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Chemical Composition and Effectiveness of *Ocimum basilicum L.* Extracts on the Adhesion of *Candida albicans* and *C. dubliniensis* on Acrylic Surfaces of Removable Orthodontic Appliances

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Abstract: The outbreak of diseases caused by *Candida* species is growing. These opportunistic pathogens can produce biofilm on non-biological surfaces, such as removable orthodontic appliances. Over the past two decades, there has been a significant increase in resistance to several types of antifungal drugs. Recently, there is a growing interest in using herbal plants and their extracts as an alternative to antifungal drugs. A common plant is basil, which belongs to *Lamiaceae* family. This study aimed to determine the effect of basil extracts on the adhesion of *C. albicans* and *C. dubliniensis* to acrylic surfaces of removable orthodontic appliances. Basil dried leaves were macerated in ethanol to extract its constituent, and then the extract was dissolved in water and fractioned by other solvents, including n-hexane and ethyl acetate, successively. The minimum inhibitory concentrations (MIC) of the extracts against standard species of *Candida* were determined by the broth microdilution method based on clinical and laboratory standard institute (CLSI) protocol. Afterward, adherence and biofilm formation tests were done on acrylic specimens in the vicinity of proper concentration of fractions with the help of the Crystal Violet and spectrophotometry method. MIC for ethyl acetate fraction, n-hexane fraction, ethanol crude extract, and the aqueous fraction was 1024, 2048, ≥2048, >2048 µg/ml, respectively. Inhibition of adherence and biofilm formation for *Candida albicans* and *Candida dubliniensis* in the vicinity of ethyl acetate fraction was 73% and 78%, respectively. In the vicinity of n-hexane fraction was 65% and 78%, respectively. Two fractions could inhibit the growth, adherence, and biofilm formation of two *Candida* species.

Keywords: Basil extract; *Candida albicans*; *Candida dubliniensis*; Adherence; Biofilm; Removable orthodontic appliances.

1. Introduction

Removable orthodontic appliances and full dentures provide an excellent environment for adhesion and colonization of pathogenic and non-pathogenic organisms that can cause

infectious ulcers in the oral mucosa [1]. Yeasts are not inherently pathogenic, which means that very few of them can cause diseases in a healthy person, and immunodeficiency is a necessity for their pathogenicity [2, 3]. Various types of *Candida* are known as opportunistic pathogens, which are located in the oral cavity [4]. Common types that lead to diseases are *Candida albicans*, and *Candida dubliniensis*, which could replicate in cases of diminished host defense and become pathogenic despite their limited virulence [5, 6]. *Candida*'s adhesion to the host's mucous membranes, acrylic surfaces of removable orthodontic appliances, and denture prostheses lead to the production of proteolysis enzymes that damages mucosal cells; resulting in denture stomatitis [7-10]. Continuous use of removable appliances, especially during the night, such as most functional and removable orthodontic appliances, predispose patients to denture stomatitis [11, 12]. Over the past two decades, there has been a significant increase in resistance to several types of antifungal drugs [13-15]. Azole-resistant types of *Candida* are amongst the pathogens that are mainly responsible for nosocomial and food-related infections [15]. Also, biofilm formation by *Candida* species has raised concerns since biofilms increase the cell's resistance to antifungal drugs by protecting them against the host's immune system [16]. One way to avoid resistance to antifungal drugs is to use herbal extracts [17].

Several reports are available on the use of plants as a treatment for oral diseases [18-20]. Many herbal-extracts are used in traditional medicine and documented in the pharmacological books as a means to deal with oral infections [21, 22]. It has been shown that some herbal extracts can effectively inhibit the production of *Candida* biofilms [23-26].

Basil (*Odium basilicum L.*) is an Ocimum genus [27]. The Ocimum genus contains 50-150 species of plants that are distributed globally and found in the tropical regions of Asia, Africa, Central America, and South America [28, 29]. Basil is a medicinal plant widely used in traditional medicine to treat headaches, cough, kidney diseases, and also as an anticonvulsant agent. It is also used as a food flavor in sauces, meats, pizzas, and salads. Also, this vegetable oil is used in the cosmetics, decorative, and perfume industries [27, 30, 31]. In Oxenham *et al*. study, it was reported that two chemotypes of basil, methyl chavicol and linalool oil, had antifungal effects against "Botrytis Fabae" at 1000 and 300 ppm (Parts Per Million), respectively [32]. In the study by Pozzatti *et al*. in 2008, it was shown that the essential oil of *Ocimum basilicum L.* did not have an antifungal effect at the concentrations which they used in their study [33]. Ferreirra *et al*. reported that *O. basilicum L*, the extract, had *in vitro* anti-biofilm activity of *Streptococcus mutans* on acrylic surfaces of removable orthodontic appliances at concentrations of 1:1 (pure) till 1:128 [34].

