Evaluation of the Antioxidant and Antibiofilm Activities of *Rosmarinus Officinalis* Essential Oil Extract

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Abstract

Biological activities of essential oils from various plants, including Rosemary, have been attributed to the presence of specific chemical compounds with antimicrobial, anti-inflammatory and antioxidant activities. The aim of this study is to estimate the antioxidant and antifungal activity of *Rosmarinus officinalis* essential oil extract. The study included the extraction of essential oil using a Clevenger apparatus. The chemical compositions were evaluated by GC-MS and High Performance Liquid Chromatography (HPLC).The rosemary essential oil extract was tested with regard to antioxidant utilizing 2, 2- diphenyl-1-picrylhydrazyl (DPPH) assay. Microtiter plate Assay used to determine the antifungal and antibiofilm activity. The result showed that the GC-MS analysis revealed that the major components determined in *R. officinalis* essential oil were linalool (17.09 %), L- Borneol (11.92 %), Verbenone (8.52 %), camphor (5.30 %), Eucalyptol (4.79 %), while the chemical compositions identified by HPLC shows four phenolic acids were identified in the essential oil, Rosmarinic acid, Caffeic acid, p-Coumaric acid, 4-hydroxybenzoic acid, and lignans (medioresinol), while Isorhamnetin was the only flavonol detected. The free radicals scavenging activity increased gradually with the increase in the concentration of essential oil which was 81.59 % when compared with BHT and V.C (92.34 and 97.42) respectively. The results of the antifungal activity revealed that Minimum Inhibitory Concentration of *C. albicans* and *C. krusei* was 3.125%, while the MIC of *C. glabrata* was 12.5% in contrast with the highest MIC which recorded for *C. tropicalis* at 25% of rosemary essential oil. The current results revealed that the reduction of bofilm formation among *C. albicans* and *C. krusei* was obvious at the lower concentration (1.56%), where the percentage of biofilm formation in *C. albican*s was (91.25%) and *C. krusei* was (84.25%), while *C. tropicalis* exhibit (86.32%) for biofilm reduction at the concentration (12.5%) of rosemary essential oil, also it was found that the effect of essential oil on *C. glabrata* biofilm formation was at the concentrations 3.125% and 6.25%. The findings of this study indicated to the significant effect of rosemary essential oil against the growth and biofilm formation of the important pathogenic yeast *C. albicans* at low concentrations.

Keywords*: Rosmarinus officinalis, Essential oil, GC-MS, HPLC, Antioxidant, Antibiofilm*

Introduction

The use of traditional herbs and medicinal plants has recently become very popular because they contain large amounts of natural products with biological properties. Medicinal plants extracts possessed strong antioxidant and antimicrobial activities against pathogenic organisms [(1)]. Medicinal and aromatic plants have been used for their bioactive compounds with potential applications in food industry, nutraceuticals, cosmetics and perfumes [(2)]. Also, some plants from the Lamiaceae family are very rich in phenolic compounds, such as flavonoids, phenolic acids and phenolic diterpenes, and possess high antioxidant activities [(3)]. These compounds

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can delay or inhibit the oxidative damage caused by free radicals and can protect us against major diseases such as coronary heart disease and cancer in human [4, 5]. Also it was found that the extracts and essential oils of *Rosmarinus officinalis* show inhibitory effects against cyanobacteria [6].

Rosemary (*Rosmarinus officinalis* L.) is a spice and medicinal herb widely used around the world. They are also used as flavouring agents in foods [7]. Additionally, various pharmacological studies have demonstrated the analgesic, anti-inflammatory, and anti-ulcerogenic properties of *R. officinalis* [8, 9]. Many compounds have been isolated from rosemary, including flavones, diterpenes, steroids, and triterpenes. Of these, the antioxidant activity of rosemary extracts has been primarily related to two phenolic diterpenes: carnosic acid and carnosol. The main compounds responsible for the antimicrobial activity are α-pinene, bornyl acetate, camphor and 1, 8-cineole [10, 11]. Thus, the purpose of this research is to estimate the antioxidant, antifungal, and antibiofilm activities of *Rosmarinus officinalis* essential oil extract

Material and Methods

Chemical reagents

The chemical reagents DPPH (2,2-diphenyl-1-picrylhydrazyl), Butylated hydroxytoluene (BHT), ascorbic acid, gallic acid monohydrate (3,4,5-trihydroxybenzoic acid) and sodium carbonate were purchased from Sigma aldrich chemicals (St. Louis, USA). Folin Ciocalteu reagent was purchased from Merck (Darmstadt, Germany), Resazurin dye (Himedia, India), Sabouraud's dextrose broth (Himedia, India) and crystal violet (Pro-Lab, Canada).

