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Assessment of Essential Oils and Honey bee Bioactivity, as Anti-biofilm Agents against *Pseudomonas aeuroginosa* and *Candida albicans*

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Abstract
Background: *Biofilms, are complex biological communities which are difficult to treat by conventional antibiotic therapy and contributing to >80% of humans infections. Natural medicines have been used for centuries and have shown remarkable effects in preventing and treating microbial biofilm infections. In recent years, there has been an increase in the number of studies on the anti-biofilm activity of natural products as plant essential oil (EOs) and honey bee.*
Materials and method: *In this study, we investigated the antibiofilm activity of different natural products (NP) including, five essential oils (EOs), cinnamon (Cinnamomum cassia), marjoram (Majorana hortensis), tea tree (Melaleuca alternifolia), clove (Syzygium aromaticum), and mint (Menth peperita), and honey bee against the biofilm of the Gram-negative pathogenic bacterium Pseudomonas aeruginosa and the dimorphic pathogenic fungi Candida albicans which are major human pathogens possessing a high risk to patients due to their biofilm development ability. Antibiofilm activity of the tested NP were evaluated by crystal violet staining assay and detected by scanning, fluorescent, and light microscopy.*
Results: *Antibiofilm activity showed a strong effect of the compounds being tested against the tested bacterial and fungal biofilms. EOs showed the strongest activities against biofilms of tested microbes. EOs showed antibiofilm activity ranging from 20.5 to 86.4% against P. aeuroginosa strains (Pa1 and Pa2) and from 0.2 to 84.1% against C. albicans strains (C1, C2, C3, and C4).Cinnamon oil showed the strongest antibiofilm activity with biofilm reduction ranged from 46.2 to 86.4% against P. aeuroginosa and from 40.4 to 79.6% against C. albicans. On the other hand, honey bee showed antibiofilm activity ranged from 6.3 to 56.7% against C. albicans and from 40.9 to 67.9% against P. aeuroginosa.*
Conclusion: *In present study, cinnamon oil showed the strongest antibiofilm activity against the tested strains of P. aeuroginosa and C. albicans, suggesting that it could be available alternative to antibiotics.*
Key Word: *antibiofilm – Eos- Pseudomonas aeruginosa - Candida albicans*

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I. Introduction

Microorganisms can live in nature in two forms, planktonic form and biofilm form, whereas planktonic form means the free living cells while biofilm means the cluster of microorganisms that adhere over the surface [1,2]. Microorganisms in a biofilm can resist host immune responses, withstand antimicrobial treatments, and generally exhibit specific modification in genetic expression by which they can be easily distinguished from their planktonic counterparts in terms of physiology and metabolism [1,3]. Biofilm formation represents a protected growth pattern, mediated by many cellular mechanisms combines with each other as bacterial motility, adhesion mechanisms, and quorum-sensing (QS) phenomenon. Surface structures, as flagella, fimbriae, and pili play the key role in the adhesion and strengthening the bond between bacteria and substrate to produce extracellular polymeric substance (EPS) or biofilm matrix, which is the main component of biofilm structure. EPS composed of a mixture of polysaccharides, extracellular DNA (eDNA), and proteins that help the biofilm to adhere to the surface, trap nutrients, and provide structural support. Moreover, the biofilm matrix (EPS) is also responsible for holding the community of biofilm cells in close proximity, enabling cell-to-cell communication (quorum sensing), and facilitating the exchange of genetic material through horizontal gene transfer, thereby, influencing the overall architecture as well as their resistance phenotype [3-6]. Biofilms can grow on different surfaces and can be found in a wide range of environments, including aquatic environments, on artificial industrial structures, on implanted medical devices, and on plant and mammalian tissues [1, 2].

Among the wide range of Gram-negative biofilm forming bacteria; *Pseudomonas aeruginosa* (*P. aeruginosa*) is an opportunistic pathogen and considered a major cause of nosocomial infections, involving urinary tract infections, pneumonia and bacteremia and also found in patients having burns, surgical, pus and accidental wounds. These infections become severe when the patients have impaired immune system. *P. aeruginosa* is ranked as the 4th among the nosocomial pathogens and it is difficult to be treated due to its resistant behavior against different antibiotic drugs [7,8]. *P. aeruginosa* can effectively colonize various surfaces such as, medical materials (urinary catheters, implants, contact lenses, etc.) [1], and food industry equipment (mixing tanks, vats and tubing) [9]. On the other hand, Candida species are the predominant fungal species isolated from infected medical devices and account for about 15% of hospital-acquired cases of sepsis [1]. *Candida albicans* (*C. albicans*) is the most common Candida species identified in clinical contexts and is one of the main causes of nosocomial infections. *C. albicans* is a dimorphic fungus that can be either commensal or an opportunistic pathogen with the ability to cause a variety of infections, ranging from superficial to life threatening [10]. Therefore, a greater understanding of the composition and structure of the biofilm underlying the antimicrobial tolerance of bacteria growing within a biofilm, are vital for the design of effective strategies to manage, prevent and more importantly to eradicate biofilm-associated infections.