This study aimed to determine the effect of basil extracts on the adhesion of *C. albicans* and *C. dubliniensis* to acrylic surfaces of removable orthodontic appliances. The necessity of conducting this research is due to the presence of porosity on the surface of acrylic appliances caused by food and microorganisms; thus, a solution with anti-adhesion properties against microorganisms can control the oral hygiene of the users of these appliances. This solution can be a proper alternative to chemicals, such as Chlorhexidine, with side effects such as changes in the color of both teeth enamel and appliances.

2. Materials and Methods

2.1. Plant materials and extraction procedure.

The basil plant, called *O. basilicum L.*, was collected from Kazerun in southern Fars province in May 2018 and was identified and approved by Mr. Mehdi Zare, the botanist of the

Medicinal and Natural Products Chemistry Research Center (MNCRC). After drying the leaves (300g) in the shade and grinding with an electric grinder, the resulting plant powder was extracted by soaking in 3 liters of 96% ethanol for 48 hours. The crude ethanol extract was filtered using filter paper and evaporate ethanol solvent, using a rotary evaporator under reduced pressure at 40 ºC. The crude ethanol extract was dissolved in 200 ml of distilled water and 200 ml of methanol.200 ml 0f n-hexane were added in 3 steps to the solution to extract the n-hexane fraction. After evaporating the methanol of the aqueous fraction, 200 ml of ethyl acetate were added to the solution to produce ethyl acetate fraction. The n-hexane and ethyl acetate fractions were placed in a rotary evaporator to remove their solvents. The remaining polar solution was named as an aqueous fraction.

2.2. Acrylic resin specimens.

150 Acrylic resin specimens were prepared using cold cure acrylic resin (Acropars). To produce a smooth surface, each coin was polished using a laboratory milled. Finally, acrylic specimens were washed with distilled water and alcohol and then autoclaved at 121°C with 15 pounds.

2.3. Preparation of yeast suspension for microdilution.

A few colonies of fresh yeast were added to 100 ml of distilled water. By adjusting the spectrophotometer at 530 nm and 0.15 Optical Density (OD); the suspensions with $1\text{-}5\times10^6$ cells were obtained. The resulting suspension was diluted 1/1000 by RPMI-1640 medium (Sigma).

2.4. Micro dilution assay.

To determine the Minimum Inhibitory Concentration (MIC) of herbal extracts which inhibited 90% of fungal growth (MIC 90), the broth microdilution method was used [35-38]. To perform this test, the 96-well microplate was used. 200 µl and 100 µl of liquid medium (RPMI-1640) were added to the column 1 and 2 to 12, respectively. We considered the first row for ethyl acetate fraction and the second to fourth row for n-hexane fraction, ethanol crude extract, and aqueous fraction. The amount of (12.5 µl) four extracts were added to column 2. The volume of column 2 reached to 200 µl using a liquid medium. The serial dilution method was done using an 8-channel sampler up to column 11. Then 100 µl of yeast suspension equal to $1\text{-}5\times10^6$ cells was added to columns 2 to12. The first column containing only 200 µl of culture medium was used as blank for controlling the sterility of the condition, and column 12 containing a fungal suspension, and no extracts were used as a positive control (growth control). The plates were incubated for 48 hours at 35°C to determine MIC. This test was done in duplicate.

2.5. Biofilm adhesion.

To determine the inhibitory effect of basil extracts on adhesion and production of biofilm of *C. albicans* and *C. dubliniensis* on acrylic specimens, 5 plates with 24 wells were used (Figure 1). Plates 1 to 5 were used for 1024 µg/ml and the four concentrations below the MIC of n-hexane and ethyl acetate extracts. The sterilized acrylic specimens were placed in all microplates. A column of these plates was used as the negative control, which included a culture medium without yeast suspensions and extracts to determine the probability of

contamination, and another column was considered as the positive control, which included the culture media and suspensions of yeast without extracts as the control group. Finally, the plates were incubated at 35°C for 48 hours for biofilm formation on acrylic specimens.