Candida spp. isolates

The isolates of *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis and C. krusei*) were obtained from specialist hospital in Baghdad, Iraq, from female patients with Vulvovaginitis and identified by Vitek2 system.

Plant material

Fresh leaves of *Rosmarinus officinalis* plant were procured from the greenhouse in Baghdad, Iraq. Authentication and identification of the plant were carried out by the specialist, Department of Biology, College of Science, University of Baghdad.

Extraction of the essential oil

Air-dried leaves of the *Rosmarinus officinalis* plant was subjected to steam distillation for 4 h using a Clevenger apparatus. The essential oil was kept at −4°C in an amber glass airtight container.

Fourier transform infrared (FTIR) assay

FTIR stands for Fourier Transform Infrared, the preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the infrared radiation is absorbed by the sample and some of it is passed through (transmitted), the resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. Like a fingerprint no two unique molecular structures produce the same infrared spectrum, this makes infrared spectroscopy useful for several types of analysis. The FTIR spectrum was recorded between 4000 and 400 cm-1 [12].

Gas chromatography mass spectrophotometer analysis

Analysis of the *Rosmarinus officinalis* essential oil was carried out on GC-MS equipment. The experimental conditions of the equipment are: HP-5MS ultra inert capillary non-polar column, dimensions: 30 mm × 0.25 mm; ID: 0.25 mm, film thickness: 0.25 μm. The flow rate of mobile gas: 1.0 ml/min. The oven temperature for the gas chromatographic part was 50°C raised to 300°C at 7oC/min for 10 min. The nature and structure of compounds were identified by the mass spectrometer. The spectrum of unidentified components was compared with the spectrum of identified components stored in the national institute standard and technology (NIST) library [13].

High-Performance Liquid Chromatography (HPLC) Analyses

HPLC analysis was performed for analysis of Rosmarinus officinalis essential oil extract. The HPLC analysis was carried out according to Adham (2015) [14] the separation was performed in reversed-phase ODS-C18 column (250 mm×4.6 mm i.d); the mobile phase consisting of 80% methanol as solvent (a) and 20% (water with 0.1% acetic acid) as solvent (b). A flow rate was set at 1 ml/min for 10 min, detected by UV at 320 nm.

Determination of total phenolic contents

Total phenolic content of *Rosmarinus officinalis* essential oil extract was determined spectrophotometrically using the Folin-Ciocalteu method described by Jayaprakasha *et al*. (2001) [15], 2 ml of Folin-Ciocalteu reagent (diluted 10 times) was mixed with 1.6 ml of 7.5% sodium carbonate solution and 0.4 ml of the essential oil extracts. The volume was completed to 5 ml by adding distilled water. The tubes were covered with parafilm for 30 min. at room temperature, and then the absorbance was read at 760 nm spectrophotometrically. The total phenolic content was calibrated against gallic acid standards and expressed the results as mg gallic acid equivalents (GAE)/g extract. The test was performed in triplicate.

DPPH assay

The DPPH free radical scavenging activity of *Rosmarinus officinalis* essential oil extract was determined following the method described by Kedare and Singh, (2011) [16], 5ml of a freshly prepared 0.004% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was mixed with 50 µl of different concentrations (0.312, 0.625, 1.25, 2.5 and 5) µg/ml of the essential oil, then the mixture was left to stand for 30 min. The absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) and ascorbic acid were used as a positive control. All tests were performed in triplicate. The percentage of DPPH reduction was calculated as:

% Reduction = (Abs DPPH – Abs Dil.) / Abs DPPH × 100

Where: Abs DPPH = average absorption of the DPPH solution, Abs Dil. = average absorption of the three absorption values of each dilution.

With the obtained values, a graphic was made using Microsoft Excel. The EC_{50} of each extract (concentration of extract or compound at which reduced 50% of DPPH) was taken from the graphic.

Determination of the antifungal activity

Antifungal activities of the rosemary essential oil towards *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis and C. krusei*) were determined by the Resazurin Microtiter plate Assay (broth dilution method) [17]. Fifty microliters of the essential oil was two-fold serially diluted (0.19-100%) with Sabouraud's dextrose broth in a microtiter plate. Fifty microliters of the *Candida* suspension was added and mixed with the oil. *Candida* cultured in the broth without the tested agents served as a positive control and the mixture of broth and the tested agents without microorganism served as a negative control. The plates were incubated for 24 h, at 37 °C. Then *Candida* growth was examined and the lowest concentration of the tested agents which inhibited the visible growth of the yeast was recorded as the minimum growth inhibitory concentration (MIC). All experiments were repeated on two of each species of *Candida* and three separate occasions, with triplicate determinations on each occasion.

