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Research Article

Antibacterial Activity of *Citrus limon* Peel Essential Oil and *Argania spinosa* Oil Against Fish Pathogenic Bacteria

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Abstract

The main objective of the study was the identification of antibacterial activity of lemon (*Citrus limon* L.) peel essential oil and argan (*Argania spinosa* L.) oil against fish pathogenic bacteria. Antibacterial activity was determined against six different fish pathogens (*Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii and Lactococcus garvieae*). Essential oil derived from lemon peel and argan oil were applied against the bacteria using the disc diffusion and micro dilution method under in vitro conditions. The disc diffusion results indicated that essential oil of naturally *C. limon* peel and argan oil significantly inhibited the growth of *Y. ruckeri, A. hydrophila, L. anguillarum* and *C. freundii*. Our results suggested that the use of lemon peel essential oil and argan oil induced a stronger antibacterial effect.

Keywords: *Essential oil, Citrus limon, Argania spinosa, Fish pathogen, Antibacterial*

Balık Patojenlerine Karşı Limon *(Citrus limon)* ve Argan *(Argania spinosa)* Yağının Antibakteriyel Aktivitesi

Özet

Bu çalışmanın amacı limon kabuğu yağı (*Citrus limon* L.) ve argan (*Argania spinosa* L.) yağının bakteriyel balık patojenlerine karşı etkisinin belirlenmesidir. Antibakteriyel aktivite altı farklı balık patojenine (*Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii and Lactococcus garvieae*) karşı belirlenmiştir. Limon kabuğundan elde edilen yağ ve argan yağının antibakteriyel etkileri in vitro koşullar altında disk difüzyon ve mikrodilüsyon metodu kullanılarak belirlenmiştir. Disk difüzyon sonuçlarına göre limon ve argan yağının önemli ölçüde özellikle *Y. ruckeri, A. hydrophila, L. anguillarum* ve *C. freundii* patojenlerinin gelişimini engellediği saptanmıştır. Çalışmada kullanılan limon ve argan yağının güçlü antibakteriyel etkilerinden dolayı kullanılabileceği önerilmiştir.

Anahtar sözcükler: *Temel yağ, Citrus limon, Argania spinosa, Balık patojeni, Antibakteriyel*

INTRODUCTION

Fish pathogens such as *Yersinia ruckeri*, *Aeromonas hydrophila*, *Listonella anguillarum*, *Edwarsiella tarda*, *Citrobacter freundii* and *Lactococcus garvieae* are known to be causes of serious disease in aquaculture with high economic losses. In aquaculture, antibiotics are widely used for treatment and control of these pathogens. Due to conscious or unconscious excessive use of antibiotics, bacteria can improve resistance of these antibiotics [1]. Also antibiotics can accumulate in soil or sediment and become harmless for environment. Medical plants are very popular products of pre-treatment, treatment and immunostimulation in fish health. The ability of natural plants to inhibit activity of bacteria having potential interest as fish pathogens has been documented [2]. Many essential oils and plant extracts have been shown to be effective against fish pathogens [3-8].

Lemon is an important medicinal plant of *Citrus* genus (Rutaceae). Citrus essential oils mainly exist in fruit peels which are usually discarded as waste. Thus, citrus essential oil could be manufactured at a more affordable price than plant essential oils [9]. Citrus fruit peels exhibiting antimicrobial activity are rich with flavonoid glycosides,

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coumarins, β and ɤ- sitosterols, and volatile oils [10]. Of course the chemical ingredients are responsible for their antimicrobial activity. Argan oil is obtained from *Argania spinosa* seeds belonging to the Sapotaceae family [11]. It contains mainly oleic (47.7%) and linoleic acid (29.3%) which are essential unsaturated fatty acids [12]. Several biological activities of *A. spinosa* such as antiproliferative [13], antiatherogenic, antiradical and anti inflammatory activities [14] and immunomodulatory activities [15] have been investigated in animals.

Lemon peel essential oil and argan oil were selected for the study, because both have different biological activities. To the best of our knowledge, though, both of them were investigated for food borne pathogens, none of them have been investigated for fish pathogens. Therefore, the aim of the present study was to investigate the antibacterial activity of essential oil of lemon peels and argan oil against six fish pathogenic bacteria; namely, *Yersinia ruckeri, Aeromonas hydrophila, Listonella anguillarum, Edwarsiella tarda, Citrobacter freundii* and *Lactococcus garvieae*. In addition, the chemical constituents of lemon pell essential oil and argan oil were analyzed by GC and GC-MS.

MATERIAL and METHODS

Chemicals

α-thujene, α-pinene, camphene, β-pinene, α-terpinene, terpinolene, borneol, terpinene-4-ol, α-terpineol, cis-geraniol, geranyl acetate, β-caryophyllene, valencene and caryophyllene oxide were obtained from Sigma-Aldrich GmbH, Sternheim, Germany. *p*-cymene, γ-terpinene, limonene and linalool were obtained from Fluka, GmbH, Sternheim, Germany. Myristic acid (C14:0), pentadecanoic acid (C15:0), palmitoleic acid (C16:1), palmitic acid (C16:0), margaric acid (C17:0), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), nonadecanoic acid (C19:0), eicosanoic acid (C20:0), heneicosanoic acid (C21:0), eruric acid (C22:1), docosanoic acid (C22:0), tricosanoic acid (C23:0), tetracosanoic acid ($C_{24:0}$) were obtained from sigma-aldrich GmbH, Sternheim, Germany. All other chemicals are in analytical grade.

Plant Material and Extraction of the Essential Oil

The lemon peels were gathered from fruit juice industry in Muğla - Ortaca, Turkey, March 1st, 2015. The citrus essential oil was obtained from fresh peels using hydro-distillation method by a Clevenger type apparatus. For this purpose, 100 g of lemon peels were used and 1 mL of essential oil was obtained after 3 h hydrodistillation. The oil was stored at 4°C in a dark bottle until usage.