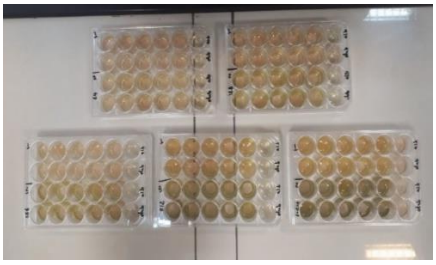

Figure 1. Five plates with 24 wells for determining adhesion and biofilm formation assay. (Three lower plates from right to left are 1024, 512 and 256 µg/ml of n-hexane and ethyl acetate fractions, and two upper plates from right to left are 128 and 64 µg/ml of n-hexane and ethyl acetate fractions which ethyl acetate fraction is in first 2 rows of each plate and n-hexane fraction is in second 2 rows of each plate.)

After incubation, the wells were washed twice using sterile PBS buffer to remove the non-stick cells from the acrylic surface. 500 µl of 0.1% crystal violet was added to wells and kept the plates at room temperature for 15 minutes. The next step was to drain the well and then again wash with PBS for 3 times. The acrylic specimens were transferred to another plate, and 500µl of 30% Acetic acid was added to dissolve the colored cells. Finally, we measured the optical density (OD) of the microplates with the Elisa microplate reader at 550 nm.

2.6. Gas chromatography (GC) analysis.

For gas chromatography analysis, a set of Agilent 6890N chromatograph (Agilent Technologies, USA) used with an HP-5 capillary column (30 m × 0.25 mm: 0.25 µm film thickness), the oven temperature condition was 60°C- 250°C at 5°C/min, and the final temperature continued for 10 minutes. Helium used as carrier gas using a flow rate of 1.0 mL/min. The temperature for the injector and detector (FID) was set at 240 and 250 °C, respectively.

2.7. Gas chromatography-mass spectroscopy (GC-MS) analysis.

The GC-MS was done with an Agilent 7890N chromatograph, coupled to an Agilent 5975C mass spectrometer (Agilent Technologies, USA) with the following specifications: operate at 70 eV ionization energy, 0.5 s/scan, and the mass range: 35-400. The capillary column was HP-5MS (phenyl- (5%) methyl-(95%) siloxane, 30 m × 0.25 mm, film thickness 0.25 µm), and the carrier gas was helium at a flow rate of 1.0 ml/min. The oven temperature was increased from 60°C to 250°C with 5°C /min rate. A 1 ml volume of the sample was injected in a split ratio of 1:10. Scan time was 45 min**.** By the n-alkanes standards and via the Van den Dool method [39], relative retention indices [5] were calculated for all of the constituents. Identification of the compounds performed by comparison of their RRI indices and mass spectra with those reported in Wiley, NIST, and Adams library and authentic literature [40, 41].

2.8. High-performance liquid chromatography (HPLC) analysis.

The analysis of ethyl acetate fraction was carried out on an analytical HPLC system using an RP C18 column (Eurospher-100, 250×4.6mm, Knauer, Berlin, Germany) at a flow rate of 1 ml/min, injection volume of 20µl, K-1000 pump, and K-2500 UV Detector at 210 nm.

3. Results and discussion

3.1. Minimum inhibition concentration (MIC).

Growth inhibition concentrations for the two species of *C. albicans* (ATCC 10261) and *C. Dublin insist* (CBS 8501) are presented in Table 1.

Table 1. MIC 90% of *Candida albicans* and *C. dubliniensis* in the vicinity of 4 extracts of *O. basilicum L.* (µg/ml) *International Unit.

