0.05$) with increasing the AMPE concentrations. Moreover, the inhibitory effects of each AMPE were similar to that of the antibiotics Nystatin range effects. The results of this study confirmed that the 100 mg from AMPE-1inhibits the *A.flavus, A.niger* and *A.ochraceus* strains. The inhibition zone dimeters values were 16, 13 and 15 mm respectively. Likewise, the extraction concentration of AMPE-2inhibits the above fungal species and the inhibition zone dimeters were 18, 16 and 17 mm, respectively. The AMPE-3 caused an inhibition to all fungal species at zone dimeters of 13, 12 and 12 mm respectively. The AMPE-4 and AMPE-5 inhibition zones diameters against all fungal species were at 19, 17, 18 mm and 17, 19, 20 mm, respectively. The results revealed that AMPE-3 as *Eugenia caryophyllus* recorded the lowest inhibition activity values compared with other AMPE types. this may be refer to the variations in the concentration of chemical composition of AMPE that's not assay in this study, which was attributed to many factors, including the environmental (Tigrine-Kordani *et al*., 2007), abiotic stress (Tounekri *et al*., 2011), genetic heritance (Jordan *et al*., 2013)..

AMPE activity on mycotoxins production: The effect of AMPE types on AFB1 or OCA produced from *A.flavus* and *A.ochraceus* were presented in table (4). The results indicated that 100 mg for both the AMPE-1 and AMPE-2 increased significantlyAFB1production, and the values were 255 and 220 ηg/ml, respectively, whereas OCA values were 123 and 119 ηg/ml, respectively. Other concentration values for AMPE were found to reduce significantlyAFB1 and OCA production as compared with the control group giving a concentration values of 213 and 104 ηg/ml respectively. The reason underlying the increased production of AFB1 or OCA in the presence of AMPE as a result of that AMPE affected on reducing and depressing fungal growth. This could resulted in an elevation of the cytoplasmic level of two-carbon precursors for mycotoxins synthesis, and accordingly increase production of the toxins (Moghtader, 2012). The degradation ability of the AMPE on AFB1 or OCA may be attributed to the AMPE compounds and concentrations contains. The previous studies referred that the effects of AMPE were as antioxidants actions, which were damages the coumarin rings and the double bonds in the structure of AF or OCA which leads to reduce the concentration of the mycotoxins (Eugénia, *et al*., 2006; Albert, *et al*., 2009).

The cytotoxic capacity of the phytochemical compositions of the medical plants based on a pro-oxidant activity can make them excellent antiseptic and antimicrobial agents for personal use such as personal hygiene, or for the preservation of foods (Tsuneki *et al*., 2005). A big advantage of phytochemical compositions is the fact that they are usually devoid of long-term genotoxic risks. Moreover, some of them show a very clear antimutagenic capacity which could well be linked to an anticarcinogenic activity (Manosroi *et al*., 2006; Wu, *et al*., 2006; Kachadourian and Day, 2006).

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