Derivatization of Argan Oil

Commercially provided argan *(A. spinosa*; Mecitefendi 20 mL, Yeşilvadi) oil was used in this study, as well. In order to analyze its constituents, argan oil was derivatized to its fatty acid methyl esters. Argan oil (10 mg) was dissolved in 2 mL methanol in a 25 mL flask on which 2 mL 0.5 M NaOH was added. After the flask was heated at 50°C using a water bath, 2 mL BF_3:MeOH was added. The mixture was boiled for 2 min. After cooled down the volume was completed to 25 mL with saturated NaCl solution. The esters were extracted with *n*-hexane. The hexane layer was washed with a potassium bicarbonate solution (4 mL, 2%) and dried with anhydrous Na_2SO_4 and filtered. The organic solvent was removed under reduced pressure by a rotary evaporator to give methyl esters [16].

Analysis of Essential Oil and Argan Oil

The essential oil and argan oil constituents were analyzed with a Shimadzu GC-17 AAF, V3, 230 V series gas chromatograph (Japan); GC–MS analyses were carried on a Varian Saturn 2100T (USA) system equipped with an ion trap analyzer (IT). The essential oil was diluted with hexane 1:50 v/v, ratio, and the methyl derivative of argan oil diluted with chloroform 1:20 v/v, ratio before injection to the GC, and GC-MS. The standards were prepared in 40 ppm. For these purpose 1 mg of standard sample was diluted in 25 mL of chloroform.

Gas Chromatography (GC) Analysis Conditions

A DB-1 fused silica capillary non-polar column containing dimethylpolysiloxane (30 m × 0.25 id., film thickness 0.25 µm; J&W Scientific) and a flame ionisation detector (FID) were used for GC analyses. The injector temperature and detector temperature were adjusted to 250 and 270°C, respectively. Carrier gas was He at a flow rate of 1.4 mL/min. Sample volume was 1.0 µL with a split ratio of 50:1. For the essential oil analysis, initial oven temperature was held at 60°C for 5 min, then increased up to 280°C with 4°C/min increments and held at this temperature for 15 min. For the methylated argan oil, however, column temperature program started at 100°C for 5 min, then increased to 238°C with the rate of 3°C/min and held at this temperature for 15 min. The percentage compositions of the essential oil were determined with the Class GC10 GC computer programme [16]. The Retention indices were calculated according to the following equation:

n, n+i = Carbon number of reference hydrocarbon, i = 1 or 2

t_{Rx} = Retention time of Analyte

t_{Rn} = Retention time of Reference hydrocarbon before analyte

t_{Rn+i} = Retention time of Reference hydrocarbon after analyte

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

Same analytical column and oven temperature program were used for the GC-MS analysis. Sample size was 0.2 µL

with a split ratio of 50:1.70 eV was used for electron ionization. Injector, transferline and manifold temperatures were adjusted to 250, 290 and 240°C respectively. For the determination of the constituents, NIST 2005 library, retention time index comparison as well as co-injection of standards were used [16].

Bacterial Fish Pathogens

Six bacterial fish strain were tested for the antibacterial activity of essential oils from lemon and argan oil. Different species of bacteria were isolated from sick fish. The antibacterial activity of essential oil of lemon and argan were tested against *Yersinia ruckeri, Aeromonas hydrophila, Lactococcus garvieae, Listonella anguillarum, Edwarsiella tarda,* and *Citrobacter freundii (Table 1)*. These isolates were stored in Triptic soya agar at 4°C for further use. Also bacterial strains were examined by phenotypic tests. Identification was carried out by conventional biochemical tests and API 20E as described by Austin and Austin [17].

Antibacterial Assay

The antibacterial effects were tested by the disc diffusion method [18]. The final concentrations of lemon and argan oil (dissolved in methanol) were at 0.5, 1%, 2.5%, 5%, 7.5%, or 10% [6]. The microorganisms used were: *Y. ruckeri, A. hydrophila, L. anguillarum, E. tarda, C. freundii*, which are Gram-negative bacteria, and *L. garvieae*, which is a Gram-positive bacteria. The previously prepared bacteria strains inoculums were adjusted to 0.5 Mc Farland standards, which are equal to $1x10^8$ CFU/mL and then the MHA plates were seeded with 100 µL of the standardized inoculum of each tested organism. The inoculum was spread evenly over plate with loop or sterile glass spreader. Afterward 25 µL of each lemon essential oil and argan oil were inoculated onto wells, plate culture of each microbial isolates were made in the spread. After incubation, each essential oil was noted for zone of inhibition for all isolates. The diameters of the zone of tested bacteria were measured by measuring scale in millimeter (mm). Thirteen different antibiotics (OTC:Oxytetracycline 20 µg, N: Neomycin 30 µg, AX: Amoxicilin 25 µg, NV: Novobiocin 30 µg, CIP: Ciprofloxacin 5 µg, SXT: Sulphamethoxazole 25 µg, CN: Gentamicin 10 µg, S: Streptomycin 10 µg, UB: Flumequine 30 µg, C: Chloramphenicol 30 µg, P: Penicillin 10 µg, TE: Tetracycline 30 µg, ENR: Enrofloxacin) were used as a positive, the methanol as a negative control. The tests were carried out in triplicate.

Table 1. *The bacterial strains and origin*
Tablo 1. *Bakteri suşları ve kökenleri*

Bacteria	**Origin**
Yersinia ruckeri	Rainbow trout *(Oncorhynchus mykiss)*, Fethiye
Aeromonas hydrophila	Common carp *(Cyprinus carpio)*, Çanakkale
Lactococcus garvieae	Rainbow trout *(Oncorhynchus mykiss)*, Fethiye
Listonella anguillarum	Mullet *(Mugil cephalus)*, Muğla
Edwarsiella tarda	Nil tilapia (*Oreochromis niloticus*), Çanakkale
Citrobacter freundii	Rainbow trout *(Oncorhynchus mykiss)*, Çanakkale

Minimum Inhibitory Concentration (MIC) Assay

The mininum inhibition concentration (MIC) of lemon peel essential oil and argan oil was determined according to the method of Eloff [19] with slight modification. The bacterial suspensions were prepared as described in antibacterial assay section. A twofold serial dilution of each oil (100 µL) in methanol was prepared in 96-well micro plates. 100 µL bacterial suspensions were added to each well. The methanol was included as negative control in each assay. The plates were incubated overnight at 22-25°C. After, incubation was measured OD=630 nm. MIC values were recorded as the lowest concentration of the oils that completely inhibited bacterial growth.

