J. Food and Dairy Sci., Mansoura Univ., Vol. 7 (5): 273 - 282, 2016

Antifungal and Antioxidant Activities of Rosemary (*Rosmarinus officinalisL.*) Essential Oil

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ABSTRACT

Using of natural antifungal preservatives, especially extracted from herbs is innovatively used, since plant matrices possesses antifungals and antioxidants compounds. The current work was undertaken to evaluate the antifungal and antioxidant activities of Rosemary (*Rosmarinus officinalis*L.)essential oil (REO). REOcontained high amount of Total phenolic compounds (TPC)exude high radical scavenging activity toward DPPH, ABTS and linoleic acid radicals as well as iron chelating activity. The TPC of REO was 198.8 mg GAE g^{-1} demonstrated 234.7µmol of TE g^{-1}DPPH radicals scavenging activity and 291.3µmol of TE g^{-1} ABTS radicals scavenging activity. The antioxidant capacity of REO exhibited 75.8% reduction when evaluated by β-carotene bleaching assay. The reducing power activity related to iron chelating was198.1 µmol of AAE g^{-1}.The CEO exhibit a high of 1,8-Cineole content (32.65%) over 20 identified components by GC-MS analysis. Furthermore, REO exhibited antifungal activity *in vitro* at low concentrations against tested food borne pathogens. The concluded results fromMIC and hyphal extension inhibition test indicated that REO was affected either growth or hyphae ofFusariumefficiently *in vitro*. The MIC was in range of 300 – 1000 mg L^{-1}. The mode of action of *in vitro* experiment exhibited that REO was effective to affect the fungal cell thenpermeabilize the cell membrane. Therefore, REO can be reliably applied in commercial applications as antioxidant, antifungal and flavoring agent as seperate or in combination with traditional preservatives for controlling the undesirable organoleptic and fungal deteriorationsas well as mycotoxins in foodstuffs.

Keywords:*Rosmarinus officinalis*, essential oil, antifungal activity, antioxidant activity, *in vitro*.

INTRODUCTION

Hence, there is a strong controversyabout the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. A considerable interest has developed on natural foods preservatives by using of the spices's essential oils to effectively retard growth and mycotoxin production as well as extend the shel-life stability. Therefore, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable preservatives has been intensively investigated (Burt and Reinders 2003, Burt 2004). The exploration of naturally occurring antimicrobials for food preservation receives increasing attention due to consumer awareness of natural food products and a growing concern of microbial resistance towards conventional preservatives (Pintore *et al.* 2009). Antimicrobial properties of plant material such as herbs and spices have been recognized for food preservation and in medicine. Conner (1993) confirmed that natural antimicrobial agents dating back more than a century. A renewed interest in natural preservation appears to be stimulated by present food safety rules. Numerous studies have documented the antifungal (Cairns and Magan 2002, Bluma and Etcheverry 2008) and antibacterial (Canillac and Mourey 2001, Dorman and Deans 2000) effects of plant essential oils.

The growth of fungi in food may cause dramatic spoilage and reduction in quality and quantity. The antimicrobial properties of essential oils derived from many plants have been empirically known for centuries, but scientifically confirmed only recently (Silva and Fernandes 2010, Fratianni *et al.* 2010, Serrano *et al.* 2008). Useful uses of these activities have long been proposed in humans and animals, but only in the last years has it been described that some essential oils are proficient of inhibiting foodborne microorganisms and extending the shelf-life of processed food (Vardar-Ünlü *et al.* 2007, Schelz; Hohmann and Molnar 2010, Suhr and Nielsen 2003). Among the aromatic plants fitting to the Lamiaceae family, the genus rosemary is noteworthy for the abundant species and varieties of wild-growing plants. In recent years, several reports have been available concerning the composition and/or the biological properties of Rosemaryessential oils (Celiktas *et al.* 2007, Angioni *et al.* 2004, Okoh; Sadimenko and Afolayan 2010, Sacchetti *et al.* 2005). These studies have stressed the existence of noticeable chemical differences among oils extracted from different species or varieties. These variations are likely to impact the antimicrobial activity of the oil and are normally a function of three factors: genetically determined properties, the age of the plant and the environment (Sacchetti *et al.* 2005).

With a view for controlling fungi and mycotoxin production, the EO from *R. officinalis* demonstrated obvious antifungal activity against *Aspergillus parasiticus* growth and its aflatoxin production. Rosemary EO was analyzed chromatographically being exhibited a major components as Piperitone (23.65%), α-pinene (14.94%), Limonene (14.89%), 1,8-Cineole (7.43%) (Rasooli *et al.* 2008).Okah*et al.*(2010) GC–MS analyses of the oils exposed the presence of 24 and 21 compounds in the essential oils obtained through hydro-distillation (HD) and solvent free microwave extraction (SFME). Higher amounts of oxygenated monoterpenes such as borneol, camphor, terpene-4-ol, linalool, a-terpeneol (28.6%) were present in the oil of SFME in comparison with HD (26.98%). However, HD oil contained more monoterpene hydrocarbons such as a-pinene, camphene, β-pinene, myrcene, a-phellanderene, 1,8-cineole, trans β-ocimene, c-terpenene, and cis-sabinene hydrate (32.95%) than SFME extracted oil (25.77%). The essential oil composition of *R. officinalis*

