Research Article

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Chemical characterization and antibacterial-antifungal activity of *Rutaceae* Family Essential oils from different plants on probiotic microorganisms

Abstract

Lemon, lime, orange, grapefruit, bergamot, mandarin and bitter orange species which have major characteristic specialities of *Rutaceae* family, have antimicrobial activities on pathogene microorganisms. Probiotic microorganisms have valuable effects on human body and inhibition of probiotics causes many diseases. In this present study, it was aimed to determine indicate probiotic resistance against natural antimicrobial agents (as essential oils) compare to pathogenes in previous studies. Analysis of essential oils (Eos) from were analyzed by GC-FID and GC/MS, analysis of Eos antimicrobial and antifungal activity from were analyzed by Microdilution test. Limonene (%95.29) and Linalool (%34.94) were found as major compounds of EOs respectively. All essential oils have antimicrobial activities on probiotic microorganisms.

Keywords: essential oil, *lamiaceae*, antimicrobial, antifungal, characterization

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Introduction

The Rutaceae are a large, widely distributed family of trees and other woody plants comprising about 150 genera and some 180 species.[1] The genus Citrus has been variously described as consisting of from 1 to 162 species.[2,3] The most widely accepted taxonomic systems today are those of Swingle (1946) and Tanaka (1977) who recognized 16 and 162 species, respectively. Relationships among taxa are complicated by several factors such as a high frequency of bud mutation, a long history of cultivation, and wide cross-compatibility. In species that are grown primarily for fruit, sports may be vegetatively propagated and maintained by budding, which can lead to small, mutation-based differences among varieties within cultivated species.[4] For example, little genetic variation was detected within the important cultivated species *C. sinensis* and *C. paradisi* when examined by microsatellite-based markers.[5-7] In medicine, Citrus fruits are used in the treatment of various diseases. Research shows that the intake of Citrus fruits can reduce the incidence of gastric cancer. In addition, some isolated compounds from these fruits have effects on the central nervous system. For example, limonene, which is present in high concentrations in Citrus aurantium, showed a strong anxiolytic effect when tested in both animals and humans (Table 1).[8]

Table 1 Pharmacological action table of widely-used *Citrus sp.* fruits in previous studies

Citrus species	Pharmacological action[8]
Citrus aurantium L.	Gastrointestinal stimulant and general tonic. Treatment of central nervous system disorders like insomnia, anxiety, and hysteria.
	Relieve stomach cramps and constipation, combat stomach acidity.
	Hypoglycemic effect.
	Anti-inflammatory.
	Anxiolytic effect.
	Sedative action.
Citrus sinensis L.	Anthelmintic properties.
	Treatment of liver cirrhosis.
	Antidiabetic properties.
	Anxiolytic effect.
Citrus bergamia L.	Antibacterial. Antifungal. Anti-inflammatory. Analgesic. Antiproliferative and anticancer properties. Neuropsychopharmacological. Neuroprotective.
	Anxiolytic activity.
	Hypoglycemic and hypolipidemic activities.

Table Continued...

Citrus species	Pharmacological action[8]
Citrus limon L.	Analgesic. Anti-anemic. Anti-sclerotic. Antipyretic. Antiseptic. Emollient and moisturizer properties. Anti-diarrheal. Diuretic. Intestinal mucosa protector. Local hemostatic. Vascular stimulant and protector. Antioxidant. Antiallergic. Antiviral. Anti-inflammatory. Antiproliferative, antimutagenic, and anticancer activities.

Antibiotics are drugs that have the ability to prevent or destroy the growth of various microorganisms. The antibiotic era began when Alexander Fleming (1881-1955) discovered penicillin in 1928. Louis Pasteur, in his work on the fermentation of lactic acid (1857), mentioned the existence of certain substances capable of showing antimicrobial effects. In that fact, probiotic microorganisms so much important for indicate pathology of infections of pathogene microorganisms. Generally probiotics are more resistant than pathogene microorganisms and they inhibit them in competetive inhibition tests (Table 2).[9]

Table 2 Antimicrobial activity of investigated *Rutaceae* essential oils on pathogene microorganisms in previous studies