Statistical Analysis

The data were expressed as arithmetic means with standard error (SEM). Statistical analysis of data involved one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison tests. Different letters in tables represent the significant difference at $P<0.05$.

RESULTS

Chemical Composition of Essential Oil and Argan Oil

The yellowish essential oil of *C. limon* with a yield of 2% was obtained by hydro-distillation. The essential oil constituents analyzed by GC-MS were given in *Table 2* along with LOD, LOQ, coefficient of determination and *m/z* values of the compounds. The major constituents of essential oil were elucidated as limonene (54.4%), γ-terpinene (%12.0), β-pinene (8.81%), α-terpineol (3.45%), myrecene (2.96%) and terpinolene (2.08%). *Table 3* shows the percentage concentration of the fatty acid composition of *A. spinosa* oil, analyzed by GC and GC-MS along with LOD, LOQ, coefficient of determination and m/z values of the compounds. Oleic acid (40.9%), linoleic acid (28.4%), palmitic acid (15.0%) and stearic acid (10.2%) were detected as major fatty acid constitutes.

Biochemical Test Results

Bacterial groups were determined on the criteria of shape, motility, catalase and oxidase reactions, oxidation-fermentation test. API® 20E system (BioMerieux, France) was furthermore used in order to identify oxidase positive and negative bacteria, respectively, at species level.

Antibacterial Activity Disc Diffusion Test Results

Results of antibacterial activity of lemon peel essential oil and argan oil against Gram positive and negative isolates by the disc diffusion method were shown on

Table 2. *Chemical composition of C. limon essential oil*
Tablo 2. *C. limon uçucu yağının kimyasal içeriği*