MIC 90%	Ethyl acetate fraction	n-Hexane fraction	Ethanol crude extract	Aqueous fraction	Nystatin
C. albicans ATCC 10261	1024	2048	≥2048	>2048	**1 (5 IU*)**
C. dubliniensis CBS 8501	1024	2048	≥2048	>2048	**1 (5 IU)**

3.2. Biofilm adhesion.

Adhesion and biofilm production of two species of *C. albicans* and *C. dubliniensis* were evaluated in the presence of ethyl acetate and n-hexane fractions, which had the lowest MIC (1024 and 2048 MIC, respectively). The inhibitory percentage of biofilm formation (IPBF) was reported in Table 2, according to the formula.

$$[(OD_{\text{Positive Control}} - OD_{\text{Sample}}) \div OD_{\text{positive control}}] \times 100$$

Table 2. Mean of Optical Density and inhibitory percentage of biofilm formation for *C. albicans* and *C. dubliniensis* in the vicinity of ethyl acetate fraction and n-hexane fraction of *O. basilicum L.*

Species of *Candida*	Fractions of OB	MIC and 4 lower concentration included MIC/2, MIC/4, MIC/8, MIC/16 (µg/ml)					
			1024	**512**	**256**	**128**	**64**
C. albicans	Ethyl acetate fraction	Mean of OD ± SD	0.194 ± 0.013	0.338 ± 0.058	0.402 ± 0.067	0.417 ± 0.074	**0.505 0.199±**
		IPBF	73.43%	53.70%	44.94%	42.88%	**30.83%**
	N-hexane fraction	Mean of OD ± SD	0.250 ± 0.035	0.322 ± 0.087	0.401 ± 0.083	0.380 ± 0.066	**0.454 ± 0.052**
		IPBF	65.76%	55.09%	45.07%	47.95%	**37.81%**
C. dubliniensis	Ethyl acetate fraction	Mean of OD ± SD	0.146 ± 0.018	0.268 ± 0.067	0.300 ± 0.045	0.379 ± 0.076	**0.444 ± 0.079**
		IPBF	78.47%	60.48%	55.76%	44.11%	**34.52%**
	N-hexane fraction	Mean of OD ± SD	0.144 ± 0.027	0.246 ± 0.016	0.271 ± 0.051	0.404 ± 0.121	**0.590 ± 0.055**
		IPBF	78.77%	63.72%	60.03%	40.42%	**12.98%**
PC of *C. albicans*	0.794	0.666	PC of *C. dubliniensis*	0.692	0.665	-	-

OB: *O. basilicum L.*, OD: Optical Density, SD: Standard Deviation, IPBF: Inhibition Percentage of Biofilm Formation, PC: Positive Control. The formula for IPBF:

$$[(OD_{\text{Positive Control}} - OD_{\text{Sample}}) \div OD_{\text{positive control}}] \times 100$$

Using three-way ANOVA, it was determined that the biofilm adhesion and production relative to the microorganisms alone, the concentration alone, the combination of the two factors of concentration and microorganisms, as well as the combination of three factors of concentration, extracts, and microorganisms, were significantly different ($P < 0.05$).

3.3. Comparison of ethyl acetate and n-hexane fraction to each microorganism.

Using three-way ANOVA, the adhesion and biofilm formation of *C. albicans* and *C. dubliniensis* were significantly different in the vicinity of ethyl acetate fraction ($P < 0.05$), but there was no significant difference between the two variables, including microorganisms and concentration ($P > 0.05$). However, there was a significant difference in adhesion and biofilm production of two species of *Candida* for the concentration variable alone ($P < 0.0001$).

The adhesion and biofilm formation of *C. albicans* and *C. dubliniensis* were not significantly different in the vicinity of n-hexane fraction ($P > 0.05$), but there was a significant difference between the two variables, including microorganisms and concentration ($P < 0.05$). The adhesion and biofilm formation of the two species of *Candida* for the concentration factor alone was significantly different ($P < 0.0001$). It means that as the concentrations increased, the adhesion and biofilm formation for every two spices of *Candida* decreased statistically significant. A comparison of ethyl acetate and n-hexane fraction with each microorganism is shown in Figure 2.

