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ORIGINAL ARTICLE

Antimicrobial activities of microbial essential fatty acid against foodborne pathogenic bacteria

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ABSTRACT

Background and Objectives: Human health and development have been related to dietary intake of essential fatty acids (omega 3, 6 and 9) and important for brain development, immune system function, and blood pressure regulation. Microbial essential oils are more natural and safer alternatives to synthetic preservatives. These oils have been demonstrated to have antibacterial activity within food systems and may be ideal additives to food formulations. Zygomycete fungi are well-known as good candidate for production of essential oils.

Materials and Methods: Essential oils of fungi *Mucor rouxii, Mucor circinelloides* and *Cuninghamella echinulata* were extracted and fatty acids were analyzed by GC, for the first, antimicrobial activity of the fungi essential oils against foodborne pathogenic bacteria *E. coli, S. aureus, B. cereus, B. subtilis,* and *S. enterica* was examined by disc diffusion and well diffusion methods and the minimal inhibitory concentrations (MIC) of oils were determined by microtiter plate.

Results: The fungi oils were exhibited the stron g antibacterial effect against Gram-positive bacteria, *B. cereus, S. aureus* and *B. subtilis* higher than Gram-negative and commercial oleic acid and linoleic acid. The MIC of the fungi oil extracts was 0.25 mg/ml for *B. cereus* and *B. subtilis* and 0.5 mg/ml about *S. aureus*. This research demonstrated microbial essential oils may be suitable for their antimicrobial properties in food.

Conclusion: Microbial essential oil with good antibacterial activity could also be used in selected cases like foodborne disease.

Keywords: Anti-bacterial agent; Fatty acid essential; Omega 6; Microbial sensitivity tests; Foodborne diseases

INTRODUCTION

Essential oil (EO) or essential fatty acids (EFA) are useful in the structure and biological functions which in turn convene fluidity and modulate the behavior of certain membrane-bound proteins (1). The body cannot produce EFAs, so they must be provided with the diet or taken as supplements. A primary function of EFAs is the production of prostaglandins, which regulate body functions such as heart rate, blood pressure, blood clotting, fertility, conception, and play a role in immune function by regulating inflammation and encouraging the body to fight infection (2). EFAs support the cardiovascular, reproductive, immune and nervous systems. EFAs are also needed for proper growth in children, particularly for neural development and maturation of sensory systems, with male children having higher needs than females (3).

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Commonly EFA obtained by fish, nuts, seeds, and vegetable oils but microorganisms are good candidate for EFA production, among these, special attention has been focused on the use of paid to various oleaginous Zygomycetes including *Mortierella alpina, Mortierella isabellina, Mucor circinelloides, Cryptococcus curvatus, Crypthecodinium cohnii* and *Yarrowia lipolytica*, have been widely studied (4).

Essential fatty acids have been well known as antibacterial additives which inhibit the growth of unwanted microorganisms. Foods can serve as vehicles for many pathogenic and toxigenic agents of disease, including bacteria, viruses, molds, and parasites (5). Microorganisms grow by increasing in number rather than size. Unlike spoilage microorganisms, Foodborne diseases are well recognized but are considered emerging because they have become more common. The most common bacterial foodborne illnesses result from *S. aureus, C. botulinum, Salmonella* species, *B. cereus*, and *L. monocytogenes* (6). Essential fatty acids are bactericidal to important pathogenic microorganisms, including methicillin-resistant *S. aureus* (7). *Helicobacter pylori* (8), and *Mycobacteria* (9). However, their primary molecular target still remains unknown. FabI is an enoyl-ACP reductase that catalyzes the final and rate-limiting step of the chain elongation process of the type II FAS. Since there is a lack of an overall sequence homology with the corresponding one of humans, FabI has been identified as a target for antibacterial drug development (10).

In this study, microbial EFA produced by Zygomycete fungi including *Mucor circinelloides, Mucor rouxii* and *Cunninghamella echinulata* and antimicrobial activities of various concentration of EFA examined in food pathogens like *E. coli, Salmonella enterica, B. cereuse, B. subtilis* and *S. areuse*.

MATERIALS AND METHODS

Microorganisms and media. *Mucor rouxii* DSM1194, *Mucor circineloides* DSM 1175 and *Cunninghamella echinulata* DSM1905 purchased from Leibniz Institute DSMZ, Germany and were used for essential oil production. These strains were maintained on potato dextrose agar (PDA) (Merck, Darmstadt, Germany). EO production medium contained (per liter of distilled water) 7.0 g KH_2PO_4, 2.5 g Na_2HPO_4, 1.5 g $MgSO_4$, 0.06 g $MnSO_4$, 0.15 g $CaCl_2$, 0.15 g $FeCl_3$, 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, pH 6.0, and 4% (V/V) oil waste as a carbon source (11). 1ml of spore suspension (around1×10^7 spores) was used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of basal fermentation medium and incubated in a rotary shaker-incubator at 180 rpm and 28°C for 72 h.