No	Analyte	Molecular ion(m/z)[a]	Fragments MS[b]	Ionization Mode	RI[c]	$R^{2,d}$	LOD/LOQ (µg/L)[e]	Concentration (%)	Identification method
1	α-thujene	136	136 (8.2), 94 (8.6), 93 (99.9), 92 (30.8), 91 (34.9), 79 (9.9), 77 (34.2), 41 (11.2), 39 (9.9), 27 (8.4)	Poz	901	0.9925	13.3/ 44.6	0.82	Co-GC[f], MS[g], RI[h]
2	α-pinene	136	121 (13.6), 105 (10.2), 93 (99.9), 92 (34.9), 91 (32.3), 79 (22.7), 77 (28.5), 41 (20.0), 39 (18.9)	Poz	914	0.9977	18.2 / 57.1	0.20	Co-GC[f], MS[g], RI[h]
3	Camphene	136	121 (58.2), 93 (99.9), 91 (37.3), 79 (39.9), 77 (28.0), 67 (29.4), 41 (31.7), 39 (33.5), 27 (19.7)	Poz	925	0.9939	16.3 / 52.7	0.26	Co-GC[f], MS[g], RI[h]
4	β-pinene	136	93 (99.9), 92 (15.2), 91 (30.8), 79 (27.4), 77 (27.5), 69 (35.0), 53 (13.4), 41 (60.9), 39 (31.8), 27 (21.4)	Poz	960	0.9912	18.3 / 54.8	8.81	Co-GC[f], MS[g], RI[h]
5	Myrecene	136	93 (85.5), 91 (95.0), 79 (13.8), 77 (11.0), 69 (79.6), 67 (11.0), 53 (14.1), 41 (99.9), 39 (29.9), 27 (28.0)	Poz	977			2.96	MS[g], RI[h]
6	α-phellandrene	136	136 (15.6), 94 (85.0), 93 (99.9), 92 (25.3), 91 (33.2), 79 (64.0), 77 (30.6), 41 (14.1), 39 (11.2), 27 (10.1)	Poz	989			0.19	MS[g], RI[h]
7	α-terpinene	136	136 (35.8), 121 (30.3), 105 (9.90), 93 (99.9), 92 (21.4), 91 (37.2), 79 (18.5), 77 (28.1), 43 (17.2), 41 (9.90)	Poz	1003	0.9951	17.2 / 57.3	0.73	Co-GC[f], MS[g], RI[h]
8	*p*-cymene	134	134 (25.4), 120 (10.0), 119 (99.9), 117 (9.10), 115 (4.40), 91 (15.8), 77 (5.20), 65 (4.70), 41 (5.90), 39 (4.80)	Poz	1007	0.9947	14.7 / 51.6	0.74	Co-GC[f], MS[g], RI[h]
9	Limonene	136	136 (16.4), 107 (18.0), 94 (22.4), 93 (50.0), 92 (18.1), 79 (16.4), 68 (99.9), 67 (44.4), 53 (17.5), 39 (22.0)	Poz	1018	0.9987	12.1 / 31.4	54.4	Co-GC[f], MS[g], RI[h]
10	β-cis-ocimene	136	93 (99.9), 92 (22.5), 91 (27.2), 80 (19.1), 79 (31.0), 77 (25.8), 53 (16.5), 41 (35.6), 39 (27.5), 27 (20.5)	Poz	1026			0.20	MS[g], RI[h]
11	β-trans-ocimene	136	93 (99.9), 92 (24.5), 91 (28.2), 80 (15.6), 79 (29.7), 77 (26.9), 53 (16.5), 41 (35.6), 39 (27.5), 27 (20.5)	Poz	1037			0.37	MS[g], RI[h]
12	γ-terpinene	136	136 (31.1), 121 (28.5), 93 (99.9), 92 (24.7), 91 (56.5), 79 (24.5), 77 (41.5), 43 (13.9), 41 (15.0), 39 (15.0)	Poz	1047	0.9963	16.3 / 55.2	12.0	Co-GC[f], MS[g], RI[h]
13	Terpinolene	136	136 (61.4), 121 (78.3), 107 (16.5), 105 (26.0), 93 (99.9), 91 (61.7), 79 (45.7), 77 (42.8), 41 (24.0), 39 (25.3)	Poz	1074	0.9969	23.9 / 73.2	2.08	Co-GC[f], MS[g], RI[h]
14	*cis-p*-mentha-2,8 dienol	152	137 (41.5), 134 (91.9), 119 (82.0), 93 (42.1), 91 (99.9), 79 (76.1), 77 (45.3), 43 (84.4), 41 (63.2), 39 (59.2)	Poz	1080			0.29	MS[g], RI[h]
15	Linalool	154	121 (20.6), 93 (59.1), 80 (24.1), 71 (99.9), 69 (38.1), 55 (46.9), 43 (64.0), 41 (64.2), 39 (21.7), 27 (19.8)	Poz	1082	0.9923	8.0 / 22.0	0.76	Co-GC[f], MS[g], RI[h]
16	Fenchol	154	84 (20.7), 82 (18.8), 81 (99.9), 80 (52.8), 72 (21.0), 71 (23.1), 69 (24.2), 67 (16.9), 43 (26.3), 41 (24.6)	Poz	1110		4.1 / 13.3	0.19	MS[g], RI[h]
17	Borneol	154	139 (8.5), 110 (19.1), 96 (8.6), 95 (99.9), 93 (8.9), 71 (7.2), 69 (7.4), 67 (7.9), 55 (10.3), 41 (15.9)	Poz	1132	0.9941	9.7 / 33.1	0.15	Co-GC[f], MS[g], RI[h]
18	Terpinene-4-ol	154	154 (14.8), 111 (49.8), 93 (43.4), 86 (27.2), 69 (21.2), 68 (15.2), 55 (17.2), 43 (29.0), 41 (23.1)	Poz	1142	0.9977	10.7 / 35.6	2.11	Co-GC[f], MS[g], RI[h]
19	α-terpineol	154	136 (47.3), 121 (58.3), 95 (22.3), 93 (67.7), 81 (31.7), 68 (27.3), 67 (21.7), 59 (99.9), 43 (32.0), 41 (19.0)	Poz	1150	0.9938	9.1 / 30.4	3.45	Co-GC[f], MS[g], RI[h]
20	*cis*-carveol	152	134 (34.8), 119 (78.3), 117 (30.0), 109 (24.1), 93 (20.8), 92 (29.4), 91 (99.9), 77 (35.4), 65 (21.1), 39 (26.3)	Poz	1189			tr[i]	MS[g], RI[h]
21	*cis*-Geraniol	154	93 (12.8), 84 (11.8), 69 (99.9), 68 (24.5),67 (15.8), 53 (12.8), 41 (93.4), 39 (20.1), 29 (12.9), 27 (13.8)	Poz	1206	0.9951	2.6 / 8.8	0.32	Co-GC[f], MS[g], RI[h]
22	β-citral	152	109 (9.4), 94 (13.0), 84 (19.7), 69 (85.3), 67 (8.8), 53 (10.7), 41 (99.9), 39 (24.8), 29 (12.0), 27 (25.4)	Poz	1208			0.37	MS[g], RI[h]
23	*trans*-Geraniol	154	123 (8.5), 93 (9.4), 84 (6.8), 70 (7.5), 69 (99.9), 68 (19.8), 67 (8.0), 55 (6.5), 41 (65.3), 29 (10.0)	Poz	1224			0.49	MS[g], RI[h]
24	Geranial	152	109 (9.4), 94 (16.2), 91 (8.9), 84 (26.7), 83 (12.5), 69 (99.9), 67 (10.8), 53 (13.1), 41 (74.6), 39 (25.5)	Poz	1242			0.15	MS[g], RI[h]
25	Citronellol acetate	198	123 (62.8), 95 (76.8), 82 (67.2), 81 (70.4), 69 (99.9), 68 (43.8), 67 (49.9), 53 (49.3), 43 (81.2), 41 (69.5)	Poz	1332			0.46	MS[g], RI[h]
26	Neryl acetate	196	136 (13.1), 121 (15.5), 93 (39.1), 80 (17.4), 69 (99.9), 68 (40.2), 67 (12.9), 43 (43.3), 41 (60.1), 39 (10.5)	Poz	1339			1.81	MS[g], RI[h]
27	Geranyl acetate	196	136 (31.7), 121 (23.0), 93 (41.1), 80 (23.0), 69 (99.9), 68 (69.3), 67 (28.1), 43 (84.0), 41 (76.6), 39 (21.3)	Poz	1358	0.9965	5.1/17.9	1.60	Co-GC[f], MS[g], RI[h]
28	β-Caryophyllene	204	133 (92.1), 120 (44.7), 107 (48.3), 105 (62.3), 93 (99.9), 91 (85.8), 79 (76.3), 77 (43.9), 69 (75.4), 41 (76.9),	Poz	1415		6.3/19.7	0.66	Co-GC[f], MS[g], RI[h]
29	α-*trans*-bergamotene	204	119 (99.9), 107 (22.9), 105 (17.1), 93 (68.6), 91 (25.7), 77 (14.3), 69 (34.3), 55 (17.1), 41 (57.1), 39 (17.2)	Poz	1434			0.79	MS[g], RI[h]
30	(*Z*)-β-Farnesene	204	133 (22.8), 120 (17.7), 93 (46.4), 91 (17.5), 81 (21.1), 79 (20.1), 69 (80.1), 67 (23.6), 53 (18.7), 41 (99.9)	Poz	1476			0.11	MS[g], RI[h]
31	Valencene	204	204 (57.6), 161 (99.9), 135 (36.6), 133 (40.9), 119 (48.0), 107 (50.6), 105 (56.9), 93 (53.5), 91 (46.1), 79 (46.1)	Poz	1486	0.994	5.2/17.5	0.46	Co-GC[f], MS[g], RI[h]
32	α-selinene	204	107 (54.0), 105 (58.1), 93 (59.6), 91 (57.0), 81 (42.3), 79 (68.5), 67 (53.5), 53 (42.6), 41 (99.9), 39 (57.9)	Poz	1489			0.16	MS[g], RI[h]
33	β-bisabolene	204	204 (24.5), 161 (17.3), 109 (23.8), 107 (17.6), 94 (26.5), 93 (69.0), 79 (23.8), 69 (99.9), 67 (25.5), 41 (68.6)	Poz	1498			1.74	MS[g], RI[h]