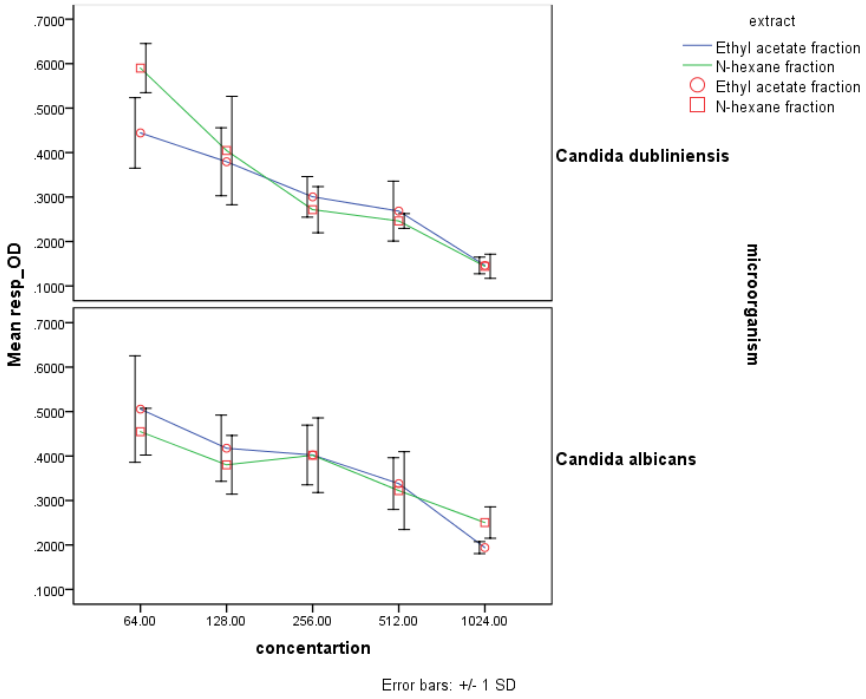

Figure 2. Comparison of ethyl acetate and n-hexane fraction to each microorganism.

3.4. Comparison of two microorganisms of C. albicans and C. dubliniensis to each extract.

Using three-way ANOVA, *C. albicans*, adhesion and biofilm production was not significantly different from that of ethyl acetate fraction and n-hexane fraction alone ($P > 0.05$), and also did not differ significantly between the two variables, including extracts and concentrations ($P > 0.05$), but adhesion and biofilm formation were significantly different between concentrations ($P < 0.0001$).

For *C. dubliniensis*, adhesion and biofilm formation did not differ significantly between the ethyl acetate fraction and N-hexane fraction alone ($P > 0.05$), but there was a significant difference between the two variables, including extracts and concentrations ($P <0.05$). Adhesion and biofilm formation were significantly different between the concentrations ($P < 0.0001$). A comparison of the two microorganisms of *C. albicans* and *C. dubliniensis* with each extract is shown in Figure 3.

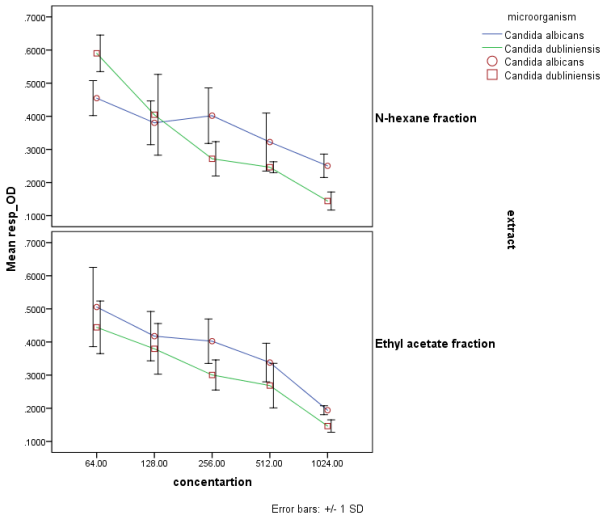

Figure 3. Comparison of two microorganisms of *C. albicans* and *C. dubliniensis* with each extract.

3.5. Identification of compounds.

3.5.1. n-hexane fraction.

Nineteen compounds were identified in the n-hexane fraction are presented in Table 3. neophytadiene (28.5%), phytol (12.0%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (isomer II) (9.3%) compounds have been detected in the extract, among which the diterpenoids were the major ones. GC-MS chromatogram of n-hexane fraction is also demonstrated in figure 4.

Table 3. Chemical compositions of n-hexane fraction.