Natural medicines have been used for centuries and have shown remarkable effects in preventing and treating microbial biofilm infections. In recent years, there has been an increase in the number of studies on the anti-biofilm activity of natural products as plant essential oil (EOs) and honey bee as they inhibit not only the formation of biofilms but also the QS system which regulates biofilms. The mechanisms of those actions depend mainly on the inhibition of the formation of the polymeric matrix, the suppression of cell adhesion and attachment, interrupting the production of the extracellular matrix, and reducing the production of virulence factors, thus blocking the development of the biofilms and the QS network [7,10,11]. Currently, natural medicines are considered a breakthrough in the development of new anti-biofilm drugs. In the present study, we investigated the effects of five essential oils; cinnamon (*Cinnamomum cassia*), marjoram (*Majorana hortensis*), tea tree (*Melaleuca alternifolia*), clove (*Syzygium aromaticum*), and mint (*Menth peperita*), and honey bee against *P. aeuroginosa* and *C. albicans* biofilms. The aim of this study was to investigate the effects of previous mentioned compounds against *P. aeuroginosa* and *C. albicans* biofilms and their potential that they could be viable alternatives to antibiotics.

II. Materials And Methods

Essential oils (EOs) and honey bee

The EOs of cinnamon (*Cinnamomum cassia*), marjoram (*Majorana hortensis*), tea trea (*Melaleuca alternifolia*), clove (*Syzygium aromaticum*), and mint (*Menth peperita*). The EOs were purchased from the essential oils and natural products unit at the National Research Centre, Dokki, Giza, Egypt. Selection of the EOs was based on their antimicrobial activities showed in other studies [12- 17]. Honey bee (*Apis mellifera*) used in this study was of clover flowers origin and was purchased from honey bee apiary situated in Minofeya-Egypt. All were tested for their inhibitory effect on both planktonic cells and biofilm formation by *Pseudomonas aeuroginosa* and *Candida albicans*.

Strains and culture media

Two reference strains of *Pseudomonas aeuroginosa* [ATCC 9027 (Pa1) and ATCC PA14(Pa2)], two reference strains of *Candida albicans* [ATCC 25922 (C1), ATCC 10231(C2)] were kindly received from Prof. Dr. Sohier Syame (Microbiology and Immunology department, National Research Centre, Dokki, Giza, Egypt). Furthermore, two confirmed clinical isolates of *Candida albicans* (C3 and C4) were obtained from two laboratories belonged to two Minofeya hospitals, Egypt also be used in this study. *P. aeuroginosa* strains were cultured in 3-5 ml of trypton soya broth media (TSB) (PH 7.5±0.2) for 24h at 37°C. *C. albicans* strains were cultured in 3-5 ml sabouraud dextrose broth medium (SDB) (PH 5.6±0.2) for 24h at 28°C. Strains were preserved in 15% glycerol stocks and frozen at 4°C.

Isolation and Identification of *Candida* isolates by morphological and biochemical criteria

A total of twelve clinical isolates were obtained from Shebin-alkaoum University hospital and Al-shohadaa central hospital laboratories, Minofeya, Egypt. The isolates were six from vagina, one from blood, one from sputum, and four from urine samples. Clinical isolates were identified by the growth characteristics on Sabouraud dextrose agar media (40gm dextrose, 10gm peptone, PH 5.6 ± 0.2) and by microscopic examination. *Candida* isolates were biochemically identified as *C. albicans* by biochemical identification test (VITEK) and by germ tube test.

Determination of MIC values of *P. aeruginosa* and *C. albicans* isolates

The minimum inhibitory concentration (MIC) was determined using standard techniques according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [18] and Clinical and Laboratory Standards Institute (CLSI) guidelines [19]. The EOs were diluted in liquid culture media in conjunction with Tween 80 (0.5%). EOs and honey bee concentrations ranged from 0.1 up to 3.2 % (v/v). Hundred microliters of cell suspension (1/100 conc.) in liquid culture medium was inoculated into each well of 96-well plate, followed by 100 µl of the diluted agents. Positive controls was contained the inoculated growth medium without any antimicrobial agent and negative controls was contained only the diluted antimicrobial agent without any growth. After 24 h incubation at corresponding temperatures, absorbance was measured at 490 nm using microplate reader. Decreases in the absorbance of lower than 50% of the positive control samples were considered the MIC values, and measurements were made in triplicates.