var. typicus and var. troglodytorum endemic to Tunisia, was determined by GC and GC–MS, were identified 1.8-Cineole (47.2–27.5%) and camphor (12.9–27.9%) as the main constituents, respectively. (Zaouali and Boussaid 2010).da Silva Bomfim*et al*.(2015a) recorded that main compounds of the REO were 1.8 cineole (52.2%), camphor (15.2%) and alpha-pinene (12.4%). Twenty compounds representing 99.93% of the oils were identified. However, the composition of the oils was p-cymene (44.02%), linalool (20.5%), gamma-terpinene (16.62%), thymol (1.81%), beta-pinene (3.61%), alpha-pinene (2.83%) and eucalyptol (2.64%). The oil consisted of monoterpenic hydrocarbons, oxygenated monoterpenes and sesquiterpene hydrocarbons. The extent of inhibition of fungal growth varied depending on the levels of essential oil used in experiment (Ozcan and Chalchat 2008, Angioni *et al.* 2004)

The mycelial growth of *Fusarium verticillioides* was reduced significantly by 150 µg mL^{-1} of REO. Significant microscopic morphological changes were visualized, such as the rupture of the cell wall and the leakage of cytoplasm at 300 µg mL^{-1} of REO (da Silva Bomfim *et al.* 2015a), *Trichophyton mentagrophytes* (Mugnaini *et al.* 2013). *Aspergillus flavus* and *A. ochraceus* (Centeno *et al.* 2010) and *Alternariaalternata*, *Botrytis cinerea* and *F. oxysporum* (Ozcan and Chalchat 2008, Angioni *et al.* 2004).

The suggested results confirmed that REO acts against *F. verticillioides* by disrupting cell wall led to a loss of cellular constitutes, subsequently inhibiting the accumulation of fumonisins and ergosterol. The degree of inhibition of fungal growth varied depending on the levels of essential oil used in experiment (Ozcan and Chalchat 2008, Angioni *et al.* 2004). The objectives of this work were: (i) to determine the susceptibility of some pathogenic and mycotoxin-producer fungal strains toward REO and (ii) to assess the antioxidant properties of REO. Moreover, understanding the potential mode of action of REO against tested fungal stains to be recommended as potential food bio-preservative to protect it from toxigenic fungal infections.

MATERIALS AND METHODS

Materials

Chemicals: DPPH, 1,1-diphenyl-2-picrylhydrazyl; ABTS, 2.2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-carboxylic acid; GA, gallic acid; TCA, Trichloroacetic acid; AA, ascorbic acid andethylenediaminetetraacetic acid (EDTA) were obtained from Sigma, Germany, while SYTOX-Green (5mM solution in DMSO) was obtained from Eugene, Oregen, U.S.A.

Essential oil: The pure essential oil of rosemary *R. officinalis* L.(REO) was obtained from the Fragrance and Extraction Factory, Sugar Industrial Integrated Company (SIIC), Cairo, Egypt. According the company procedure, REO was extracted from the matured herbs after harvesting using the hydro-distillation system. The rosemary herbs were imported from India as a yield of season 2013.

Fungal strains: Fungal strains, *Fusarium sporotrichioides* DSM 62425, *F. oxysporium* IfGB 4287, *F. moniliforme* DSM 4236, *F. poae* DSM 62376, *F. tricinctum*DSM 62446, *F. acuminatum* DSM 62148, *F. graminaerum* DSM 4527, *F. proliferatum* DSM 6546 were obtained from the international brewing services, research and technology, VLB Berlin (Versuchs und LehranstaltfürBrauerei in Berlin) and Institute for ferentation (Institut für Gärungsgewerbe, Berlin), Germany.

Methods

Determination of total phenolic compounds (TPC): The total phenolic compounds of REO was analyzed using the Folin–Ciocalteu reagent according to the method of Dewanto*et al.***(**2002) modified by Bettaieb *et al.* (2010).The measurements were compared to a prepared standard curve of gallic acid (GA) solution in range of 50-500mg ml^{-1}(R^2=0.999), and the total phenolic compounds content was expressed as milligrams of gallic acid equivalents (GAE) per gram of REO (mg of GAE g^{-1}). At least triplicate measurements were taken for each sample.

Antioxidant activity:

DPPH radical scavenging assay: Radical scavenging activity by donation capacity of REO was determined spectrophotometrically based on the bleaching of the purple-colored solution of DPPH radicals according to modified method by Lu *et al.*(2007). Aliquot of 0.1 ml from each REO dilution was added to 2.9 ml of $6x10^{-5}$ mol methanolic solution of DPPH. The absorbance at 517 nm was measured after the solution had been permitted to stand for 60 min in the dark. The Trolox calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The antiradical activity was expressed to Trolox equivalents (TE) per gram of REO as (µmol TE g^{-1}).