Table 2. *Chemical composition of C. limon essential oil* ***(Continue...)***
Tablo 2. *C. limon uçucu yağının kimyasal içeriği* ***(Devam...)***

No	Analyte	Molecular ion(m/z)[a]	Fragments MS[b]	Ionization Mode	RI[c]	$R^{2,d}$	LOD/LOQ (µg/L)[e]	Concentration (%)	Identification method
34	Caryophyllene oxide	220	95 (42.0), 93 (66.1), 91 (57.1), 81 (37.3), 79 (88.5), 69 (40.7), 67 (37.7), 55 (39.3), 43 (99.9), 41 (92.7)	Poz	1561	0.9981		tr[i]	Co-GC[f], MS[g], RI[h]
35	α-bisabolol	222	119 (39.7), 109 (50.0), 95 (27.6), 93 (38.5), 69 (80.3), 67 (28.1), 55 (35.3), 43 (99.9), 41 (89.3), 39 (26.1)	Poz	1653			tr[i]	MS[g], RI[h]
				Monoterpene hydrocarbons: 83.7 Monoterpenoids: 12.2 Sesquiterpene hydrocarbons: 3.9 Total identified: 99.8					

[a]***Parent ion (m/z):*** *Molecular ions of the standard compounds (mass to charge ratio),* [b]***MS:*** *Fragments for the related molecular ions,* [c]***RI:*** *Retention index on DB-1 fused silica column,* [d]***R^2:*** *coefficient of determination,* [e]***LOD/LOQ (µg/L):*** *Limit of detection/Limit of quantification,* [f]***Co-GC:*** *co-injection of standards,* [g]***MS:*** *Mass spectra comparison with NIST 2005 library,* [h]***RI:*** *Retention Indices on literature,* [i]***tr:*** *Trace*

Table 3. *Fatty acid composition of A. spinosa oil*
Tablo 3. *A. spinosa yağının yağ asidi içeriği*