Biofilm formation of *Candida* spp.

Biofilm formation was determined by crystal violet assay [18]. After the growth of *Candida* spp. (*C. albicans, C. glabrata, C. tropicalis and C. krusei*) with serial dilutions of sub-inhibitory concentrations of Rosemary essential oil (0.78-25%) in the wells of microtiter plate, the content of each well was carefully removed and the plate was washed five times with sterile saline solution to remove any unattached cells. Pure methanol (200 *µL*) was added to each well and incubated for 15 min.

Methanol was removed, the plates were thoroughly dried at room temperature, and 200 μL of 0.5% crystal violet was added for 15 min. Then, the stain was removed and the wells were washed in tap water and dried. Two hundred microliters of 95% ethanol was added to each well. Finally, the absorbance (OD450) was read using an microplate reader.

Inhibition of biofilm was determined from the formula described by Jadhav *et al*. (2013) [19].

$$\% \text{ Inhibition} = 100 - \left\{ \frac{(\text{OD450 nm sample})}{(\text{OD450 nm growth control})} \times 100 \right\}$$

Results and Discussion

Fourier Transform Infra-Red (FTIR)

Figure (1) shows the infrared spectra of the *Rosmarinus officinalis* essential oil, the results revealed that the presence of different functional groups such as phenolic– OH group stretching, C-H stretching, C≡C stretch, Aromatic C=C, P=O stretch, N-O stretch and Aliphatic C–O (Table 1).

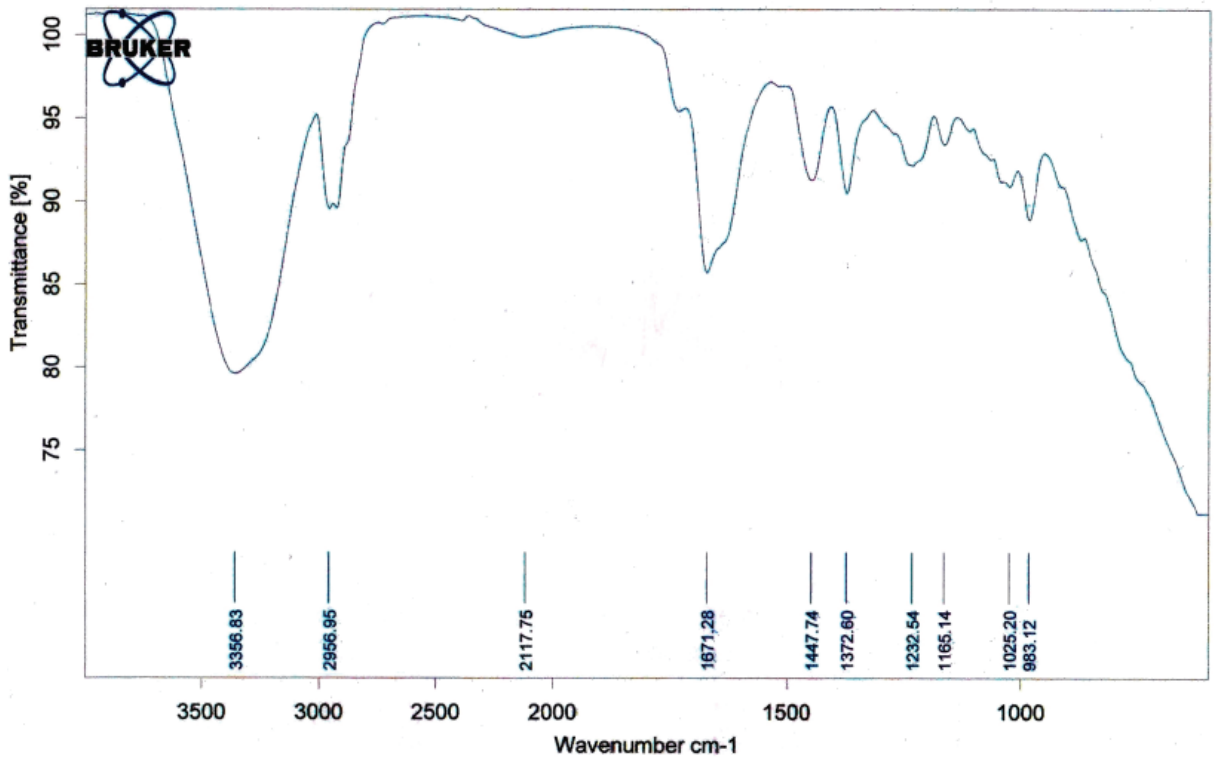

Figure 1: Infrared spectrum of the *Rosmarinus officinalis* essential oil

Table 1: The IR Frequencies region for the functional groups of the *Rosmarinus officinalis* essential oil