ABTS radical cation scavenging activity: The radical scavenging activity of REO against ABTS radical cation was measured using the modified method of Lu *et al.*(2007). Trolox calibration curve was plotted as a function of percentage of ABTS-RCS activity. The final results were expressed as micromoles of Trolox equivalents (TE) per gram of REO (µmol of TE g^{-1}).

β-carotene–linoleic acid bleaching assay: A modified spectrophotometric method described by Koleva *et al.* (2002)with minor modification was employed. A stock solution of β-carotene–linoleic acid mixture was prepared as follows: Half mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade); 20 mg linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated under vacuum evaporator at 40ºC. Then, 100 ml of oxygenated distilled water was added then the emulsion was vigorously shaken. Aliquots (2.5 ml) of this reaction mixture were dispensed to test tubes and 0.5 ml of REO dilution prepared at 4 g l^{-1} concentration was added and the prepared emulsion then was incubated for up to 48 h. at RT. The same procedure was repeated with the synthetic antioxidant [BHT] as positive test, and a blank containing only 0.5 ml of methanol was applied.

Afterward, the absorbance was determined at 490 nm. The antioxidant activity (%) of REO was evaluated in terms of the bleaching of the β-carotene relating to BHT, the results were expressed as BHA-related percentage.

Chelating effect on ferrous ions: The ferrous ion chelating activity of REO was assessed as described by Zhao *et al.* (2006). Aliquot of different REO dilutions were added to 0.05 mL of FeCl2.4H2O solution (2 mM) and left at room temperature for 5 min for incubation. Then, the reaction was initiated by adding 0.1 mL of ferrozine (5 mM), and the mixture was adjusted to 3 mL with de.H_2O, shaken vigorously, and left standing at room temperature for 10 min. The solution absorbance was then measured spectrophotometrically at 562 nm. The inhibition percentage of ferrozine-Fe2+ complex formation as metal chelating activity was calculated and expressed as (mg mL^{-1}) when EDTA was used as a positive control.

Reducing power assay: The determination of reducing power was carried out as described by Oktay*et al.*(2003). Briefly, 1 ml of methanolic diluted REO was mixed with 2.5 ml phosphate buffer (0.2 mol, pH 6.6) and 2.5 ml ($K_3Fe(CN)_6$, 1%). The mixture was incubated in screwed cap tubes at 50ºC for 20 min. A portion (2.5 ml) of TCA (10%) was added to the mixture, which was then centrifuged at 10000 *xg*for 10 min The upper layer of the solution (2.5 ml) was mixed with deionized water (2.5 ml) and 0.5 ml ($FeCl_3$,0.1%) then the absorbance was measured at 700 nm. The measurements were compared to a standard curve of prepared ascorbic acid (AA) solution, and the final results were expressed as micromoles of ascorbic acid equivalents (AAE) per gram of REO (µmol of AAE g^{-1}). Triplicate measurements were taken for all samples.

Gas chromatography mass spectrometry (GC–MS): The composition of the essential oil was analyzed using GC–MS technique according to Cosentino*et al.* (1999). The essential oils were chromatographed using a Shimadzu gas chromatograph QP2010-GC-MS with auto-sampler. A DB5MS fused silica column (5% phenyl-methylpolyxiloxane, 30mx0.32 mm, film thickness 0.25 µm) was employed. The injector and interface were operated at 200°C and 280°C, respectively. The oven temperature was programmed as follows: 60 °C raised to 180 °C (3 °Cmin^{-1}), and held for 15 min. Helium was the carrier gas at 1 ml min^{-1}; the sample (1 µl) was injected in the split mode (1:20). MS conditions were as follows: ionization voltage of 70 eV, scan rate 1.6 scan sec^{-1}, mass range 40–1000, and ion source temperature 180 °C. The essential oil components were identified by comparing their relative retention times and mass spectra with those known compounds stored in the internal library as well as some recent literature have been used.

Antifungal activity:

Propagationof fungal strains: The fungal strains were cultivated on sporulation medium (Yoder and Christianson 1998). A 7-10 days spore suspensions were obtained by washing the media surface with 0.05% Triton-x-100 solution by sterilize cotton buds. Then spore suspensions were filtrated with sterilized nylon films to remove the mycelium derivates and the spore's counts were counted by Thoma's cell with light microscopy to calculate the inoculums volume.

Fungal growth inhibition assay: The fungal growth inhibition activity of REO was assayed as described by Tripathi*et al.* (2009) with some modification. The PDA was autoclaved and cooled down tell 45ºC then mixed with different concentrations of REO in the presence of tween 80. After media solidification, 5 µl from each test strain spore suspension were spotted on the center of PDA incorporated with different REO, then inoculated dishes were incubated at 28ºC. During an incubation period for 9 days, the diameter of fungal colony was measured by micrometer ruler and data of triplicates were expressed in mm.

Minimal inhibitory concentration (MIC) determination: The *in vitro* activity of REO was determined by measuring the absorbance at 600 nm of fungal cultures in 96-well microtiter plates according to described method by Theis*et al.*(2003). Measurements were carried out in triplicates. MIC was defined as the lowest REO concentration which resulted in complete inhibition of growth. Percentage of growth inhibition was calculated according to Hu and Reddy (1997).