Food poisoning bacteria including *Staphylococcus aureus* ATCC6538, *Bacillus cereus* ATCC11778, *Bacillus subtilis* ATCC12711, *Escherichia coli* ATCC15223, and *Salmonella enterica* ATCC14028 obtained from Iranian Research Organization for Science and Technology (IROST).

Analytical methods: extraction and modification of lipids. Lipid extraction was performed according to the modified procedure of Bligh and Dyer, 1959 (12). The extracted fatty acids were modified to fatty acid methyl esters (FAMEs) according to the method of Christie (1993) (12, 13).

FAMEs analysis by gas chromatography (GC). GC was performed on Agilent 19091J-413 Series Gas Chromatograph equipped with an FID and the capillary column HP5 (30 m, 0.25 mm i. d., 0.25 µm film thickness; USA). Injector and detector temperatures were maintained at 260°C and 300°C, respectively. The oven was programmed for 2 min at 100°C, then increased to 160°C at 3 min, maintained for 2 min at 215°C, increased further to 217°C at 2 min, then maintained for 2 min at 218°C and finally increased to 260°C at 2 min. The carrier gas, nitrogen, was used at a flow rate of 1.5 ml/min. The injection volume was 1 µL, with a split ratio of 50:1(14).

Antimicrobial activities assay: gar-well diffusion method. Antibacterial activity was checked by the agar-well diffusion method with bacteria grown on Luria broth (LB) agar (Merck, Germany). Two hundred microliter suspension of the bacteria (10^5 cells/ml) were plated on the agar layer in Petri dishes (10 cm in diameter). Five wells per dish were prepared, each 5 mm in diameter. One hundred microliters of each sample, concetration 5% in DMSO. The antibacterial activity was estimated by the diameter of inhibitory zones in the agar layer after incubation at 37°C for 24 has the experiments were carried out in triplicate. Control experiments were carried out with the pure solvent (15).

Disc diffusion method. Antibacterial tests were

carried out by disc diffusion method using 10 ml of suspension containing 10^5 CFU/ml of bacteria, which was poured on LB agar. The discs (6 mm in diameter) were impregnated with 2.5 µl bioconverted microbial oil (concetration 5% in DMSO) and placed on the inoculated agar. The inoculated plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the diameter of inhibition zones against the test strains. Each assay in this experiment was performed in triplicate (16).

Minimum inhibitory concentration. The MICs were determined using the *in vitro* broth microdilution method by using 96-well microtiter plates (Greiner Bio-One, Germany). Briefly, the essential oil was dissolved in DMSO in four concentration range 5, 10, 15 and 20%. The inoculum was prepared from overnight cultures so that the initial CFU concentrations in the microplates were 10^5 for each bacterium. The inoculated plates were examined after 24 h of incubation at 37°C. The microbial growth was measured spectrophotometrically at 600nm. MICs were expressed as the lowest concentrations able to inhibit ≥ 80% of bacterial growth compared to the positive growth control. The experiments were performed in triplicate in three independent tests and median values were used for MICs calculation. Positive growth controls were each bacterium minus EO and the negative control was different DMSO concentrations with bacterium minus EO. Tetracycline and Gentamycin were used as reference antibiotics for bacteria (16).

RESULTS

The analysis of FAME extract of fungi oils by gas chromatography revealed that fungi oil including essential fatty acid especially linoleic acid (C18:2), gamma-linolenic acid (C18:3), as omega3 and alpha-linolenic acid (C18:3) as omega 3. Table 1 shows the comparison of lipid, biomass, yields (% lipid/ biomass w/w), essential fatty acid production (mg/g) (%) by three strains of Zygomycetes in media containing food oil wastes as carbon sources.

According to the results given in Table 2, different potential of antibacterial activity against the strains were tested. The bioconverted fungi oils were exhibited a strong antibacterial effect against Gram-positive bacteria, *Staphylococcus aureus* ATCC6538, *Bacillus cereus* ATCC11778, *Bacillus subtilis* ATCC12711 higher than oleic acid and linoleic acid lonely (Tables 2 and 3). The mean zone of inhibition of the extract, assayed against the test organisms ranged between 5.5 and 18 mm. The Tetracycline (30 µg/disc) antibacterial positive control produced zones of inhibition that ranged from to 22 mm on gram-positive bacteria. Gentamicin (10 µg/disc) positive control produced zones of inhibition that ranged from to 19 mm on Gram-negative. Comparison of ZOI preferred that Mucoral essential oils were a good candidate for inhibition of Gram-positive and suitable for antimicrobial properties in food.