Biofilm formation of *P. aeruginosa* and *C. albicans* isolates

P. aeruginosa isolates (Pa1 and Pa2) and *C. albicans* isolates (C1, C2, C3, and C4) were grown overnight at 37°Cc for *P. aeruginosa* and at 28°Cc for *C. albicans*. Then, the OD_{490} of the bacterial and yeast cultures were adjusted to a 0.5 McFarland standards. Polystyrene 96-wells microtiter plates were prepared and inoculated with 200 µL of 24-h-old culture of *P. aeruginosa* isolates (Pa1 and Pa2) in TSB at concentration of 1/100 and incubated for 72 h at 37°C .200 µL of 24-h-old culture of *C. albicans* isolates (C1, C2, C3, and C4) in SDB at concentration of 1/100 was incubated for 72 h at 37°C at 28°C *C. albicans*. Then, the supernatant was removed, and the plates were rinsed three times with distalled H_2O to remove detached planktonic cells.

Detection of biofilm formation

Crystal violet assay

The crystal violet (CV) assay was used for the quantification of biofilm formation by *P. aeuroginosa* and *C. albicans*. In brief, microtiter plates were stained by adding 200 µL of 0.1% crystal violet (CV) solution to each well for 15- 20 min at room temperature. After that, the excess dye was removed by washing the plates three times under running tap water. The remaining CV was resolubilized by adding 200 µL of 99.9% ethanol for 30 min at room temperature without shaking and the OD was measured at 490 nm using microplate reader (Infinite® F50, Tecan, Austria), measurements were made in triplicate.

Scanning Electron Microscopy (SEM)

Biofilms were prepared in six-well tissue culture plate, where sterile 1× 1 cm copper-carbon coupons were submerged in bacterial and fungal suspension and served as the attachment surfaces to which the cells attached. After 24 h of incubation at appropriate temperature, the coupons were rinsed with sterile dis.H_2O, then, fresh medium containing the EO was added and positive control coupons contained biofilm without treatment. The plates were further incubated for 48 h at 37 and 28°C for *Pseudomonas* and *Candida* respectively. Preparation of the coupon-biofilm samples for SEM was performed as described previously [20] and photographs were taken from relevant areas with scanning electron microscope (FE-SEM-FEI, Quanta FEG 250, Netherlands).

Fluorescent microscopy

Detection of biofilm using fluorescent microscope was performed using acridine orange fluorescent stain. Biofilm samples to be examined were prepared using the same method described above for scanning microscopy examination with some modifications. After biofilm samples preparation, the covers were rinsed with distilled H_2O and stained with acridine orange fluorescent stain for 30 minutes in the dark ,then, the covers were rinsed, air dried , and examined and photographed with fluorescent microscope (Leica, DM IL LED FLUO, Germany).

Light microscopy

For light microscopy examination, samples were prepared and then photographed using the bright field filter of Leica, DM IL LED FLUO, Germany microscope.

Biofilm treatment of *pseudomonas* and *candida*

Ninety-six-well microtiter plates were inoculated with 200 µl of 24 h old liquid culture of *P. aeruginosa* isolates (Pa1 and Pa2) in TSB and 200 µL of 24 h and old liquid culture of *C. albicans* isolates (C1, C2, C3, and C4) in SDB at 1/100 conc. Following 24 h of cell adhesion at corresponding temperatures, the supernatant was removed from each well, and the plates were rinsed with sterilized distalled H2o. Subsequently, 200 µl of fresh medium (TSB and SDB) containing the EOs (cinnamon, marjoram, tea tree, clove, and mint oils) or honey bee with concentrations ranged from 0.1 to 3.2% (v/v) added to each well, and the plates were further

incubated for 48 h. Positive controls contained only the inoculated growth medium and negative controls contained diluted EOs or honey bee in growth medium. After 48 h, the supernatant was removed, and the wells were rinsed two times with sterile dis.H2o. Then, 200µ of 0·1% crystal violet (CV) solution was added to each well. After 20 minutes incubation at room temperature, the plates were washed under running tap water to remove excess dye. Finally, bound crystal violet was resolubilized by adding 200µ of 99.9% ethyl alcohol to each well. The absorbance was measured at 490 nm using plate reader (infinite F50, TECAN) and measurements were made in triplicates.

Statistical analysis

Experiments were performed in triplicate. Data are represented as a mean with standard deviation. For statistical analysis, ANOVA was performed followed by a Tukey's HSD post hoc test, and a *P* value< 0.05 was considered to be significant.

III. Results

Isolation and identification of *C. albicans* Isolates

Identification of *C. albicans* isolates by biochemical and phenotypical criteria

Out of a total of twelve clinical samples, 11 isolates (three from urine, one from blood, one from sputum, and six from vagina) with percentage of 91.6 % were identified as Candida by their morphological characteristics on Sabouraud dextrose agar media and microscopic examination. Two isolates were confirmed as 99% *C. albicans* by biochemical identification test (VITEK) as seen in (Table 1), and by forming a germ tube after incubation in human serum for 2 h at 37°C and chlamydospores after 24-48 h at same conditions as seen in (Figure1).