Determination the mode of action: SYTOX-Green uptake assay was applied as described by Theis*et al.*(2003).1000 spores of the tested fungal strains were cultivated in 96-well microtiter plate containing 200 µl PDB medium. After 42 h at 28°C, REO and SYTOX-Green were added to final concentration of 50 µl and 0.2 µM in the presence to Tween 80, respectively. After 2 h, fungal mycelium were washed with Tris buffer (pH, 6.0) and fixed with Poly-L-Lysine (PLL) on glass slid and covered with slid cover. The results were recorded by Microscope (ZEISS, Axioskop 50) equipped with an Olympus digital camera C-4000. A fluorescence images were captured using [Argus X1 software] under fluorescence light in both untreated and treated samples with REO, subsequently merged with Adobe Photoshop 0.7.

Statistical analysis: Pearson's correlation analysis was done and obtained correlation results were compared to critical values of Pearson's *r* table under levels of significance with one-tailed test according to (Barakat and Rohn 2014).

RESULTS AND DISCUSSION

Total phenolic compounds content and antioxidant activity of REO:

The amounts of total phenolic compunds (TPC) in REO was determined spectrometrically and calculated as milligrams of Gallic acid equivalents (GAE) per gram as well as the antioxidant activities of REO by the DPPH radical scavenging, ABTS, the β-carotene–linoleic acid bleaching and reducing power were investigated. As seen in Table (1), TPC of REO was very high reached to 198.8 mg GAE g^{-1}. Whereas, obtained results exhibited that the DPPH radical cation scavenging activity (RSA) of REO was 234.7 µmol of

TE g^{-1}. Moreover, ABTS-RSA was used to determine the evolution of antioxidant activity of REO, and results are presented in the same table. Comparing with the DPPH radical scavenging activity, the ABTS-RSA of REO samples was affected similarly to present 291.3 µmol of TE g^{-1}. Furthermore, the relative antioxidative activity (RAAs) of REO is given in Table (1). The inhibition values of linoleic acid radicals were estimated as 75.8% when compared to BHA. A relationship between the DPPH scavenging ability, ABTS and β-carotene bleaching extent was found.Data in Table (1), illustrated the evolution of reducing power of REO which was 198.1 µmol of AAE g^{-1}. It is worth mentioning that, according to those results, there is a relationship between total phenol compounds content and antioxidant activities. The phenolic compounds, biologically active compounds, are the main agents donate hydrogen to free radicals and break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals explained by phenolic hydroxyl groups (Sawa *et al.* 1998).Chelating power is imperative because transition-metal ions can stimulate peroxidation by two mechanisms, namely, by contributing in the generation of initiating species and by accelerating hydro-peroxide production from lipid oxidation. Generally, a positive association between the phenolic compounds content and antioxidant capacity was reported by Thaipong*et al.* (2006). Thus, this high performance of the methanolic REO extract is linked to their phenolic composition. Recently, it has been shown that the antioxidant activity of extracts is unevenlyrelated to their phenolic composition and strongly depends upon their phenolic constructions. This phenolic acid has been stated as an efficient antioxidant compound, scavenging reactive oxygen species (ROS), counting superoxide anion, hydrogen peroxide, and hydroxyl radical (Gülçin 2006, Erkan; Ayranci and Ayranci 2008). Moreover, Andjelkovic*et al.* (2006) confirmed the capacity of several phenolic acids for complex formation with iron *in vitro*.

Table 1.Total phenolic content and potential antioxidant activities of rosemary EO (mean±SE).

Item	Rosemary EO
TPC (mg GAE g^{-1})	198.8±3.6
DPPH (µmol of TE g^{-1})	234.7±7.1
ABTS (µmol of TE g^{-1})	291.3±6.7
B-carotene bleaching* (RAA) %	75.8±8.3
Reducing power (µmol of AAE g^{-1})	198.1±4.8

***: relatively calculated based on BHA activity as100%**

Pearson's correlation coefficients of TPC and different antioxidant activities of REO:

Pearson's correlation coefficients were obtained to determine a possible association between TPC and different antioxidant activities (Table 2). Very high significant correlations have been observed between TPC and potential antioxidant activities of REO. Indeed, this varied significant correlation exhibited the efficiency of REO to combat different synthetic radicals which assayed by $DPPH^{\bullet}$, $ABTS^{\bullet+}$, β-carotene bleaching and reducing power assays. Significant correlation was found between TPC and both reducing power activity and $DPPH^{\bullet}$. Similar finding had been cinfirmed by (Barakat and Rohn 2014).

Composition of *R. officinalis* EO by GC-MS:

Twenty separated components were identified by GC-MS in *R. officinalis*EO, data were illustarted in Table (3). There are eleven components were observed in a percentage of higer than 1%. These identifiablecompounds were considered to be 97.03% of REO composition while 2.97% as unidentifiable compounds were recorded. The major compounds of REO were 1,8-Cineole (32.65%), whereasα-Pinene (24.46%), β-Pinene (10.16%), α- Terpineol(6.78%), Camphor (3.52%) and Bornyl acetate (5.31%)were observed in valuable contents. Essential oils rich in phenolic compounds such as 1,8-Cineole, α-Pinene, β-Pinene, α- Terpineol and Camphor are widely reported to possess high levels of antioxidant and antifungal activities (Soković *et al.* 2002, Shukla *et al.* 2012).