	RT(min)	n-hexane fraction	RRI	Area %
1	4.5	decane	1001	5.8
2	5.5	2- ethyl-1-hexanol	1044	3.8
3	9.3	dodecane	1201	6.5
4	9.6	estragol	1211	2.8
5	10.6	geranial	1250	1.5
6	11.4	ß-citral	1281	2.4
7	14.5	terta decane	1401	4.7
8	15.0	(E)-β-caryophyllene	1422	2.3
9	15.3	trans-α.-bergamotene	1435	t
10	15.9	α-humulene	1459	1.6
11	16.5	γ-muurolene	1485	1.5
12	17.9	α-(Z)-bisabolene	1544	3.4
13	19.0	caryophyllene oxide	1591	1.2
14	19.3	n-hexadecane	1601	2.0
15	23.7	2,6,10-trimethyl-Pentadecane	1802	1.0
16	24.5	neophytadiene	1841	28.5
17	25.3	3,7,11,15-tetramethyl-2-hexadecen-1-ol (isomer I)	1882	5.0
18	25.3	3,7,11,15-tetramethyl-2-hexadecen-1-ol (isomer II)	1882	9.3
19	30.0	phytol	2025	12.4
-		-	-	95.7

t=trace (0.05%), RT=Retention time

3.5.2. Ethyl acetate fraction.

Thirteen compounds were identified in the ethyl acetate fraction (Table 4). The major compounds were caryophyllene oxide (10.7%) and thymol (5.1%) in this fraction. Based on the GC chromatogram data (Figure 5), ten compounds were suggested for ethyl acetate fraction (Table 4).

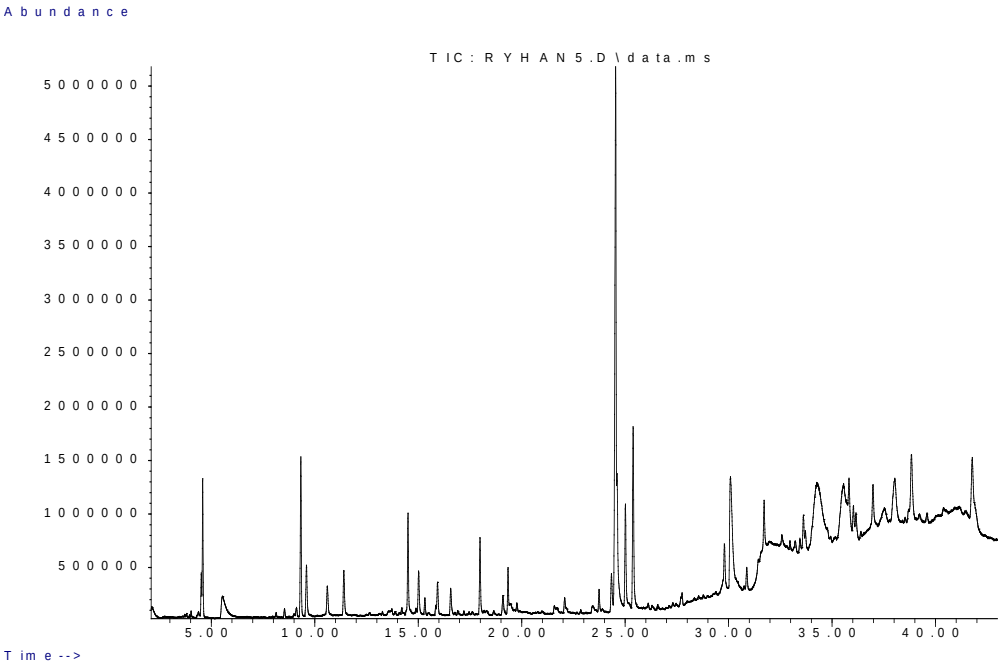

Figure 4. GC-MS chromatogram of n-hexane fraction.

Table 4. Chemical compositions of ethyl acetate fraction.

No	RT(min)	compounds	RRI real	RRI	Area%
1	6.1	decane	1001	1000	0.5
2	6.4	1,8- Cineol	1031	1018	1.8
3	7.1	linalool	1099	1068	1.4
4	8.1	terpinen-4-ol	1174	1172	1.2
5	9.2	thymol methyl ether	1232	1234	1.2
6	9.8	2-decenal	1260	1262	1.7
7	10.8	thymol	1290	1302	5.1
8	12.3	eugenol	1356	1364	1.0
9	12.8	(E)-methyl cinnamate	1381	1386	1.4
10	15.7	α-(Z)-bsabolene	1507	1512	0.5
11	16.1	eugenol acetate	1522	1530	1.2
12	17.2	caryophyllene oxide	1582	1579	10.7
13	18.9	α-cadinol	1652	1655	1.3
					29.0