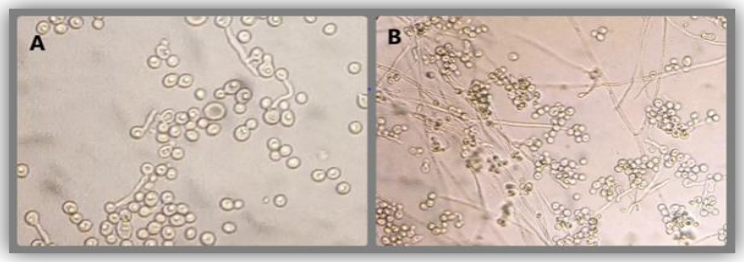

Figure 1: (A) germtube of candida isolates after 2h in serum at 37°C, and (B) chlamydospores of candida isolates after 24 h in serum at 37°C.

Table 2: Biochemical test (VITEK) result of candida isolates.

Biochemical tests of candida isolates																	
3	LysA	-	4	IMLTa	+	5	LeuA	+	7	ARG	+	10	ERYa	-	12	GLYLa	-
13	TyrA	-	14	BNAG	-	15	ARBa	-	18	AMYa	-	19	dGALa	+	20	GENa	-
21	dGLUa	+	23	LACa	-	24	MAdGa	+	26	dCELa	-	27	GGT	-	28	dMALa	+
29	dRAFa	-	30	NAGAI	+	32	dMNEa	+	33	dMELa	-	34	dMLZa	-	38	ISBEa	-
39	IRHAa	-	40	XLTa	+	42	dSORa	+	44	SACa	+	45	URE	-	46	AGLU	+
47	dTURa	+	48	dTREa	+	49	NO3a	-	51	IARAa	-	52	dGATa	+	53	ESC	-
54	IGLTa	+	55	dXYLa	+	56	LATa	+	58	ACEa	+	59	CITa	+	60	GRTas	+
61	IPROa	+	62	2KGa	+	63	NAGa	+	64	dGNTa	+						

Determination of MIC values of *pseudomonas* and *candida*

The minimum inhibitory concentrations (MIC) of the five essential oils and honey bee were determined by the broth micro-dilution method. Cinnamon oil had the highest antibacterial and antifungal activity against tested microbes, with MIC ranged from 0.1 to 0.2% (v/v) against all tested *P. aeuroginosa* strains (Pa1 and Pa2) and antifungal activity with MIC of 0.1 %(v/v) against all tested *C. albicans* isolates (C1,C2,C3, and C4). Tea tree oil showed antibacterial activity with MIC ranged from 0.4 to 1.6 % (v/v) against all tested *P. aeuroginosa* strains and antifungal activity with MIC ranged from 0.2 to 0.8 % (v/v) against all tested *C. albicans*. Marjoram oil showed antibacterial activity with MIC of 1.6 % (v/v) against all tested *P. aeuroginosa* strains and antifungal activity with MIC ranged from 0.1 to 0.8 % (v/v) against all tested *C. albicans*. Clove oil showed antibacterial

activity with MIC ranged from 1.6 to 3.2 % (v/v) against tested *P. aeuroginosa* strains and antifungal activity with MIC of 0.1 % (v/v) against all tested *C. albicans*, Mint oils showed antibacterial activity with MIC ranged from 1.6 to 3.2 % (v/v) against tested *P. aeuroginosa* strains and antifungal activity with MIC of 0.1 to 0.8% (v/v) against all tested *C. albicans*. Honey bee showed antibacterial activity with MIC ranged from 0.2 to 3.2% (v/v) against tested *P. aeuroginosa* and antifungal activity with MIC ranged from 0.8 to 3.2% (v/v) against tested *C. albicans*.

Biofilm treatment

Biological treatment of *pseudomonas aeuroginosa* biofilm

A high biofilm inhibitory effect was observed for most of the tested essential oils. Interestingly, cinnamon oil exhibited the most effective antibiofilm activity against tested pseudomonas strains (Pa1 and Pa2). Tested oils showed reduction of biofilm from 20.5 to 86.4% against tested *P. aeruginosa* strains (Pa1 and Pa2). Cinnamon oil showed the strongest biofilm reduction activity ranged from 52.1 to 86.4% with minimum biofilm inhibitory concentration (MBIC) of 0.1% (v/v) against Pa1 and biofilm reduction ranged from 32.3 to 72.3% and MBIC of 0.2% (v/v) against Pa2. Marjoram oil showed biofilm reduction activity ranged from 39.4 to 80.8% with MBIC of 0.4% (v/v) against Pa1 and biofilm reduction ranged from 25.6 to 68.9% with MBIC of 3.2% (v/v) against Pa2. Tea tree showed biofilm reduction activity ranged from 45 to 82.1% with MBIC of 0.4% (v/v) against Pa1 and biofilm reduction ranged from 30.1 to 74.4% with MBIC of 3.2% (v/v) against Pa2. Clove showed biofilm reduction activity ranged from 43.8 to 78% with MBIC of 0.8% (v/v) against Pa1 and biofilm reduction ranged from 24.6 to 73.8% with MBICof 0.8% (v/v) against Pa2. Mint oils showed biofilm reduction activity ranged from 35.6 to 82.3% with MBICof 0.4% (v/v) against Pa1 and biofilm reduction ranged from 20.5 to 67.8% with MBICof 3.2% (v/v) against Pa2.