The Functional Groups	I.R Frequencies Standard Groups (cm-1)	I.R. Frequencies of essential oil
Phenolic–OH group stretching	3650-2500	3356.83
C-H stretching	3100-2850	2956.95
C≡C stretch	2230-2100	2117.75
Aromatic C=C	1680-1600	1671.28
P=O stretch	1260-1230	1232.54
N-O stretch	1390-1300	1372.60
Aliphatic C-O	1300 -1000	1025.20

Gas chromatography mass spectrophotometer

The phytochemical constituents present in the essential oil extract of *Rosmarinus officinalis* showed twenty four constituents (Figure 2), the major components such as Eucalyptol, Linalool, Camphor, Borneol, Verbenone, *Caryophyllene* and molecular formula with retention time are shown in Table 2.

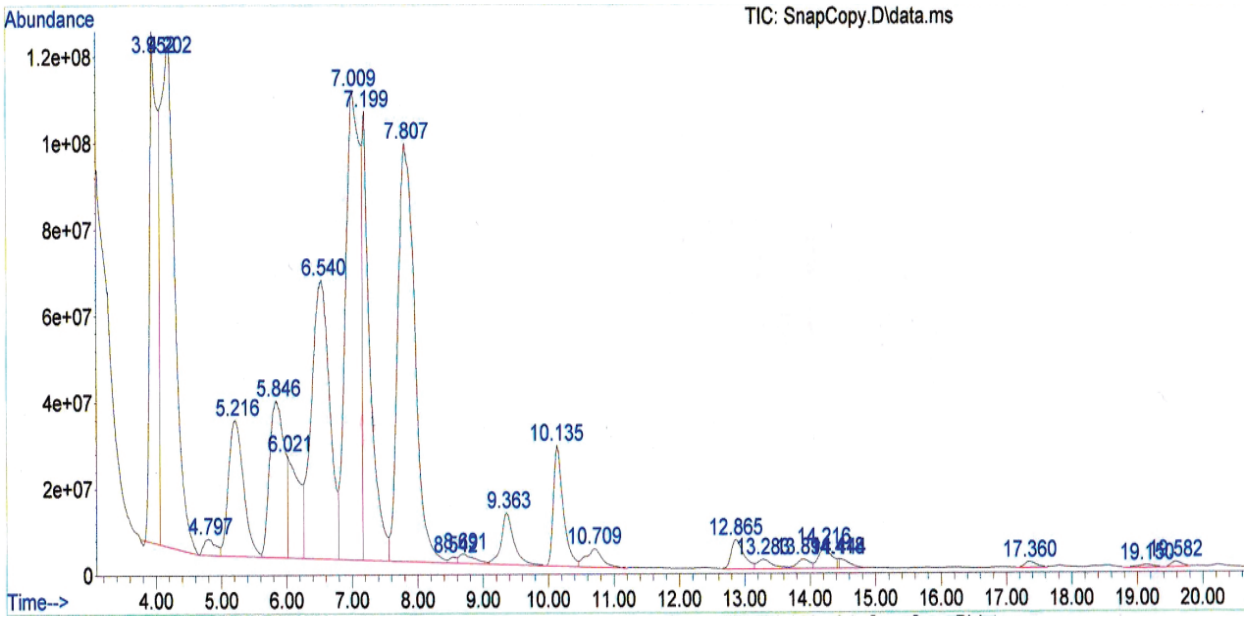

Figure 2: GC-MS Chromatogram of *Rosmarinus officinalis* essential oil

Table 2: GC-MS report of *Rosmarinus officinalis* essential oil

No.	Name of Compound	Ret. time (min)	Area (%)	M. weight (g/mol)
1	α-Linalool	4.200	17.09	154
2	Eucalyptol	5.215	4.79	154
3	Camphor	5.847	5.30	152
4	L- Borneol	6.540	11.92	154
5	Verbenone	7.200	8.52	150
6	*Camphene*	8.690	0.31	136
7	Geranyl acetate	9.361	1.50	196
8	*Caryophyllene*	10.135	2.75	204
9	Humulene	10.708	0.66	204
10	*Patchoulane*	14.418	0.03	206
11	D-limonene	14.445	0.21	152
12	*Farnesol*	19.584	0.11	222

Abdullah *et al*. (2010) [20] mention the GC analysis revealed that the major components determined in *R. officinalis* essential oil were 1,8-cineol, camphor, α-pinene, limonene, camphene and linalool, while Christiane *et al*. (2016) [21] revealed the major components of the essential oil indicated three compounds: cineole, camphor and alpha-pinene. The variation in the chemical compositions of *R. officinalis* essential oil across countries might be due to different ecological conditions. Our results are in agreement with the findings of Jan *et al*. (2017) [22] that also identified eucalyptol, α-Linalool, Camphor, L- Borneol, Verbenone, Caryophyllene, etc. as major components of *R. officinalis* essential oil.