Table 2. Pearson's correlation coefficients of TPC and different antioxidant activities of REO.

Item	TPC	DPPH	ABTS	B-carotene bleaching	Reducing power
TPC	1.00	0.61*	0.68**	0.82**	0.55*
$DPPH^{\bullet}$		1.00	0.94***	0.94***	0.88***
$ABTS^{\bullet+}$			1.00	0.97***	0.99***
B-carotene bleaching				1.00	0.96***
Reducing power					1.00

Asterisks (*, and ***) represent a significant difference at (p<0.05,p<0.01and p<0.005), respectively.**

Table 3. Identification of *R. officinalis* essential oil components by using GC-MS.

N°	Compound[a]	R_t	K.I.	%	Method of identification
1	α-Thujene	10.21	928	0.17	RI
2	α-Pinene	10.94	936	24.46	MS, RI, Lit.
3	Camphene	11.64	951	0.41	RI, Lit.
4	β- Pinene	12.64	981	10.16	MS, RI, Lit.
5	Myrcene	13.77	988	1.99	RI, Lit.
6	Limonene	14.73	1029	0.69	RI, Lit.
7	1,8-Cineole	15.46	1030	32.65	MS, RI, Lit.
8	Linalool oxide	16.28	1085	0.18	MS, RI
9	Linalool	16.82	1099	0.15	MS, RI, Lit.
10	β -Thujone	17.50	1112	0.37	RI, Lit.
11	Camphor	18.40	1141	3.52	MS, RI, Lit.
12	Terpin-4-ol	19.47	1177	0.90	MS, RI, Lit.
13	α- Terpineol	19.70	1190	6.78	MS, RI, Lit.
14	Verbenone	20.30	1203	3.62	RI, Lit.
15	trans-Myrtanol	21.04	1255	2.01	RI, Lit.
16	Bornyl acetate	21.26	1284	5.31	RI, Lit.
17	α- Terpinyl acetate	23.37	1347	0.12	RI, Lit.
18	β -Caryophyllene	24.13	1420	1.62	MS, RI, Lit.
19	gamma- Cadinene	28.33	1510	0.22	RI, Lit.
20	(-)-Caryophyllene oxide	34,883	1579	1.70	MS, RI, Lit.
				97.03	
Unidentifiable compounds				2.97	
Total				100	

[a] : Tentatively identified compounds;R_t: Retention time in minutes; RI: Retention index; MS: Mass spectrum; Lit.: Literature review.

In present studies, 1,8-Cineole is a major volatile constituent (32.65%) of REO which is a phenolic

component was found as the major compound, therefore the REO showed high antioxidant and antifungal activities (Celiktas *et al.* 2007, Soković *et al.* 2002, Vilela *et al.* 2009, Pina-Vaz *et al.* 2004). Over many recent literatures, the variations in chemical composition of essential oils depending on climatic, seasonal, and geographic conditions (Celiktas *et al.* 2007). Our study supports the view that 1,8-Cineole is a major component for the essential oil REO.

Antifungal activity of REO *in vitro*:

Recently, there has been considerable interest in essential oils with antimicrobial activities for controlling pathogens and/or toxin producing microorganisms in foods (Alzoreky and Nakahara 2003, Valero and Salmeron 2003). A few preservatives containing essential oils are already commercially available such as 'DMC Base Natural', 'Protecta One' and 'Protecta Two' which are classified as generally recognized as safe (GRAS) food additives (Burt 2004, Oke *et al.* 2009). The antifungal activity of REO against some fusarium pathogenic strains to be used latterly as a natural antifungal preservative has been investigated. To study the antifungal susceptibility of REO, PDA had been impregnated with diffferent REO as 0, 500 and 750 mg/L and the sporse's suspension of some fuzarium fungi had been spotted on the solidified PDA, resultes of 6 days incubated Petri dishes were illustrated in Fig. 1.

Generally, inoculated fungal spores were not grown at 750 mg L^{-1} except *F. acuminatum*, whereas, the most of the tested strains were sensitive to REO at 500 mg L^{-1}. However, to precisely determine the effect of REO on fungal growth, the tested strains has been inoculated on solidified PDA after they impregnated by different REO at 0, 250, 500 and 1000 mg L^{-1}, the diameter of fungal colony was measured by micrometer ruler, data were illustrated in Fig. 2.