Essential oil	Inhibited Pathogene Microorganisms
Citrus limon L.	Bacillus cereus, Mycobacterium smegmatis, Listeria monocytogenes, Micrococcus luteus, Escherichia coli, Klebsiella pneumoniae, Pseudococ cusper, Aspergillus niger, A. flavus, Penicillium verrucosum, P. chrysogenum, Kluyveromyces fragilis, Rhodotorula rubra, Candida albicans, Hanseniaspora guillermoni[10,11]
Citrus aurantifolia L.	Bacillus subtilis ATCC 6633, Enterococcus durans ED010, Enterococcus hirae ATCC 10541, Listeria monocytogenes ATCC 7644, Staphylococcus aureus ATCC 6538, Staphylococcus epidermidis ATCC 49134, Enterobacter cloacae EC02, Proteus mirabilis PM02, Pseudomonas aeruginosa ATCC 9721, Escherichia coli ATCC 10536, Serratia marcescens ATCC 19980 and Salmonella tiphi ATCC 13311, Candida albicans ATCC 10231, Candida parapsilosis ATCC 2219[12]
Citrus sinensis L.	Staphylococcus aureus, Listeria monocytogenes, Vibrio parahaemolyticus, Salmonella typhimurium, Escherichia coli, Pseudomonas aeruginosa, Aspergillus flavus, A. fumigatus, A. niger, A. terreus, Alternaria alternata, Cladosporium herbarum, Curvularia lunata, Fusarium oxysporum, Helminthosporium oryzae, Penicillium chrysogenum, P. verrucosum, Trichoderma viride[13,14]
Citrus paradisi L.	Bacillus cereus, Enterococus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudococcus sp., Shigella flexneri, Staphylococcus aureus, Cladosporium cucumerinum, Penicillium digitatum, P. italicum, P. chrysogenum[11,15]
Citrus bergamia L.	Escherichia coli, Staphylococcus aureus, Bacillus cereus, Salmonella enterica, S. typhimurium, Pseudomonas putida, Arcobacter butzleri, Enterococcus faecium, E. faecalis, Listeria monocytogenes, Hanseniaspora guilliermondii, Debaryomyces hansenii, Kluyveromyces fragilis, Rhodotorula rubra, Candida albicans, Aspergillus niger, A. flavus, Penicillium italicum, Fusarium solani, F. sporotrichioides, F. oxysporum, Curvularia lunata, Verticillium dahliae, Phomopsis sp., Phoma sp., Myrothechium verrucaria[16]
Citrus reticulata L.	Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Penicillium italicum, P. digitatum, P. chrysogenum, Aspergillus niger, A. flav. Alternaria alternata, Rhizoctonia solani, Curvularia lunata, Fusarium oxysporum, Helminthosporium oryzae[17]
Citrus aurantium amara L.	Bacillus subtilis, B. cereus, Staphylococcus aureus, S. epidermis, Enterococcus faecalis, Micrococcus luteus, Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Aspergillus niger, A. flavus, A. nidulans, A. fumigatus, Fusarium graminearum, F. oxysporum, F. culmorum, Alternaria alternata[18,19]

Materials and methods

Plant material

Pharmacopeae essential oils were used as standarts of Rutaceae plants. EOs were selected from Anadolu University, Faculty of Pharmacy, Pharmacognosy Research Laboratory essential oil collection. Microorganisms were bought from Christian Hansen©.

GC-MS analysis

The GC-MS analysis was carried out with an Agilent 5975 GC-MS system. Innowax FSC column (60m, 0.25mm film thickness) was used with helium as carrier gas (0.8ml/min). GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min. Then, programmed to 240°C at a rate of 1°C/min. Split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70eV. Mass range was from m/z 35 to 450.

GC analysis

The GC analysis was carried out using an agilent GC system. FID detector temperature was 300°C to obtain the same elution order with GC-MS, simultaneous auto-injection was done on a duplicate of the same column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Identification of components

Characterization of the essential oil components was carried out by comparison of their retention times with those of authentic samples or by comparison of their Linear Retention Indices (LRI) to a series of n-alkanes. Computer matching against commercial Wiley GC/MS library (MacLafferty and Stauffer, 1989), MassFinder 3 Library (Koenig et al., 2004) and in house "Baser Library of Essential Oil Constituents" built up by genuine compounds and components of known oils, as well as MS literature data (Joulain and Koenig, 1998: ESO, 2000) was used for the identification.