R. officinalis is an aromatic plant popularly known as rosemary which has important biological properties, especially due the phenolic and the essential constituents, such as carnosol, carnosic acid and rosmarinic acid present in the extract of rosemary and α-pinene, bornylacetate, camphor and eucalyptol present in the essential oil of this species [23].

High-Performance Liquid Chromatography (HPLC)

Individual phenolic compositions of *R. officinalis* essential oil were analyzed by HPLC method. Figure (3) shows that the essential oil of *R. officinalis* contains 16 polyphenolic compounds, and 6 compounds were identified depending on retention time according Hossain (2010) [24], Romo-Vaquero (2012) [25] and Meziane-Assami *et al*., 2013 [26], the result shows four phenolic acids were identified, Rosmarinic acid (compound 1), Caffeic acid (compound 2), p-Coumaric acid(compound 4), 4-hydroxybenzoic acid(compound 6), and lignans (medioresinol), while Isorhamnetin was the only flavonol detected.

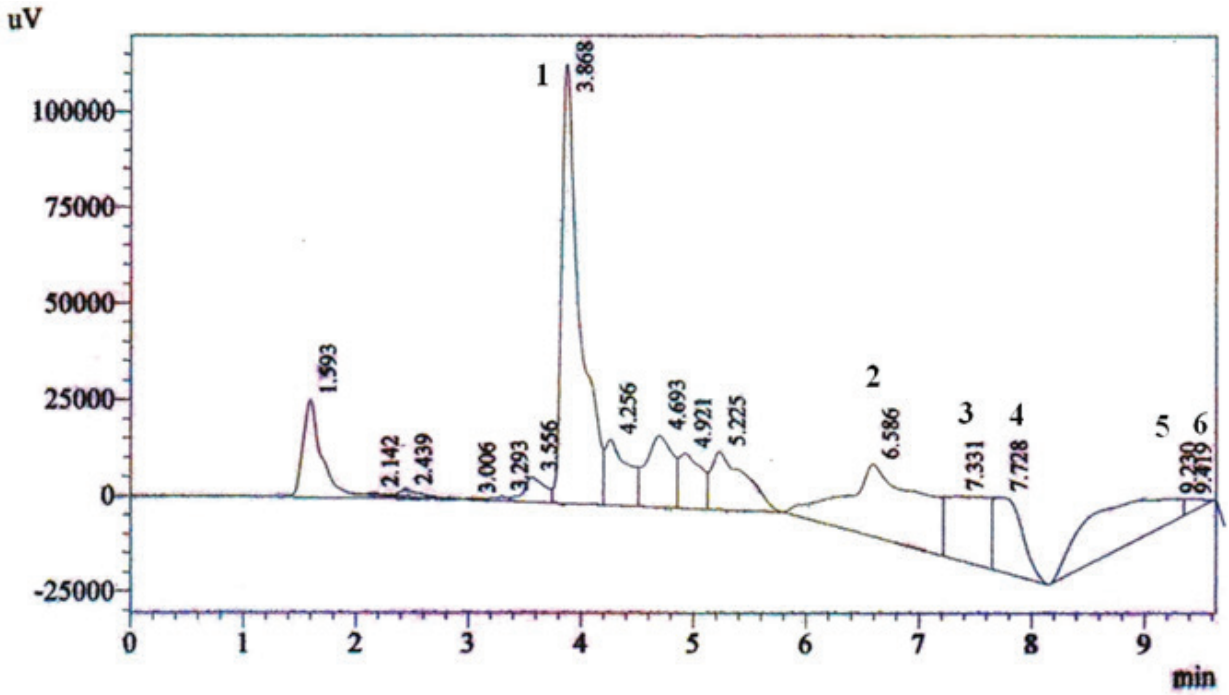

Figure 3: HPLC chromatogram of the *Rosmarinus officinalis* essential oil.

Total phenolic content of *Rosmarinus officinalis* essential oil extract

Phenolic compounds are a class of antioxidant agents which act as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals [27].