REO inhibited the mycelial growth of *Fusarium spp.* in a dose-dependent manner (Table 4). High concentrations of essential oils (1000 mg mL^{-1}) exhibited strong inhibition of fungal starins, whereas the levels of inhibition was relatively correlated to the REO concentration. Daferera et al. (2003) observed that 72% inhibition of the mycelial growth of *Fusarium spp.* at 1000 µg mL^{-1} of REO and 67% had been recorded by adding 600 µg mL^{-1} of REO (da Silva Bomfim *et al.* 2015b). In the current study, the same result was obtained using a lower concentration 1000 mg mL^{-1}. Subsequently, the MIC have been determined using PDB media and data were tabulated in the same table. The MIC was ranged from a low of 300 mg L^{-1} for *F. proliferatum* to a high of 1000 mg L^{-1} for *F. acuminatum.* These results are in agreement with mentioned recently by (da Silva Bomfim *et al.* 2015b, Ng; Cheung and Wong 2013, Mugnaini *et al.* 2013, Centeno *et al.* 2010, Pinto *et al.* 2009, Ozcan and Chalchat 2008, Angioni *et al.* 2004).

Potential mode of action of *R. officinalis* EO

The REO mode of action in term of the membrane permeabilisation by REO against foodborne pathogenic fungi was detected. In a qualitative tactic, based assay on the fluorogenic SYTOX-Green dye uptake to check the attack of REO to the cell wall and plasma membrane of sensitive fungal strains followed by membrane permeabilizationwas applied.

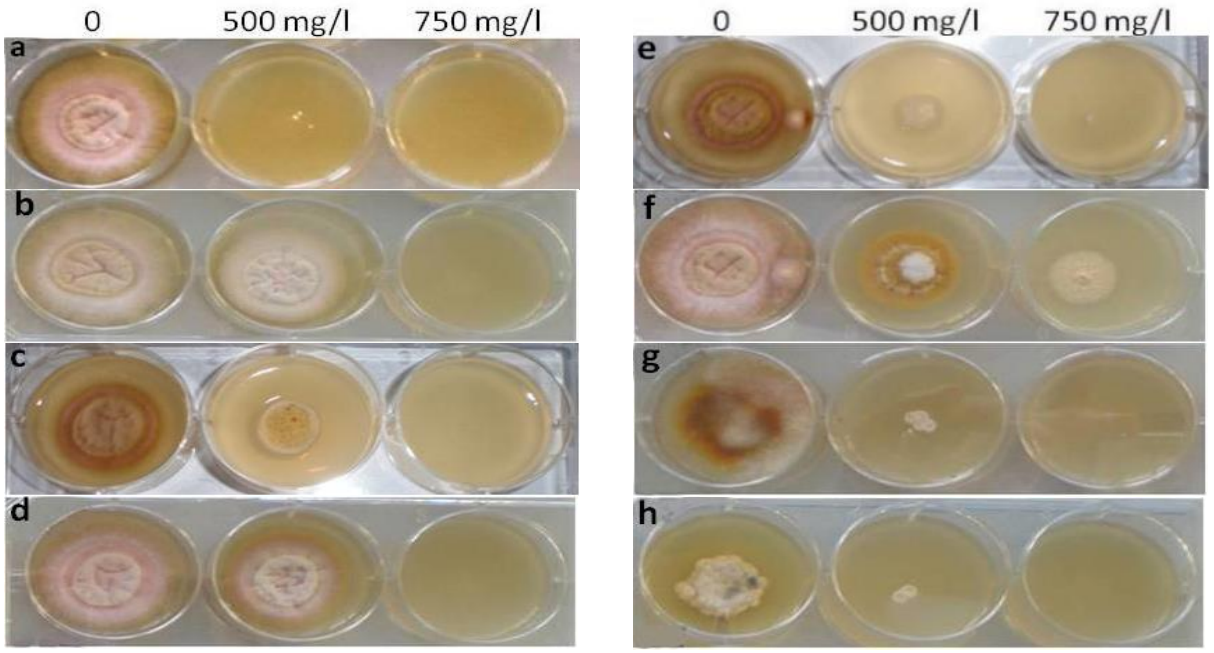


Fig. (1). The antifungal activity of *R. officinalis*EO at 0, 250 and 750 mgL^{-1}against (a) *F. sporotrichioides,* (b) *F. oxysporum,* (c) *F.moniliforme,* (d) *F. poae,* (e) *F. tricinctum,* (f) *F. acuminatum,* (g) *F. graminearum,* (h) *F. proliferatum.* The tested fungal strains have been inoculated and incubated at 28ºC for 6 days, then the 6-microtitter plates were photographed using digital Olympus camera [8 MP model FS-32].