Antimicrobial and antifungal activities with microdilution methods

This technique helps to determine MIC (minimal inhibitory concentration) and MLK (minimal lethal concentration) values of antimicrobial drugs. For this purpose, 2 or 10-fold dilutions of antimicrobial drug in Mueller-Hinton Broth are made and dilutions of dense concentrations of drugs are obtained. Ex. drug 256, 128, 64, 32, 16, 8, starting at 256 32g in 1 ml. 4, 2, 1, 0.5, 0.25, 0.12 /g/mL are gradually diluted in three layers. The isolated test is seeded in 100µL of the 24-48 hours liquid culture of the microorganism and incubated at 37°C for 24-48 hours. The reproduction in the tubes is evaluated by the eye. Thus, the final dilution without reproduction is accepted as MIC value. However, in order to be precise, it is appropriate to perform the test in three parallel. The average of the most recent results is the MIC or MLK obtained. Essential oil fractions of *Citrus sp.* were dissoluted in %10(v,v) DMSO(Merck©, CAS: 67-68-5) and emulsufied in distilled water. Resazurin sodium(Sigma-Aldrich©,CAS No:62758-13-8) is used as indicator for determination of MIC values. Chloramphenicol (Sigma-Aldrich©, CAS: 57-75-7) was used as positive control as indicated in the Clinical Laboratory Standards Institute guide.[20,21] In this study we calculated MIC values as other MIC studies in the literature.

Results and discussion

As shown in Table 2, in total 36 constituents were identified. The main components were limonene and linalool in Eseintial Oils as 68,7%, 95,299%, 73,101% and 34,94% respectively. β-pinene, myrcene, γ-terpinene and linalyl acetate were the second major component in EOs 10,644%, 1,417%, 16,048% and 13,561% resp. The third major component were γ-terpinene, α-pinene, p-cymene and α-pinene in EOs 1,921%, 0,503%, 2,819% and 12,032% resp. The contents of these EOs show us limonene is most widely chemical compound in this study (Table 3 & 4).

Table 3 Chemical components of *Rutaceae* essential oil

Compound Name (EOs)	*C. limonum*	*C. sinensis*	*C. reticulata*	*C. aurantium*
α-pinene	1.63	0.503	1.923	12.032
linalyl acetate	-	-	-	13.561
β-pinene	10.644	-	1.524	-
sabinene	1.734	0.383	0.275	1.198
myrcene	1.424	1.417	1.691	1.748
caryophylene oxide	-	-	-	-
β-caryophylene			-	-
camphora	-	-	-	-
thymol	-	-	-	-
α-thujene		-	0.78	-
limonene	68.7	95.299	73.101	9.692
limonene-4-ol		0.064	-	-
1,8-cineole	-	-	-	-
carvacrol	-	-	-	-
(Z)-β-ocimene	-	-	-	-
(E)-β-ocimene	-	-	-	5.553
p-cymene	1.921	-	2.819	-
terpinolene	-	-	0.748	-
methyl acetate	-	-	-	-
bicyclogermanilen	-	-	-	-
carvacrol	-	-	-	-
linalool	-	0.239	-	34.94
Δ-3-karnen	-	0.265	-	-
Δ-terpineol	-	-	-	-
γ-muurolan	-	-	-	-
α-terpinene	-	-	0.293	-
bornyl acetate	-	-	-	-
geranyl acetate	0.669	-	-	3.185

Table Continued...

Compound Name (EOs)	***C. limonum***	***C. sinensis***	***C. reticulata***	***C. aurantium***
terpinen-4-ol	-	-	-	-
β-caryophyllene	0.363	-	-	-
geranyl isobutirate	-	-	-	-
geraniol	1.414	-	-	2.784
geranial	-	0.08	-	-
β-phellandrene	-	0.168	0.212	-
p-cymene-8-ol	-	-	-	-
neryl acetate	-	-	-	1.657
nerol	-	-	-	1.035
neral	0.777	-	-	-
(E)-nerolidole	-	-	-	2.494
menthone	-	-	-	-
dimethyl antranilate	-	-	0.585	-
germacrene D	-	-	-	-
isomenthone	-	-	-	-
neomenthole	-	-	-	-
isopulegon	-	-	-	-
menthole	-	-	-	-
cis-p-mentha-1-ol	-	0.157	-	-
trans-p-mentha-2,8-diene	-	0.153	-	-
pulegon	-	-	-	-
menthofurane	-	-	-	-
isopulegol	-	-	-	-
camphene	-	-	-	-
α-kapaen	-	-	-	-
γ-terpinene	9.178	-	16.048	-
trans-carveol	-	0.07	-	-
cis-carveol	-	0.178	-	-
tricyclene	-	-	-	-
α-tuyen	-	-	-	-
cis-1,2-limonene-epolisite	-	0.311	-	-
trans-1,2-limonene-epolisite	-	0.177	-	-
terpineolene	-	-	-	-
trans- sabinene- hydrite	-	-	-	-
camphor	-	-	-	-
γ-terpineol	-	-	-	-
α-humulene	-	-	-	-
α-terpineol	-	0.037	-	3.657
α-terpinyl acetate	-	-	-	-
decanal		0.05	-	-
menthyl acetate	-	-	-	-

Table Continued...