The results of total phenolic content in the *Rosmarinus officinalis* essential oil were (30.52, 41.80 and 54.47 mg/g) in (50, 100 and 200 µg\ml) respectively as shown in (Table 3).

Table (3): Total Phenolic content of *Rosmarinus officinalis* essential oil

Concentration µg/ml	**Total phenol (mg/g)**
50	30.52 ± 0.21
100	41.80 ± 0.09
200	54.47 ± 0.18
LSD value	0.601 **
** (P≤0.01).	

Genena *et al.* (2008) [28] mention the amount of phenolic compounds content ranged from 7.45 to 13.51g of TAE/100 g of extract, with an overall mean of 10.06 g of TAE/100 g of extract. Phenolics are a class of compounds, which act as free radical scavengers and are responsible for the antioxidant activity in many medicinal plants [29]

Determination of Antioxidant Activity Using (DPPH) Radical Scavenging Method

The antioxidant activity of essential oil was assessed by the DPPH (2, 2-diphenyl-1-picryl hydrazyl) free radical scavenging method. The free radicals scavenging activity increased gradually with the increase in the concentration of essential oil as shown in Table (4). Furthermore, the antioxidant activity is expressed as an effective concentration (IC50). The half-maximal effective concentration (IC_{50}) often refers to the concentration of a drug, toxicant, or antibody which induces a response halfway between the baseline and maximum after a specified exposure time is commonly used as a measure of the potency of a drug [30]. In this study, the radical scavenging capacity (IC50) of vitamin C and BHT were (0.2 and 0.4 µg\ml) respectively, while the essential oil was (1.8 µg\ml) as shown in Figure (4). Lee *et al.* (2007) [31] confirmed if the IC_{50} value of the extract was less than 10 µg/ml it indicates the extract is an effective antioxidant. In this study, the IC_{50} value of *Rosmarinus officinalis* essential oil is less than 10 µg / ml this indicates the extract was effective antioxidant.

Elansary *et al*. (2012) [32] revealed monoterpenes hydrocarbons are known to have noticeable antioxidant activities. Our study was higher than study presented by Benyoucef *et al*. (2018) [33] which concluded that *R. officinalis* essential oil showed the greatest antioxidant activity with an IC50 of 2.6 mg/L.

Table 4: Radical scavenging activity of *Rosmarinus officinalis* essential oil

Concentration µg/ml	essential oil	BHT	Ascorbic acid	LSD value
0.312	14.06 ±0.04	47.16 ±0.02	89.70 ±0.03	0.113 **
0.625	28.34 ±0.14	70.33 ±0.01	91.80 ±0.01	0.295 **
1.25	39.16 ±0.19	86.39 ±0.13	94.82 ±0.05	0.484 **
2.5	60.76 ±0.08	90.51 ±0.09	96.44 ±0.04	0.258 **
5	81.59 ±0.14	92.34 ±0.05	97.42 ±0.14	0.431 **
LSD value	0.420 **	0.248 **	0.231 **	---
** (P≤0.01).				

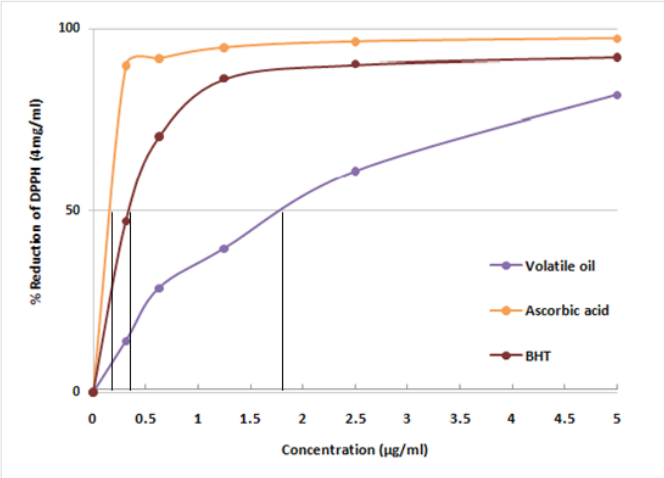

Figure 4: IC_{50} of *Rosmarinus officinalis* essential oil

Determination the antifungal activity

In this study, the antifungal activity of rosemary essential oil was evaluated. The minimal inhihibitory concentrations (MICs) of rosemary oil against four species of *Candida* were detected by Resazurin Microtiter plate Assay as showed in Figure 5.