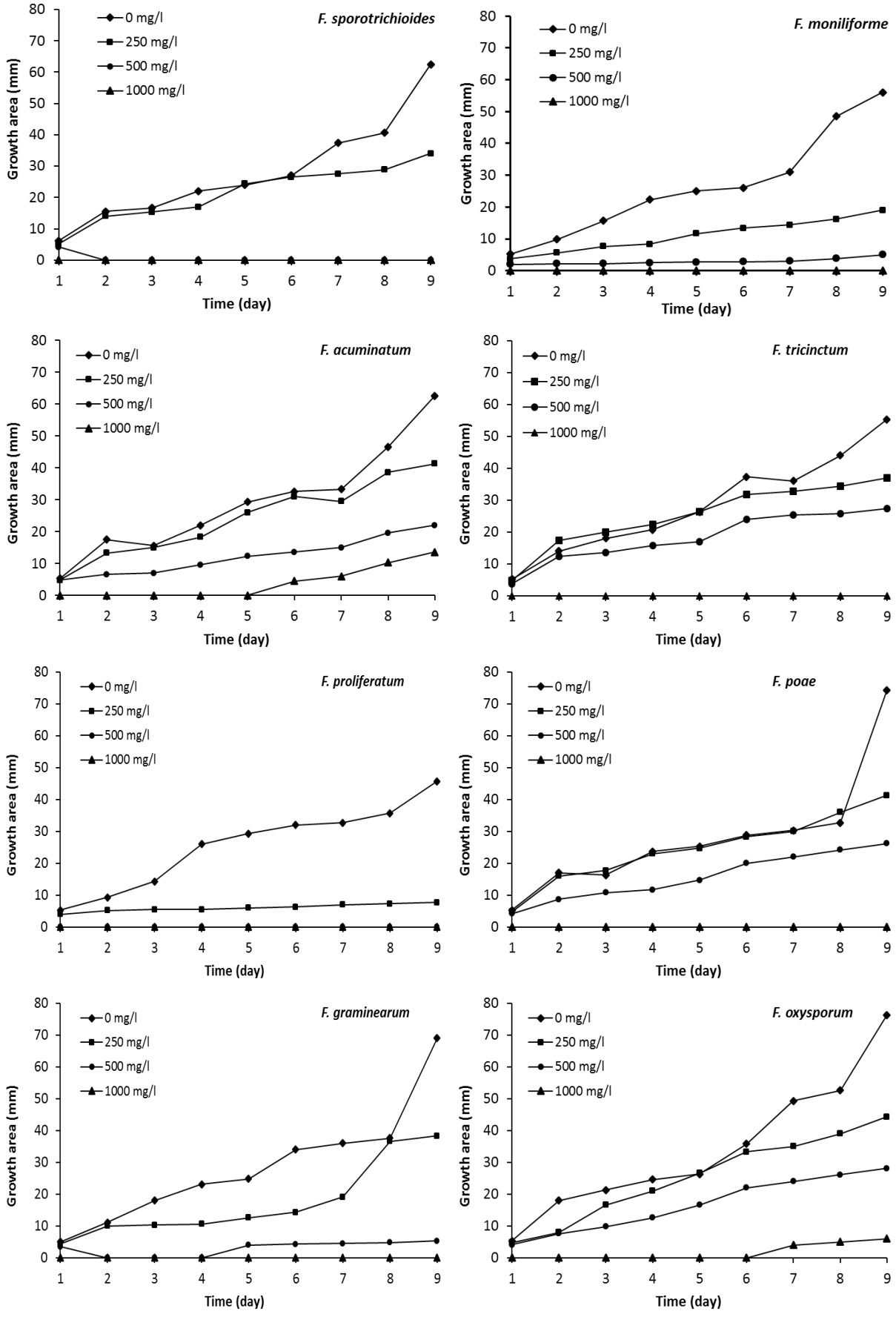


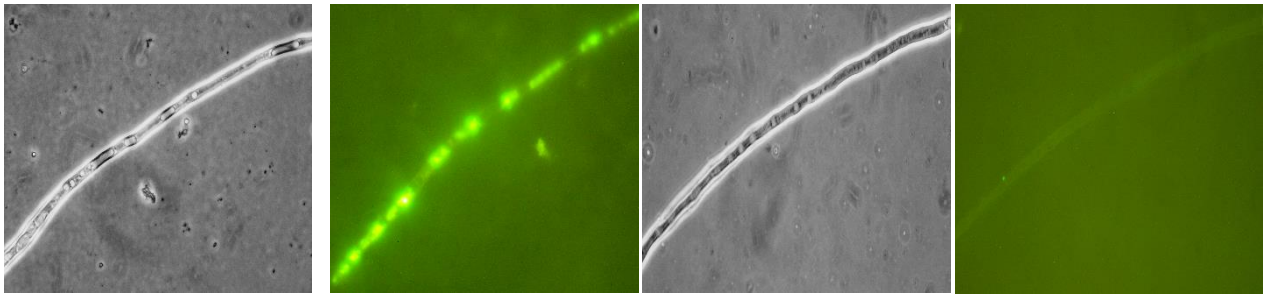
Fig. 2. The effect of REO at 0, 250, 500, and 1000 mg/L on the mycelium growth of different fungal starins *in vitro* at 28°C for 9 days.

Table 4. Inhibition effect of Rosemary essential oil (ROE) on mycelial growth of *Fusarium spp.* The cultures were incubated for 9 days at 28 ºC.