Compound Name (EOs)	*C. limonum*	*C. sinensis*	*C. reticulata*	*C. aurantium*
borneole	-	-	-	-
farnesol	-	-	-	3.477
octanal	-	0.09	-	-
valensen	-	0.165	-	-
p-cymen-8-ol	-	-	-	-
bicyclogermanilene	-	-	-	-
Imalol	-	-	-	-
Imalil acetate	-	-	-	-
sabinyl acetate	-	-	-	-
Total %	98.454	99.806	99.999	97.013

Table 4 MIC table of *Rutaceae* family Eos

Microorganism / Essential oil (mg/L)	La-5	La-14	L.reu.	L.rh.	L.fer	B.coa.	B.N.	B.cl.	S.sal.	S.ther.	S.b.	S.c.	BB-12
C. limonum	>128	0.25>	>128	>128	>128	>128	>128	0.25>	>128	0.25>	>128	>128	96
C. aurantifolia	>128	>128	128	64	64	64	>128	96	0.25>	>128	0.25>	>128	64
C. sinensis	>128	>128	>128	128	96	>128	>128	0.25>	0.25>	>128	0.25>	>128	>128
C. paradisi	>128	>128	128	32	32	>128	>128	2	0.25>	>128	0.25>	96	0.5
C. bergamia	>128	>128	>128	>128	>128	>128	8	12	>128	8	>128	32	>128
C. aurantium	>128	>128	>128	>128	>128	>128	>128	0.25>	>128	0.25>	>128	>128	>128
Ketoconazole	4	4	8	0,37	12	16	12	0.5	12	0.25>	-	-	0.25
Chloramphenicol	-	-	-	-	-	-	-	-	-	-	1	1	-

La-5, *Lactobacillus acidophilus La-5*; La-14, *Lactobacillus acidophilus La-14*; L.fer., *Lactobacillus fermentum CECT- 5716;* L.reu., *Lactobacillus reuteri DSM 17938*; L.rh., *Lactobacillus rhamnosus GG*; B.coa., *Bacillus coagulans SNZ 1969*; B.cl., *Bacillus subtilis var. clausii ATCC9799*; B.N., *Bacillus subtilis var. natto BN*; S.sal., *Streptococcus salivarius K12*; S.ther., *Streptococcus thermophilus TH-4*; S.b., *Saccharomyces cerevisae var. boulardii ATCC–MYA976*; S.c., *Saccharomyces cerevisae ATCC–MYA9763;* BB-12, *Bifidobacterium bifidum BB-12*

For these results, *Citrus aurantifolia*, *Citrus paradisi* and *Citrus bergamia* essential oils are most effective EOs against probiotic microorganisms. If they are used on gastrointestinal microflora directly, they can inhibit many microorganisms and cause many gastroinstestinal problems. All of the EOs in this study effect *Saccharomyces cerevisae var. boulardii ATCC–MYA976*. This microorganism isn't resistant against EOs without *Citrus limonum, Citrus bergamia* and *Citrus aurantium* . *C. aurantium* didn't show any antimicrobial activity against probiotic microorganisms without *Streptococcus thermophilus* and *Bacillus clausii*. When compared all data's about this study probiotic microorganisms generally resistant against Rutaceae EOs. As indicated in the pathogens microorganism's table section, many microorganisms inhibited with Rutaceae family EOs but probiotic microorganisms are generally resistant on related EOs. This is important to protecting human body against bacterial and fungal infections with symbiotic microorganisms and their fundamental seconder metabolites. This study shows us probiotic microorganisms abilities to protect human body when natural antimicrobial compounds are taken.

In the other hand, probiotic microorganisms can use with antimicrobial agents in the same drug formulations to solve resistant pathogens super infectious agents in the future.

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None.

Conflicts of interest

Author declares that there is no conflict of interest.

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