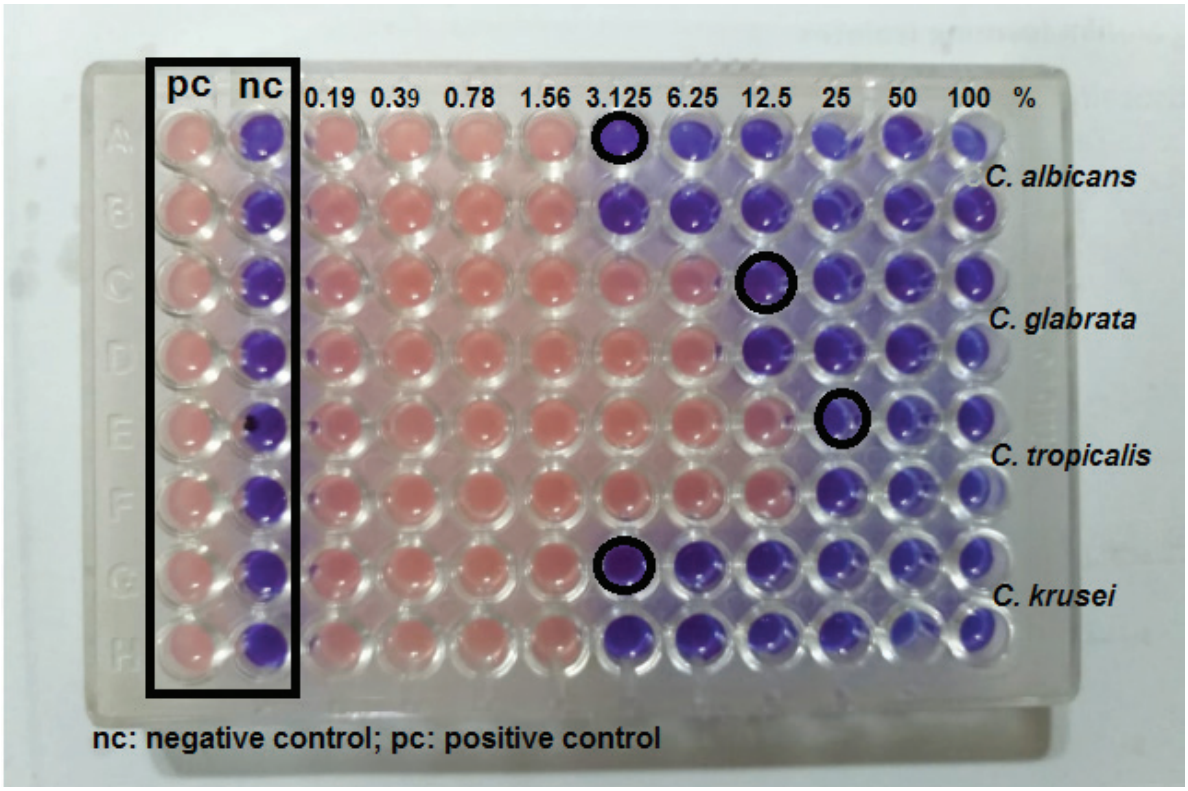

Figure 5: Minimal inhibitory concentration of serial dilutions of rosemary oil against four species of *Candida* by Resazurin Microtiter plate Assay

The results revealed that MIC of *C. albicans* and *C. krusei* was 3.125%, while the MIC of *C. tropicalis* was 12.5% in contrast with the highest MIC which recorded for *C. tropicalis* at 25% from rosemary oil. The findings of the current study indicated to the significant effect of rosemary oil toward the important pathogenic yeast *C. albicans* at low concentrations. Fu *et al*. (2007) [34] investigated the antimicrobial activity of the essential oils from rosemary (*Rosmarinus officinalis* L.) and studied the Minimum inhibitory concentrations (MICs) against three Gram-positive bacteria, three Gram-negative bacteria and two fungi were determined for the essential oil The essential oil possessed significant antimicrobial effects against all microorganisms tested, where the MICs of rosemary oil ranged from 0.125% to 1 % (v/v).

In a previous study by Abdulaziz *et al*. (2015) [35], the diameter of inhibition zone with rosemary essential oil was (11.8±2.8) and the serial two-fold dilutions of the tested essential oil showed exhibited antifungal activities even at very low concentrations. In a study by Tavassoli and Emamdjomeh (2011) [36] the antimicrobial activity of rosemary leaf extract against *some of bacteria, Saccharomyces cerevisiae,* and *Candida krusei* was determined by MIC measurements and the results indicated that the rosemary extract had a stronger inhibitory effect against the bacteria.