Peak no	Compounds	Molecular ion peak (m/z)[a]	Fragments MS[b]	$R^{2,c}$	LOD/LOQ (µg/L)[d]	Concentration (%)	Identification Methods
1	Myristic acid ($C_{14:0}$)	228	228 (44.1), 129 (43.7),73 (99.9), 71 (37.6), 60 (95.2), 57 (72.7), 55 (56.9), 43 (86.3), 41 (72.9), 29 (41.6)	0.9922	13.3/35.7	0.49	Co-GC[e], MS[f]
2	Pentadecanoic acid ($C_{15:0}$)	242	242 (30.2), 73 (92.2),71 (32.7), 69 (33.9), 60 (91.9), 57 (64.4), 55 (68.1), 43 (99.9), 41 (87.1), 29 (54.1)	0.9944	13.2/38.1	0.19	Co-GC[e], MS[f]
3	Palmitoleic acid ($C_{16:1}$)	254	97 (29.8), 83 (43.8), 69 (69.1), 67 (32.4), 56 (32.4), 55 (99.9), 43 (54.2), 41 (84.2), 29 (34.2), 28 (33.3)	0.9970	3.4/9.7	0.33	Co-GC[e], MS[f]
4	Palmitic acid ($C_{16:0}$)	256	73 (90.5), 71 (28.5), 69 (31.0), 60 (83.0), 61 (21.8), 57 (63.4), 55 (61.6), 43 (99.9), 41 (74.9), 29 (41.4)	0.9939	33.4/95.9	15.0	Co-GC[e], MS[f]
5	Margaric acid ($C_{17:0}$)	270	270 (67.5), 129 (50.7), 73 (99.9), 71 (46.3), 69 (31.6), 60 (85.8), 57 (78.4), 55 (43.0), 43 (76.3), 41 (44.9)	0.9946	31.8/96.1	0.30	Co-GC[e], MS[f]
6	Linoleic acid ($C_{18:2}$)	280	96 (54.7), 95 (62.7), 82 (73.0), 81 (87.8), 69 (35.9), 68 (60.2), 67 (99.9), 55 (59.5), 54 (44.9), 41 (54.1),	0.9928	33.2/99.0	28.4	Co-GC[e], MS[f]
7	Oleic acid ($C_{18:1}$)	282	97 (44.3), 83 (59.3), 70 (32.0), 69 (75.7), 67 (35.3), 57 (43.9),56 (34.5), 55 (99.9), 43 (54.9), 41 (75.2),	0.9919	30.5/89.2	40.9	Co-GC[e], MS[f]
8	Stearic acid ($C_{18:0}$)	284	129 (31.8), 73 (84.0), 71 (37.1), 69 (35.2), 60 (80.6), 57 (75.8), 55 (63.7), 43 (99.9), 41 (69.5), 29 (38.1)	0.9948	10.3/27.5	10.2	Co-GC[e], MS[f]
9	10-Nonadecenoic acid ($C_{19:1}$)	296	97 (40.5), 87 (38.6), 84 (37.6), 83 (50.7), 74 (58.4), 69 (65.2), 57 (33.8), 55 (99.9), 43 (59.4), 41 (75.8)	0.9952	14.8/43.1	0.10	MS[f]
10	Nonadecanoic acid ($C_{19:0}$)	298	298 (40.6), 73 (74.9), 71 (33.7), 69 (33.6), 60 (68.6), 57 (71.9), 55 (61.3), 43 (99.9), 41 (69.8), 29 (38.8)	0.9973	13.8/38.6	0.15	Co-GC[e], MS[f]
11	10,13-Eicosadienoic acid ($C_{20:2}$)	308	109 (44.3), 96 (71.3), 95 (81.7), 82 (74.7), 81 (98.2), 69 (52.1), 68 (53.0), 67 (99.9), 55 (74.7), 41 (49.5)	0.9971	4.1/13.6	0.10	MS[f]
12	11-Eicosenoic acid ($C_{20:1}$)	310	292 (52.0), 97 (46.0), 84 (39.0), 83 (56.0), 74 (57.0), 69 (73.0), 57 (39.0), 55 (99.9), 43 (63.0), 41 (72.0)	0.9937	4.5/13.9	1.52	MS[f]
13	Eicosanoic acid ($C_{20:0}$)	312	85 (26.1), 73 (70.9), 71 (42.9), 69 (35.2), 60 (60.5), 57 (78.7), 55 (65.9), 43 (99.9), 41 (68.8), 29 (30.8)	0.9921	5.4/15.9	1.42	Co-GC[e], MS[f]
14	Heneicosanoic acid ($C_{21:0}$)	326	326 (99.9), 129 (27.3), 73 (53.6),71 (32.1), 69 (26.0), 60 (45.3), 57 (58.7), 55 (39.7), 43 (65.5), 41 (36.1)	0.9929	*4.4/12.3*	*tr*[g]	Co-GC[e], MS[f]
15	Eruric acid ($C_{22:1}$)	338	97 (36.7), 83 (48.4), 70 (25.8), 69 (63.5), 57 (41.7), 56 (29.1), 55 (99.9),43 (60.1), 41 (78.1)	0.9936	*4.2/13.5*	*tr*[g]	Co-GC[e], MS[f]
16	Docosanoic acid ($C_{22:0}$)	340	340 (99.9), 129 (38.3), 97 (28.9), 83 (26.3), 73 (41.3), 71 (35.4), 60 (28.1), 57 (51.3), 55 (29.2), 43 (39.3)	0.9940	4.2/12.9	0.48	Co-GC[e], MS[f]
17	Tricosanoic acid ($C_{23:0}$)	354	354 (75.2), 129 (35.4), 73 (62.2), 71 (36.9), 69 (37.8), 60 (63.2), 57 (67.5), 55 (70.8), 43 (99.9), 41 (68.8)	0.9939	*3.5/8.8*	*tr*[g]	Co-GC[e], MS[f]
18	15-Tetracosenoic acid ($C_{24:1}$)	366	348 (28.0), 97 (26.0), 83 (41.0), 74 (54.0), 69 (57.0), 67 (25.0), 57 (35.0), 55 (99.9), 43 (60.0), 41 (65.0)	0.9933	*4.4/11.5*	*tr*[g]	MS[f]
19	Tetracosanoic acid ($C_{24:0}$)	368	368 (39.8), 129 (50.7), 73 (79.7), 71 (55.6), 69 (41.6), 60 (61.3), 57 (99.8), 55 (58.2), 43 (99.9), 41 (50.6)	0.9927	4.3/11.7	0.17	Co-GC[e], MS[f]
	Saturated Unsaturated Total identified					28.8 71.4 99.8	

[a]***Parent ion (m/z):*** *Molecular ions of the standard compounds (mass to charge ratio),* [b]***MS:*** *Fragments for the related molecular ions,* [c]***R^2:*** *coefficient of determination,* [d]***LOD/LOQ (µg/L):*** *Limit of detection/Limit of quantification,* [e]***Co-GC:*** *co-injection of standards,* [f]***MS:*** *Mass spectra comparison with NIST 2005 library,* [g]***tr:*** *Trace*

Tables 4 and *5* respectively. The lemon peel essential oil produces a maximum zone of inhibition 19.00±0.58 mm against *Y. ruckeri* followed by *L. anguillarum* (18.00±1.15), *A. hydrophila* (17.00±1.20), *C. freundii* (17.00±0.57), *E. tarda* (11.00±0.57). The minimum were 10.33±0.28 mm zone inhibition produced against *L. garvieae*. The strongest

Table 4. *Antibacterial activity of C. limon peel essential oil against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 4. *C. limon kabuğu yağının farklı bakteriyel balık patojenlerine karşı antibakteriyel aktivite (inhibisyon zon çapı, mm)*

Bacteria	Lemon (C. limon) peel essential oil different concentration (%)				
	1%	2.5%	5%	7.5%	10%
Y. ruckeri	8.33±0.33	10.33±0.88	12.00±0.57	13.00±0.57	19.00±0.58
A. hydrophila	9.00±0.57	10.66±0.66	12.33±0.33	14.00±0.57	17.00±1.20
L. anguillarum	8.00±0.57	9.00±0.56	11.66±1.45	13.66±1.33	18.00±1.15
C. freundii	8.33±0.33	10.00±0.57	12.66±0.66	14.00±0.46	17.00±0.57
E. tarda	7.33±0.33	8.00±0.57	8.33±0.76	9.33±0.33	11.00±0.57
L.garvieae	7.33±0.33	8.34±0.42	10.00±0.57	11.00±0.33	10.33±0.28

Inhibition zones>15 mm were declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities (M±SE; indicates Mean ± Standard error)

Table 5. *Antibacterial activity of A. spinosa oil against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 5. *A. spinosa kabuğu yağının farklı bakteriyel balık patojenlerine karşı antibakteriyel aktivite (inhibisyon zon çapı, mm)*