Honey bee showed antibiofilm activity ranged from 48.4 to 70.3% with MIBC of 0.2% (v/v) against Pa1 and antibiofilm activity ranged from 40.9 to 69.4% with MIBC of 0.4% (v/v) against Pa2.

Table 2: The mean of OD ± Standard deviation of *P. aeuroginosa* biofilm after treatment with EOs and honey bee at minimum biofilm inhibitory concentration (MBIC).

	Pa1 (ATCC9027)		**Pa2 (ATCC PA14)**	
	OD±SD	MBIC (%v/v)	OD±SD	MBIC (%v/v)
Cinnmon oil	0.392±0.07	0.1	0.298±0.003	0.2
Marjoram oil	0.388±0.08	0.4	0.188±0.02	3.2
Tea tree oil	0.379±0.04	0.4	0.154±0.05	3.2
Clove oil	0.262±0.05	0.8	0.248±0.029	0.8
Mint oil	0.391±0.07	0.4	0.194±0.01	3.2
Honey bee	0.341±0.04	0.2	0.229±0.03	0.4

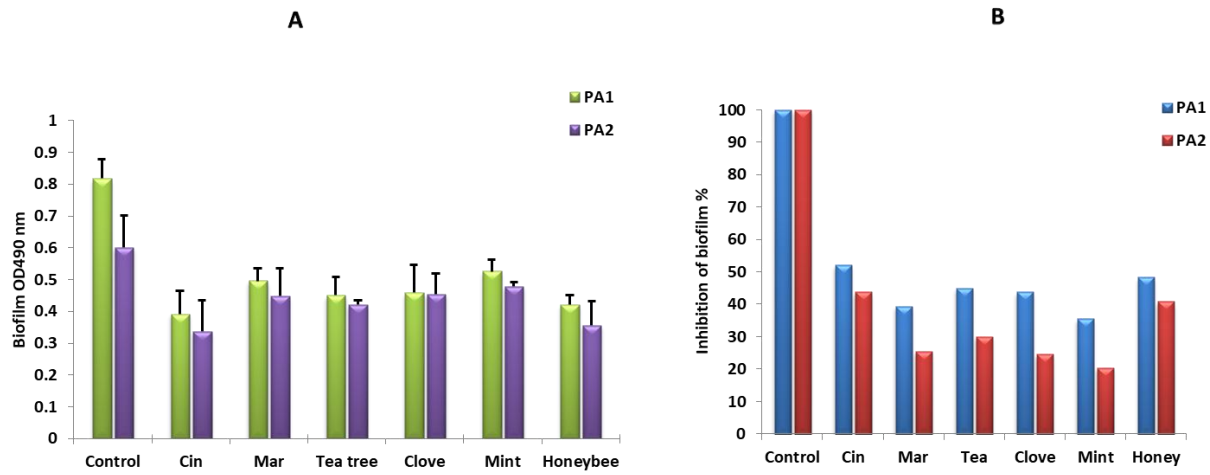

Figure 2: Effect of essential oils and honey bee on *P. aeuroginosa* biofilm (A)Mean optical density (OD) ± standard deviation (SD) of biofilm before (control) and after treatment with 0.1%(v/v) of EOs and honey bee, and (B) Percentage % of biofilm inhibition with EOs and honey bee.