The mechanism of action of essential oils is related to changes in cell membrane permeability. The liposoluble nature of essential oils and their constituents facilitates their interaction with cellular structures that

have lipid components, resulting in increased membrane permeability, which in turn can cause electrolyte imbalance and cell death [37]. It was found that the antifungal action of the *M. alternifolia* essential oil against *C. albicans*, *C. glabrata* and *Saccharomyces cerevisiae*, is related to the changing in the permeability and membrane fluidity of these fungi [38].

The effect of rosemary essential oil on the biofilm formation of the local isolates of *Candida* spp. was investigated as showed in table 5.

Table 5: The percentages of biofilm reduction by rosemary oil against four *Candida* spp at different sub-inhibitory concentrations

	Biofilm reduction (%)*					
Yeast Species	**Rosemary oil concentration (%)**					
	0.78	**1.56**	**3.125**	**6. 25**	**12.5**	**25**
Candida albicans	50.75 – 55.25	85.75 – 91.25	-	-	-	-
Candida glabrata	19.75 – 24.25	38.24 – 41.28	66.83 – 68.27	72.34 – 76.08	-	-
Candida tropicalis	9.75 – 14.25	29.75 – 34.25	48.95 – 50.71	71.44 – 75.18	81.01 – 86.32	-
Candida krusei	41.90 – 53.51	79.75 – 84.25	-	-	-	-

***The reduction of biofilm included 3 isolates of each species.**

The current results revealed that the reduction of bofilm formation among *C. albicans* and *C. krusei* was obvious at the lower concentrations (1.56%), where the percentage of biofilm formation in *C. albican*s was (91.25%) and *C. krusei* was (84.25%), while *C. tropicalis* exhibit (86.32%) for biofilm reduction at the concentration (12.5%) of rosemary essential oil, also it was found that the effect of essential oil on *C. glabrata* biofilm formation was at the concentrations 3.125% and 6.25%. The local study of Raheem and Ghaima (2021) [39] revealed that the Nystatin had the inhibitory activity against *C. albicans* at the concentrations 6.25 and 12.5 µg/ml, while the highest antibiofilm activity by Nystatin were demonstrated at the subinhibitory concentration 50 µg/ml with biofilm eradication percent (75.80%).

As the findings of the present study, Cavalcanti *et al.* (2011) [40] found that the essential oil of *R. officinalis* had an anti-adherent effect on *C. albicans*, where the oil at the concentration 2.25 mg/mL caused significant cell disruption and inhibition of adhesion and theintermediate effect was observed at 1.12 mg/mL. The rosemary essential oil was found to be more active against the gram-positive pathogenic bacteria and drug-resistant mutants of *E. coli*, similarly, it was found to be more active toward nonfilamentous, filamentous, dermatophytic pathogenic fungi and drug-resistant

mutants of *Candida albicans* [41]. *The results of the the previous study revealed that R. officinalis* extract provided a significant biofilms reduction after 5 min treatment, with rates of 99.96 ± 0.07% for *C. albicans*; 67.84 ± 12.05% for *S. aureus*; 77.64 ± 15.67% for *E. faecalis*; 79.32 ± 7.34% for *S. mutans*; and 98.23 ± 2.17% for *P. aeruginosa*. In the biofilm of *C. albicans* with *S. mutans* was also observed reductions of 92.04 ± 5.24% and 64.55 ± 15.12%, respectively [42].

Rosmarinus officinalis essential oil when coated to nanoparticles strongly inhibited the adherence ability and biofilm development of *C. albicans* and *C. tropicalis* to the catheter surface, as shown by viable cell counts and scanning microscopy examination and the sub-inhibitory concentrations of these substances resulted in a reduction of the amount of sterol extracted as well as the capsule size, suggesting that they play an important role, in particular by causing the cell wall destruction and membrane irregularities, the presence of vesicles and cell wall thickening in *C. albicans* [43, 44].

Conclusion

The phytochemical constituents present in the essential oil extract of *Rosmarinus officinalis* showed twenty-four constituents such as Eucalyptol, Linalool, Camphor, Borneol, Verbenone, and *Caryophyllene* and contains 16 polyphenolic compounds with 6 compounds were identified. The free radicals scavenging activity increased gradually with the increase in the concentration of essential oil and it was found that the essential oil was effective antioxidant. The findings of this study exhibited that the essential oil has antifungal activity against pathogenic *C. albicans* isolates at low concentrations. Also, the current results revealed that the reduction of biofilm formation among *C. albicans* and *C. krusei* was obvious at the lower concentrations.

Source of Funding: Authors

Ethical Clearance: Yes

Conflict of Interest: Nil

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