Fungal strains	REO concentration [mgL^{-1}]			MIC*
	250	500	1000	
F. sporotrichioides,	45.60	100	100	400
F. oxysporum	41.92	63.10	92.14	950
F.moniliforme,	66.07	91.07	100	550
F. poae,	44.39	64.80	100	700
F. tricinctum,	33.13	50.60	100	750
F. acuminatum,	34.04	64.89	78.19	1000
F. graminearum	44.44	92.27	100	650
F. proliferatum.	83.21	100	100	300

***: MIC is the lowest REO concentration that completeley inhibited thefungal growth.**

The obtained results are shown in Fig. 3. Strong SYTOX-Green fluorogenic dye and staining of the nucleic acid was observed when the strainwas incubated with SYTOX-Green and 1000 mg L^{-1} of REO (Fig. 3B). In contrast, it has no effect of SYTOX Green dye on fungal growth which only displays a very faint fluorescence when not bound to DNA. No DNA-SYTOX-Green fluorescence was detected when *F. graminearum* was incubated with SYTOX-Green in the absence of REO (Fig. 3D). This observation confirmed that REO cause intercellular uptake of the dye under these conditions. The obtained results are in agreement with confirmed results by (Barakat 2014, Pinto *et al.* 2009, Lopez *et al.* 2005, Ng *et al.* 2013).



A B C D

Fig. 3.Theuptake of*F. graminearum* SYTOX-Green in absences or presence of REO.(A)–Light photo and (B)–Florescence photo for treated strain with 1000mg/L REO. (C)–Light photo and (D)–Florescence photo without REO.

CONCLUSIONS

Rosemary (*R. officinalis* L.) essential oil (REO) exhibited high content of TPC which harmonies to high RS activity against DPPH, ABTS and chelating activity as well as linoleic acid radicals toward iron element. The composition of REO exhibit a high 1,8-Cineole content (32.65%) over 20components were identified by GC-MS. The REO exhibited antifungal activity at minor concentrations toward tested pathogenic fungi*in vitro*. REO exhibited MIC in range of 300-100 mg L^{-1}. The mostly active and appear to act principally as membrane permeabilisersare the phenolic components which established using staining-DNA fluorescence dye. The REO can be reliably used in commercial applications as antifungal and flavoring agent in separate or in combination with the traditional preservatives for regulation of the undesirable organoleptic and microbial deterioration in some food modules.

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النشاط المضاد للفطريات والمضاد للأكسدة لزيت إكليل الجبل العطرى (*Rosmarinus officinalis* L.)

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يعد إستخدام المواد الحافظة الطبيعية كمضادات لنمو للفطريات مبتكر حديثاً، خصوصا المستخرجة من الأعشاب منذ ان ذكر انها تمتلك خواصاً مضادة للنشاط الفطرى ومضادة للاكسدة. أجريت هذه الدراسة لتقييم النشاط المضاد لنمو للنشاط الفطرى والنشاط المضاد للاكسدة لزيت إكليل الجبل العطرى (*Rosmarinus officinalis* L.). أظهر زيت إكليل الجبل العطرى إحتوائة على محتوى عال من المركبات الفينولية الكلية والتي تمتلك نشاطاً عالياً لكسح الشقوق الحرة تجاه DPPH، ABTS والشقوق المستحدثة من حمض اللينوليك وأيضا نشاطها الخالب للحديد. بلغ محتوى زيت إكليل الجبل العطرى ١٨٩.٨ ملجم/جم مقدرة كمكافئ لحمض الجاليك، سجلت مايكافئ ٢٣٤.٧ ميكرومول/جم مقدرة كمكافئ الترولوكس تجاه الشقوق الحرة DPPH و سجلت مايكافئ ٢٩١.٣ ميكرومول/جم مقدرة كمكافئ الترولوكس تجاه الشقوق الحرة ABTS. كما أظهرت نتائج القدرة المضادة للأكسدة لزيت إكليل الجبل ٧٥.٨٪ انخفاضا عند تقييمها بإختبار القدرة على منع تبييض البيتاكاروتين. قدرت أيضا القدرة الخالبة للحديد بمايكافئ ١٩٨.١ ميكرومول/جم مقدرة كمكافئ لحمض الأسكوربيك. أظهر تفريد المركبات الفعالة أن 1,8-Cineole مثل ٣٢.٦٥٪ من بين أكثر من ٢٠ مركباً تم التعرف عليها فى تحليل GC-MS ليكون المركب الفعال السائد. وزيادة على ذلك، أظهر زيت إكليل الجبل العطرى نشاطاً مضاداً في المختبر في تركيزات منخفضة ضد الكائنات الحية الدقيقة الممرضة. في الواقع، أشارت النتائج المستخلصة من اختبارى أقل تركيز مثبط (MIC) وتثبيط نمو واستطالة هيفات الفطريات إلى كفاءة تأثير زيت إكليل الجبل على فطريات الفيوزاريوم في المختبر. كانت نتائج اختبار أقل تركيز مثبط (MIC) فى مدى من ٣٠٠ - ١٠٠٠ ملجم زيت/لتر. بدراسة ميكانيكية تأثير زيت إكليل الجبل وجد ان له تأثير قوياً فى إحداث خللاً فى نفاذية الجدر للخلايا موضع الإختبار. ولذلك، فإن زيت إكليل الجبل يمكن إستخدامه في التطبيقات التجارية ضمن مضادات الأكسدة والمضادات الفطرية القوية ومكسبات النكهة سواء منفردة أو بالإشتراك مع المواد الحافظة المستخدمة للسيطرة على التغيرات الحسية والفطرية الغير مرغوب فيها وأيضا للسيطرة على انتاج السموم