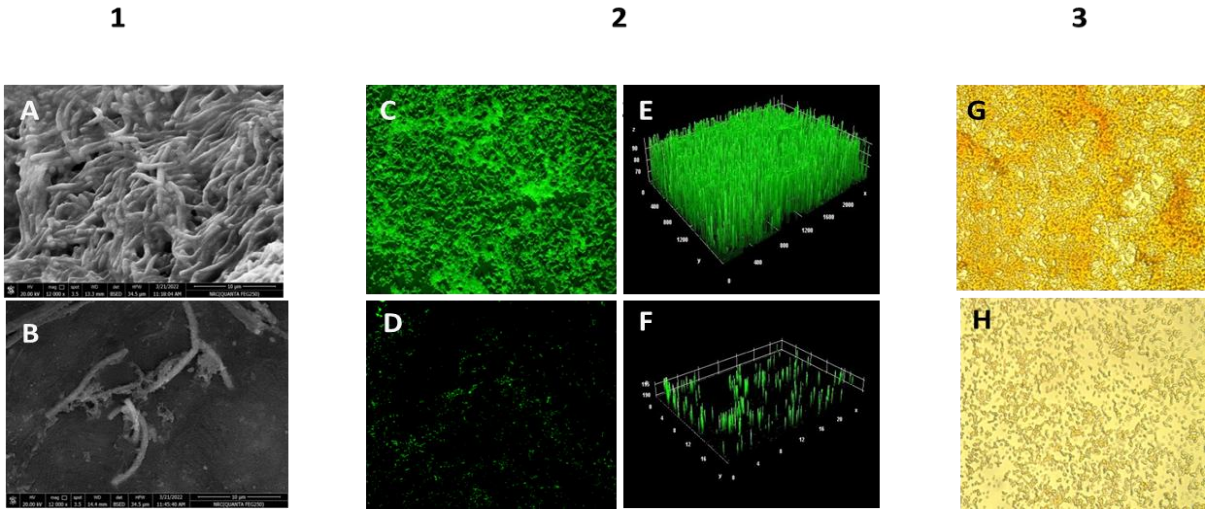

Figure 3: 1- Microscopic images of biofilm of *P. aeuroginosa* under scanning electron microscope (SEM) [(A) control untreated biofilm and (B) biofilm treated with cinnamon oil]

2- Microscopic images of biofilm of *P. aeuroginosa* under fluorescent microscope [C and D are biofilm before and after treatment with cinnamon oil, E and F are 3D- fluorescent images of biofilm before and after treatment with cinnamon oil].

3- Microscopic images of biofilm of *P. aeuroginosa* under optical microscope [(G) control untreated biofilm and (H) biofilm treated with cinnamon oil].

Biological treatment of *Candida albicans* biofilm

All tested EOs demonstrated antibiofilm activity against tested candida isolates with biofilm reduction ranged from 4.6 to 84.1 %. Cinnamon oil showed the strongest antibiofilm effect with biofilm reduction ranged from 44.9 to 80.7% and MBICranged from 0.1 to 0.2% (v/v) against tested candida isolates (C1, C2, C3, and C4). Marjoram oil showed biofilm reduction activity ranged from 10.1 to 80.5% with MBIC ranged from 0.1 to 0.8% (v/v) against tested candida isolates. Tea tree showed biofilm reduction activity ranged from 15.4 to 76.2%with MBIC ranged from 0.4 to 1.6% (v/v) against tested candida isolates (C1, C2, C3, and C4). Clove showed biofilm reduction activity ranged from 25.1 to 79.8% with MBIC ranged from 0.1 to 0.8% (v/v) against tested candida isolates. Mint oils showed biofilm reduction activity ranged from 4.6 to 78% with MBIC ranged from 0.2 to 1.6% (v/v) against tested candida isolates.

On the other hand, honey bee demonstrated less antibiofilm activity against tested candida isolates with biofilm eradication percentage ranged from 6.3 to 57.6 % and MBIC from 1.6 to 3.2% (v/v) against C1,

C2, and C3, while demonstrated good antibiofilm activity against C4 with biofilm inhibitory activity ranged from 52.2 to 57.6% and MBIC of 0.1% (v/v).

The results in table 4 clearly showed the potential activity of the tested EOs and honey bee against the biofilm of tested microorganisms. Cinnamon oil exhibited the strongest antibiofilm activity against all tested bacterial and fungal isolates with the least MBIC values.

Table 4: The mean of OD ± Standard deviation of *Candida* biofilm after treatment with EOs and honey bee at minimum biofilm inhibitory concentration (MBIC).

	C1(ATCC 25922)		C2(ATCC10231)		C3		C4	
	OD±SD	MBIC(%v/v)	OD±SD	MBIC(%v/v)	OD±SD	MBIC(%v/v)	OD±SD	MBIC(%v/v)
Cinnamon oil	0.128±0.003	0.1	0.107±0.005	0.1	0.151±0.02	0.1	0.127±0.022	0.2
Marjoram oil	0.108±0.01	0.4	0.117±0.01	0.1	0.118±0.02	0.1	0.141±0.05	0.8
Tea tree oil	0.157±0.08	0.4	0.136±0.01	0.8	0.144±0.07	0.4	0.135±0.07	1.6
Clove oil	0.141±0.03	0.1	0.138±0.02	0.4	0.124±0.06	0.8	122±0.06	0.8
Mint oil	0.132±0.07	1.6	0.146±0.04	0.4	0.156±0.05	0.2	0.148±0.06	0.4
Honey bee	0.151±0.04	1.6	0.147±0.01	3.2	0.152±0.03	1.6	0.141±0.02	0.1