Bacteria	Argan (Argania spinosa) oil different concentration (%)				
	1%	2.5%	5%	7.5%	10%
Y. ruckeri	9.00±0.57	10.33±0.88	12.00±0.57	13.00±0.66	18.33±0.88
A. hydrophila	9.00±0.57	10.33±0.88	12.33±0.33	14.00±0.57	17.00±0.57
L. anguillarum	7.00±0.57	9.33±0.33	12.00±1.52	12.33±1.45	17.00±0.57
C. freundii	8.33±0.33	10.00±0.57	12.66±0.66	14.00±0.57	17.00±0.56
E. tarda	7.33±0.33	8.00±0.57	8.33±0.33	10.00±0.57	9.66±0.88
L. garvieae	7.33±0.33	8.33±0.33	10.00±0.57	11.00±0.57	11.33±0.88

Inhibition zones>15 mm were declared as strong (bold), from 8 to 15 mm as moderate and from 1 to 8 mm as weak activities (M±SE; indicates Mean ± Standard error)

Table 6. *Antibiotic suspectibility test results against different bacterial fish pathogens (The diameter of the zone of inhibition, mm)*
Tablo 6. *Farklı bakteriyel balık patojenlerine karşı antibiyotik duyarlılık testi*

A	Bacteria					
	Y. ruckeri	***A. hydrophila***	***L. anguillarum***	***C. freundii***	***E. tarda***	***L. garvieae***
OTC	25.00±0.57	21.66±1.20	26.00±0.57	22.33±1.45	20.66±0.88	10.33±0.88
N	19.66±0.88	16.33±0.88	18.66±0.88	12.00±1.15	10.33±0.88	12.00±0.57
AX	20.33±0.88	10.00±1.15	18.00±0.57	10.00±0.57	24.00±0.57	23.66±0.88
NV	12.66±1.45	19.00±0.57	24.33±1.20	0	26.33±0.88	25.00±0.57
CIP	28.66±1.20	30.00±0.57	20.00±0.57	30.33±0.88	15.66±1.20	16.33±0.88
SXT	29.66±0.88	35.00±0.57	25.00±0.57	30.00±0.57	10.00±0.57	10.33±0.88
CN	15.33±0.88	13.66±0.88	16.33±0.88	15.00±0.57	9.66±0.88	13.00±0.57
S	16.33±0.88	13.00±0.57	20.00±0.57	15.33±0.88	8.00±0.57	9.33±0.88
UB	30.66±1.76	27.00±0.57	38.00±1.15	31.00±0.57	12.66±1.20	8.33±0.88
C	25.33±0.88	35.00±0.57	30.00±1.15	20.00±0.57	19.66±0.88	20.33±1.45
P	0	0	0	0	15.66±0.88	15.66±0.88
TE	21.00±1.54	11.00±0.57	30.66±0.88	29.66±0.88	21.33±0.88	10.00±0.57
ENR	30.00±0.57	25.00±0.57	27.00±3.60	17.92±1.68	19.66±0.88	22.00±1.15

*(A: Antibiotics, **OTC:** Oxytetracycline 20 µg, **N:** Neomycin 30 µg, **AX:** Amoxicilin 25 µg, **NV:** Novobiocin 30 µg, **CIP:** Ciprofloxacin 5 µg, **SXT:** Sulphamethoxazole 25 µg, **CN:** Gentamicin 10 µg, **S:** Streptomycin 10 µg, **UB:** Flumequine 30 µg, **C:** Chloramphenicol 30 µg, **P:** Penicillin 10 µg, **TE:** Tetracycline 30 µg, **ENR:** Enrofloxacin 5 µg)*

antibacterial activities were obtained by lemon essential oil with inhibition zones of 19 mm against *Y. ruckeri (Table 4)*. The argan oil produces a maximum zone of inhibition 18.33±0.88mm against *Y. ruckeri* followed by *L. anguillarum* (17.00±0.57), *A. hydrophila* (17.00±0.57), *C. freundii* (17.00±0.56), *L. garvieae* (11.33±0.88) whereas the minimum were 9.66±0.88 mm zone inhibition produced against *E. tarda*. The strongest antibacterial activities were obtained by lemon essential oil with inhibition zones of 18.33 mm against *Y. ruckeri (Table 5)*. Summarizing the results, it can be concluded that the most antibacterial effective lemon and argan oil were against *Y. ruckeri*, *L. anguillarum*, *A. hydrophila* and *C. freundii*.

Inhibition zone profiles against different antibiotics of bacterial isolated from fish was shown on *Table 6*. Antibiotic susceptibility test showed that *Y. ruckeri, L. anguillarum* and *C. freundii* isolates were susceptible to enrofloxacin but the isolates were resistant to penicillin. *A. hydrophila* isolates were found to be susceptible to sulphamethoxazole and chloramphenicol and to be resistant to penicillin. Also *E. tarda* and *L. garvieae* isolates were susceptible to Novobiocin.

Minimum Inhibitory Result (MIC)

The result of minimum inhibitory concentration (MIC) of oil of lemon and argan is shown in *Tables 7* and *8*. The lemon peel essential oil against *Y. ruckeri* and *L. anguillarum* showed a higher MIC values (62.5 µL/mL), followed by *A. hydrophila* and *C. freundii* (125 µL/mL), *E. tarda* and *L. garvieae* (250 µL/mL) *(Table 7)*. Argan oil against *Y. ruckeri* showed a higher MIC values of 62.5 µL/mL followed by *L. anguillarum, A. hydrophila* and *C. freundii* (125 µL/mL), *E. tarda* and *L. garvieae* (250 µL/mL) *(Table 8)*.