RT=Retention time

3.6. HPLC analysis.

The RP18 HPLC chromatogram of the ethyl acetate fraction of the ethanol crude extract was eluted with H_2O (solvent A) and acetonitrile (solvent B) as the mobile phase. The elution was gradient 10-100% acetonitrile in water with the following conditions: at first 5 min in 10% B then increased linearly to 60 %B in 5 min and was kept in 60% B for the next 10 min then increased to 100% B in 10 min and returned to 10% B solvent at the end.

The ethyl acetate presented major peaks at RT 12.267 and 12.767 min (Figure 6). The nonvolatile compounds in the ethyl acetate fraction of basil were reported as a phenolic compound such as rutin, caffeic acid, kaempferol, chlorogenic acid, gallic acid, and quercetin [39, 42].

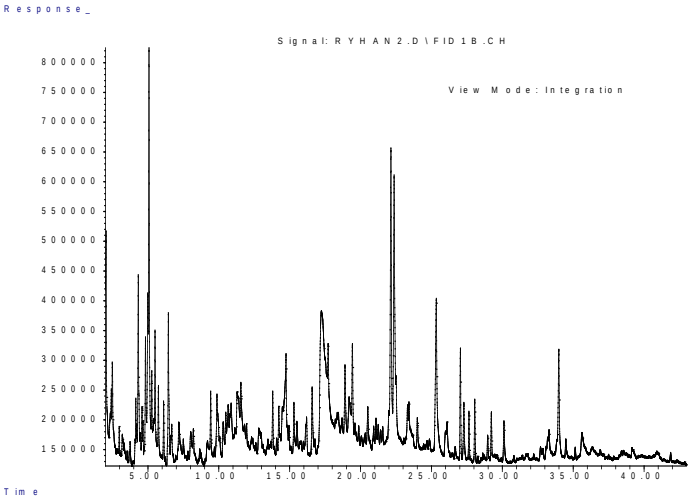

Figure 5. Gas chromatogram of ethyl acetate fraction.

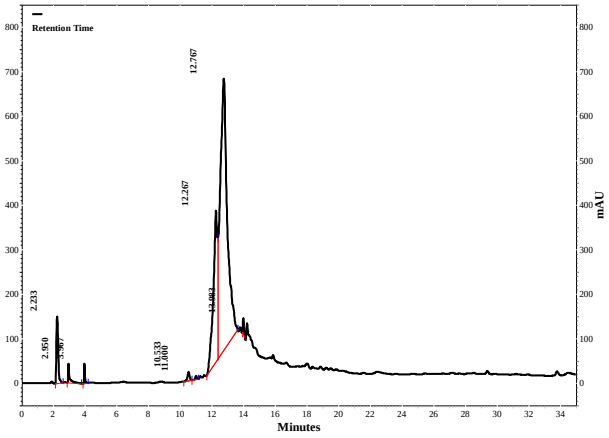

Figure 6. HPLC chromatogram of ethyl acetate fraction.

3.7. Discussion.

The ever-increasing prevalence of drug-resistant microorganisms, as well as the toxicity of existing antifungal compounds, has led toward more attention to the antimicrobial activity of natural products or other means to treat these microorganisms. The limited number of available drugs to treat fungal infections, which are mostly fungistatic, as well as the emergence and existence of resistant species to antifungal agents, requires the exploration of alternative treatments [26]. Plants can be potential options for obtaining a wide range of medications, which have traditionally been used in medical treatment as well as in pharmacy [21]. In the present study, the goal was to use natural compounds of *O. basilicum L.* against fungal agents such as *C. albicans* and *C. dubliniensis* on the acrylic surface of removable orthodontic appliances. Since these microorganisms are the natural flora of the oral environment and are one of the opportunistic pathogens, acquiring a natural compound that can restrict and inhibit yeast growth as well as controlling its pathogenic factors would be useful by replacing common antifungal drugs.