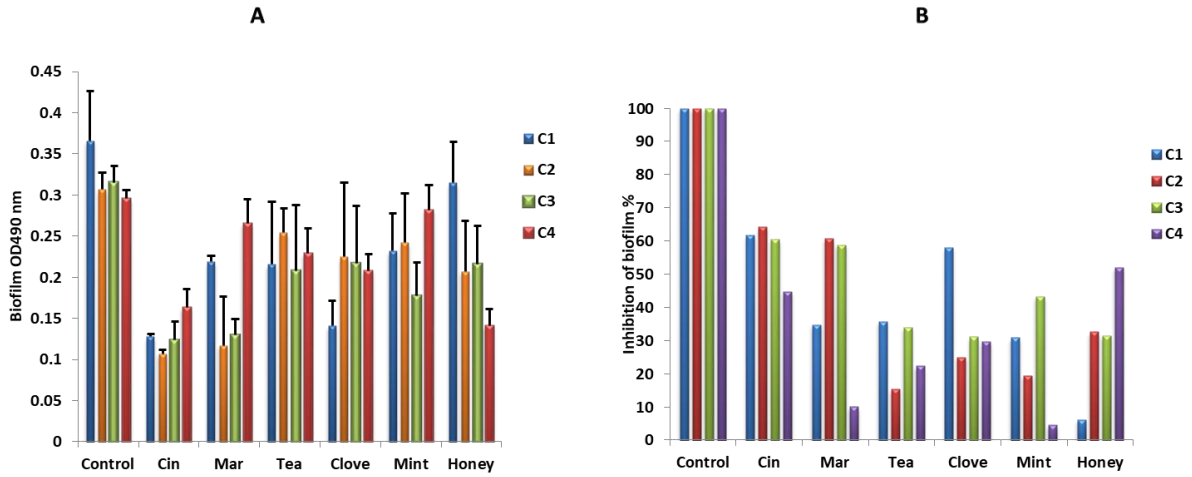

Figure 4:
Effect of essential oils and honey bee on *C. albicans* biofilm (A) Mean optical density (OD)± standard deviation (SD) of biofilm before (control) and after treatment with 0.1%(v/v) of EOs and honey bee, and (B) Percentage % of biofilm inhibition with EOs and honey bee.

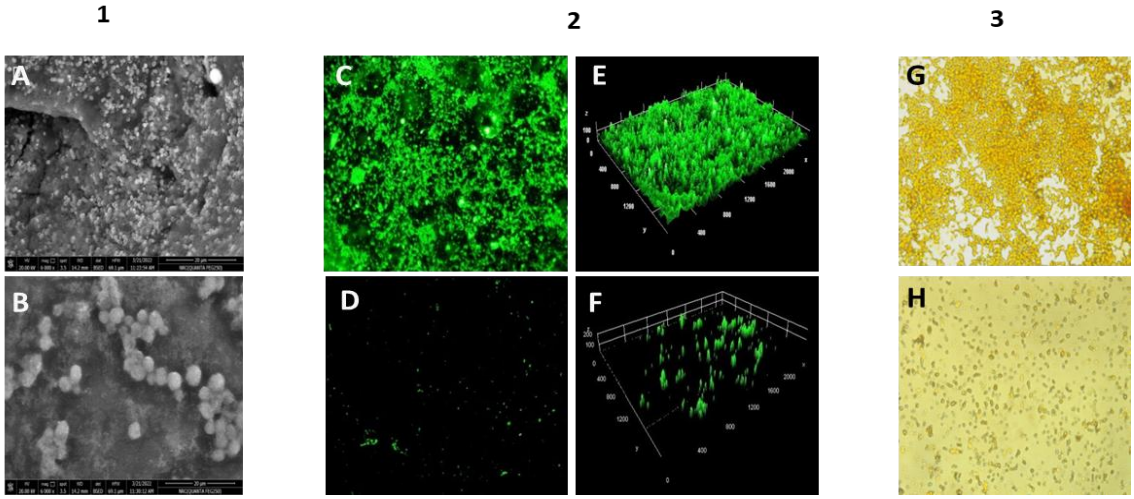

Figure 5:

1- Microscopic images of biofilm of *C. albicans* under scanning electron microscope (SEM): [(A) Control untreated biofilm and (B) biofilm treated with cinnamon oil].
2- Microscopic images of biofilm of *C. albicans* under fluorescent microscope [C and D are biofilm before and after treatment with cinnamon oil, E and F are 3D- fluorescent images of biofilm before and after treatment with cinnamon oil].
3- Microscopic images of biofilm of *C. albicans* under optical microscope [(G) control untreated biofilm and (H) biofilm treated with cinnamon oil].

IV. Discussion

Bacterial biofilms are clusters of microorganisms attached to a surface and to each other and are embedded in a self-produced extra-polymeric matrix (EPS). Biofilm formation represents a protected growth pattern, mediated by many cellular mechanisms combines with each other as bacterial motility, adhesion mechanisms, and quorum-sensing (QS) phenomenon. Biofilms have great importance for public health due to their role in many infectious diseases and device-related infections. [14]. In this study, five essential oils (cinnamon, marjoram, tea tree, clove, and mint) as well as honey bee were used for exploring their antibiofilm activities against the biofilm of two isolates of *P. aeuroginosa* and four isolates of *C. albicans*. The effect of these oils on biofilms has been investigated in several studies demonstrating sufficient antibiofilm activity against *P. aeruginosa* and *C. albicans* [12-17].