DISCUSSION

Essential oils can inhibit pathogenic bacteria because of its chemical compounds which are thymol, carvacrol, phenolic acids, ascorbic acid, polyphenols and dietary fiber [20]. Numerous studies have confirmed that the citrus show antimicrobial, antioxidant and anticancer activities [21,22]. In this study major essential oil components identified from *C. limon* essential oil were limonene, γ-terpinene, β-pinene, α-terpineol, myrecene and terpinolene. Various trials have documented the inhibitory effects of citrus against different pathogens [23,24]. Argan oil contains vanillic acid, syringic acid, ferulic acid, tyrosol, catechol, resorcinol, epicatechin, catechin [25]. In the current study, important fatty acid ingredients identified from *A. spinosa* were oleic acid, linoleic acid, palmitic acid and stearic acid. Fatty acids, particularly oleic, linoleic and linolenic acids which are the long chain fatty acids attribute to inhibit growth of bacteria as antimicrobial agents [26,27]. The effect of dietary argan oil on the immune system was also evaluated on rats. Those studies showed that argan oil effects on immune cells, which is similar to that of olive oil [28].

This study demonstrates the antibacterial activity of lemon *(C. limon)* peel essential oil and argan oil against fish pathogenic bacteria. The isolated strains from diseased fish were used in this study. Because, reference bacteria which are clinical strains were possible to lost their pathogenicity caused by subculturing. For this purpose, essential oils derived from lemon peel and argan oil were applied against isolated bacteria using the disc diffusion and micro dilution method *in vitro* conditions. Lemon essential oil and argan oil inhibited the growth of all

Table 7. *Minimum inhibitory concentrations of lemon peel essential oils against different fish pathogens*
Tablo 7. *Limon yağının farklı balık patojenlerine karşı MİK değerleri (µL/mL)*

Amount (µL/mL)	***Bacteria***					
	Y. ruckeri	***A. hydrophila***	***L. anguillarum***	***E. tarda***	***C. freundii***	***L. garvieae***
500	-	-	-	-	-	-
250	-	-	-	+	-	+
125	-	+	-	+	+	+
62.5	+	+	+	+	+	+
31.25	+	+	+	+	+	+
15.62	+	+	+	+	+	+
7.8	+	+	+	+	+	+
3.9	+	+	+	+	+	+
1.95	+	+	+	+	+	+
0.975	+	+	+	+	+	+
0.48	+	+	+	+	+	+
0.24	+	+	+	+	+	+
0.12	+	+	+	+	+	+
0.06	+	+	+	+	+	+
Control	+	+	+	+	+	+

(+): *Reproduction,* ***(-):*** *No reproduction*

Table 8. *Minimum inhibitory concentrations of A. spinosa essential oil against different fish pathogens*
Tablo 8. *Argan yağının farklı balık patojenlerine karşı MİK değerleri (µL/mL)*

Amount (µL/mL)	Bacteria					
	Y. ruckeri	***A. hydrophila***	***L. anguillarum***	***E. tarda***	***C. freundii***	***L. garvieae***
500	-	-	-	-	-	-
250	-	-	-	-	-	-
125	-	-	-	+	-	+
62.5	-	+	+	+	+	+
31.25	+	+	+	+	+	+
15.62	+	+	+	+	+	+
7.8	+	+	+	+	+	+
3.9	+	+	+	+	+	+
1.95	+	+	+	+	+	+
0.975	+	+	+	+	+	+
0.48	+	+	+	+	+	+
0.24	+	+	+	+	+	+
0.12	+	+	+	+	+	+
0.06	+	+	+	+	+	+
Control	+	+	+	+	+	+

(+): Reproduction, (-): No reproduction

bacteria. Among the strains tested, both oils were possessed remarkable activity against *A. hydrophila*, *L. anguillarum* and *C. freundii*. However, they exhibited lesser activities against *Y. ruckeri*. Both also demonstrated more or less trivial activity against *E. tarda* and *L. garvieae*. Both essential and fatty acid extracts indicated inhibitory effects on same pathogents, mentioned in this study, which is parallel to other several reports. Hindi and Chabuck [8] reported antimicrobial effect of different aqueous lemon extracts against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus agalactiae* (6 Gram-positive) and *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*,

Salmonella typhi, *Proteus spp*., *Moraxella catarrhalis*, *Acinetobacter spp*. (8 Gram-negative) and *Candida albicans* isolates. Hayes and Markovic [29] reported the antimicrobial activity of lemon against *Escherichia coli, S. aureus, P. aeruginosa* and *C. albicans*. The argan oil inhibited the growth of Gram positive and Gram negative bacteria along with yeasts and molds [30]. Antibacterial effect of *C. limon* against *S. aureus, P. aeruginosa* and *P. vulgaris* were revealed [5]. Furthermore, some essential oil, except citrus family members, was investigated antibacterial activity against fish pathogens. Ekici et al.[6] investigated the antibacterial properties essential oils of thyme *(Origanum vulgaris)*, melissa *(Melissa oleum)*, lavandula oil *(Lavandulae romanae oleum)*, rosemary oil *(Rosmarinus officinalis)* and ginger *(Zingiber officinale)*. Essential oils possessed significant antibacterial activity against *Yersinia ruckeri, Aeromonas hydrophila, Vibrio anguillarum, Flavobacterium psychrophilum* and *Lactococcus garvieae*. Starliper et al.[31] were reported that cinnamon *(Cinnamomum cassia)*, oregano *(Origanum vulgare)*, lemongrass *(Cymbopogon citratus)* and thyme *(Thymus vulgaris)* oils were reduced growth of *Aeromonas salmonicida subsp. salmonicida*.

In the present study, limonene was the main ingredient of essential oil. In a recent paper, the essential oil containing limonene as the major compound obtained from sweet orange peel were mixed to fish fed [32]. This prepared fish fed indicated resistance against *Streptococcus iniae* in Mozambique tilapia *in vivo*. Hematologic and immunologic parameters of the recent study also exhibited that orange peel oil showed no negative effect to fish health and growth performance [32].

As a result, this study showed that lemon essential oil and argan oil had antibacterial potentials against some fish pathogens. Since the lemon essential oil mainly obtained from fruit peels which are discarded as waste, the essential oil to be used for fish fed will be produced economically cheaper than those of other plants. Therefore, both can be used to prevent fish diseases by adding to fish fed or to prepare solutions for immersion treatment. However, further studies, particularly *in vivo* studies are necessary.

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