In the study by Elsherbiny *et al.*, it was shown that there are 4 main components found in ethyl acetate extract of *O. basilicum L.* including methyl cinnamate, 1,8-cineol, linalool, and terpinen-4-ol that is in line with our study results [39]. According to a study by Stefanovic *et al.* found that methyl cinnamate had a significant effect on bacterial and fungal pathogens and has the potential to become an antimicrobial agent. They reported MIC of 1000 µg/ml for methyl cinnamate, which confirms MIC results of ethyl acetate extracts in our study [43]. Another ingredient in the ethyl acetate extracts is 1-8-cineol. Its mechanism involves

preventing adhesion of the cell and also causing significant cellular impairment, which confirms the results of our tests for the production of *C. albicans* and *C. dubliniensis* biofilms in the presence of ethyl acetate extracts. The other substance is terpinen-4-ol, which has an anti-candidiasis mechanism that involves altering the properties of the cell membrane, as well as preventing cell respiration through its effect on mitochondria. The first mechanism can affect the cell membrane and its properties that can impair their adhesion ability. This can justify the results of biofilm adhesion in this research. The other substance contained in the ethyl acetate extract is the linalool, its anti-*Candida* mechanism involves preventing the extrusion of H + through its effect on proton pumps [44]. In the study by Braga *et al*., it was shown that eugenol and thymol had an effect on the surface of *C. albicans* membrane, and these two substances made this surface rough and rugged, which was initially smooth, showing the ability to change the properties of the *Candida*'s membrane that would affect the adhesion ability of this yeast. In their study, thymol had a more noticeable effect (40-50%) on the cell membrane than eugenol [45]. He *et al*., as well as Dalleau *et al*., reported that terpenoids such as eugenol, thymol, 1,8-cineol, and linalool can effectively inhibit the formation of *C. albicans* biofilm [46, 47], which confirms our test results.

We report here the bioactive compounds such as phytol, neophytadiene, and caryophyllene oxide in the n-hexane fraction. Phytol is a diterpene that has antimicrobial properties [48, 49]. Neophytadine is also shown to be a good analgesic, antipyretic, anti-inflammatory, antimicrobial, and antioxidant compound. Caryophyllene oxide is an oxygenated sesquiterpene that exhibits antimicrobial and antifungal activity against onychomycosis, *Candida albicans*, and *Cryptococcus neoformans* [40]. Since n-hexane and ethyl acetate fraction have almost the same effect as anti-biofilms, it can be suggested that compounds like methyl cinnamate, thymol, linalool, terpinen-4-ol, 1,8- cineol and eugenol are bioactive agents as well as neophytadine and phytol on the biofilm formation process and ethyl acetate may have nonvolatile substances that are responsible for the bioactivity that should be isolated and identified in the future. Zore *et al*., in their study, concluded that MIC for linalool is much stronger than eugenol, and they affect *Candida*'s membrane integrity to induce their anti-candida properties [50]. The results of Tanrıkulu *et al*. study [26] is in line with our results; hence, it can be concluded that the secondary compounds affecting *C. albicans* are higher in non-polar solvents and include more percentages. The results of the Ferrierra *et al*. study [34] and our study indicated that basil with a strong effect on the growth, adhesion, and biofilms formation of 3 major human oral pathogens, including *Streptococcus mutans* and *C. albicans*, and *C. dubliniensis*, can affect the health of a patient with poor oral hygiene as well as patients who use removable orthodontic appliances and dentures. Furthermore, as our results demonstrated, the n-hexane fraction contains estragole, which is reported to be a carcinogen [51].

Purifying the active compounds in both ethyl acetate and n-hexane fraction and evaluating their anti-biofilm activity on *Candida* species is one of the suggestions for future studies. Also, mixing two fractions to create a synergistic effect, and by testing these extracts on other common oral pathogens could be done in future studies. Testing the biocompatibility of these extracts is necessary before using them in clinics.

4. Conclusions

Two extracts (ethyl acetate and n-hexane fraction) can inhibit the growth, adherence, and formation of *C.albicons* and *C.dubliniensis* biofilm on acrylic surfaces of removable plates.

Hence, these extracts can be selected as antifungal solutions or even mouthwash. Using ethyl acetate fraction is better than n-hexane fraction due to the lack of estragole compound in ethyl acetate fraction, which is carcinogenic. Also, MIC of ethyl acetate fraction for *Candida* species is lower in comparison with other extracts.

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Conflicts of Interest

The authors declare no conflict of interest.

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