In our study, EOs showed strong antibiofilm activity on tested strains of *P. aeuroginosa* (Pa1 and Pa2) and *C. albicans* (C1, C2, C3, and C4) as it was described above by results. All tested oils have been showed reduction of biofilm with percentage ranging from 20.5 to 86.4% against *P. aeruginosa* and from 0.2 to 84.1% against *C. albicans*. The different activities of tested EOs against biofilm may be due to their chemical composition as reported by many authors who investigate that the major component of cinnamon oil is cinnamaldehyde (52–80%) and the main constituent in clove oil is eugenol with concentration ranges from 68 to 88% [21-26]. Mint oils contains mostly of menthol and menthone [22]. Ramage et al. reported that the most effective antimicrobial component in tea tree oil is the derivatives included terpinen-4-ol (T-4-ol) [24]. Several studies reported that the effectiveness of antibiofilm compounds depend mainly on the inhibition of the formation of the polymeric matrix, the suppression of cell adhesion and attachment, interrupting the gene expression, and reducing the production of virulence factors, thus blocking the development of the biofilms and the QS network [10-12]. In present study, cinnamon oil, followed by tea tree oil, clove oil, and honey bee showed the strongest antibiofilm effect against tested *P. aeuroginosa* strains. Cinnamon oil showed the strongest antibiofilm effect against *P. aeuroginosa* strains (Pa1 and Pa2) at the least MBIC of 0.1 and 0.2% (v/v) for Pa1 and Pa2 respectively. Topa et al. reported that cinnamaldehyde, which is the greater component of cinnamon oil disrupts biofilm formation and swarming motility of *P. aeuroginosa* [27]. KIM et al. and Utchariyakiat et al. reported that cinnamon oil and its component cinnamaldehyde, reduce pyocyanin production, quorum sensing ability, the swarming motility, and the hemolytic activity of *P. aeuroginosa*, resulting in inhibiting biofilm formation [28, 29]. Our results also are consistent with the findings of other studies that demonstrated cinnamon oil as one of the strongest antibiofilm agents [14, 22-26].Tea tree oil showed antibiofilm activity ranged from 30.1 to 82.1% against tested *P. aeuroginosa* strains (Pa1 and Pa2) with MBIC of 0.4 and 3.2% (v/v) against Pa1 and Pa2 respectively, which is consistent with other studies demonstrated that tea tree oil have good effect on *P. aeuroginosa* biofilm [30,31]. On the other hand, all investigated EOs showed good activity against biofilm of

tested *candida* isolates (C1, C2, C3, and C4). Cinnamon oil, followed by clove oil exhibited the strongest effectiveness against biofilm of all tested *candida.* MBIC was ranged from 0.1 to 0.2% (v/v) for cinnamon oil and from 0.1 to 0.8% (v/v) for clove oil. These results are consistent with other studies about the effectiveness of these oils against *C. albicans* biofilm [30-34]. A recent study demonstrated that cinnamon and clove oils showed significantly higher efficacy against vulvo-*vaginal C. albicans* isolates [35]. Condò et al. reported that cinnamon and clove oils gave the best results showing a significant activity against both *P. aeuroginosa* and *C. albicans* biofilms, indicating that cinnamon oil gave the strongest effects against tested strains [38].

Our microscopic examination of treated biofilms (Figures 3, 5) showed the strong effect of cinnamon oil on EPS (biofilm matrix) formation, which is consistent with other reports about mode of action of EOs against biofilms [13-18]. SEM analysis showed few biofilm cells attached to copper-carbon coupons and few EPS in the presence of cinnamon oil with *P. aeuroginosa* biofilm, which is consistent with other studies on mode of action of cinnamon oil effect on *P. aeuroginosa* biofilm [28, 29]. SEM analysis of treated *C. albicans* biofilm showed reduction in the number of biofilm cells attached to copper-caboncoupons, changes in cell shape, and reduction in EPS production in the presence of cinnamon oil with *C. albicans* biofilm, which is consistent with other studies on mode of action of cinnamon oil effect on *C. albicans* biofilm [35, 39].

Honey bee is one of the most natural compounds that has been mentioned for its antimicrobial activities against wide range of microbes. Several reports demonstrated the effectiveness of honey in the treatment of various bacterial and fungal biofilms have been published [40-42]. Activity of honey bee against biofilms of *P. aeuroginosa* and *C. albicans* has been demonstrated in other studies [43-46]. Honey bee showed antibiofilm activity against both Pa1 and Pa2 at MBIC ranged from 0.2 to 0.4% (v/v) respectively. Activity of honey bee against *P. aeuroginosa* biofilm was reported [47]. On the other hand, tested isolates of candida exhibited different reactions to honey bee with different concentrations of MIC and MBIC. These differences are likely to be due to the different strains tested and their relative biofilm-forming abilities [46].

V. Conclusion

In summary, the present study conclusively demonstrates the anti-biofilm potential of selected plant oils and honey bee shown to be antibiofilm agents for *C. albicans* and *P. aeuroginosa*, and may find use in future therapeutic strategies. Results suggested that cinnamon oil *(Cinnamomum cassia*), and honey bee may be useful approaches to control the biofilm production by *P. aeuroginosa* and *C. albicans*.

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