DISCUSSION

Omega 3 and 6 fatty acids have been extensively studied for their useful effects on human health, especially the human growth, brain, and cardiovascular system. However, their application as antimicrobial agents have not been commonly appreciated perhaps because of little understanding of antimicrobial mechanism. Nonetheless, the efficacy of these agents on microbial cell membranes and their antioxidant properties have been shown to inhibit the growth of microorganisms and thereby promote human health and animal health. Omega fatty acids can be deliberated as potential alternative therapeutic agents because of their antimicrobial and immunomodulatory properties (2).

Production of microbial essential oil reported previously in medium containing different carbon sources (11). A filamentous fungus *Mortierella alliacea* collected arachidonic acid particularly in the form of triglyceride in its mycelia and yields about 46.1 g/l dry biomass. Mamatha and Venkateswaran (2010) showed 30% (w/w) lipid in *Mucor rouxii* GFR-G15 in which 14.2% accounted to be GLA content of the total fatty acids. In this research, about 9.5% GLA and 14.7% Linoleate produced by *M. rouxii* DSM1194 by using of cheap substrate oil waste (17). Acceptable value of Omega 3 and 6 obtained by fungal *Mucor rouxii*, *Mucor circineloides* and *cuningammella echinulate* in this study.

In recent years, there has been an increased interest in the use of natural antimicrobial agents. The antimicrobial activity of fatty acids was stated to be dependent on chain length and unsaturation degree (18). Long-chain unsaturated fatty acids exhibit inhibitory activity against many bacteria even methi-

Table 1. Comparison of lipid, biomass, yields (% lipid/ biomass w/w), essential fatty acid production (mg/g) (%) by three fungi in media contains oil waste as carbon sources.

Fungal strain	Biomass (g/L)	Total lipid (g/g)	Yield (w/w %)	Omega 6/3 concentrations in total lipid (mg/g)				
				C18:2 Linoleate (n-6)	C18:3 (GLA) (n-6)	C 18:3 Linolenate (n-3)	C20:3 ARA (n-6)	C22:6 DHA (n-3)
Mucor rouxii DSM1194	8.3	3.43	41.37	504.56	327.5	88.06	-	15.98
Mucor circineloides DSM 1175	10.3	4.2	40.7	424.22	116.1	128.6	16.78	71.6
Cunninghamella echinulat DSM1905	11.25	3.85	34.2	602.38	157.85	-	92.16	96.78

GLA: gamma linolenic acid, ARA: arachidonic acid, DHA: docosahexaenoic acid

Table 2. Antibacterial activity of fungal essential oils and free unsaturated fatty against foodborne pathogen

	Zone of inhibition (ZOI) in disc diffusion method					Zone of inhibition (ZOI) in well diffusion method				
	S. aureus	*S. enterica*	*B. cereus*	*B. subtilis*	*E. coli*	*S. aureus*	*S. enterica*	*B. cereus*	*B. subtilis*	*E. coli*
M.rouxii EO5%	15	4.25	16	11	N.A	17	N.A	15.5	9.25	4.6
M.circinelloides EO5%	18	6.5	14	14	N.A	24.5	N.A	12.25	12	NA
C.echinulata EO5%	10.25	12.25	8	10.5	11	13.5	11	8.5	12.5	705
Oleic acid EO5%	7	6.75	6.5	9	N.A	8.2	6.5	N.A	7.25	6
Linoleic acid 5%	N.A	6	7.35	7	N.A	7.75	N.A	8	6.8	6.25
Tetracyclin	22	9	19	17	11	20.5	7	18	19.5	10
Gentamycin	N.A	18	7.25	8	20	8	21	7.5	6	19

EO: essential oil, NA: not active

Table 3. The MIC value (mg /mL) of fungal essential oils and the free unsaturated fatty acids against several food-borne pathogens All the MIC tests were performed independently and in triplicate ($p<0.01$).

	MIC (mg/ml)				
	S. aureus	*S. enterica*	*B. cereus*	*B. subtilis*	*E. coli*
M. rouxii oil	0.25	N.A	0.25	0.25	N.A
M. circinelloides oil	0.25	N.A	0.25	0.25	N.A
C. echinulata oil	0.5	0.25	N.A	0.5	0.5
Oleic acid oil	0.5	N.A	0.5	0.5	0.5
Linoleic acid oil	0.5	0.5	0.5	0.5	N.A

The MIC of the *M. rouxii* and *M. circinelloides* oils were 0. 25 mg/ml for gram-positive bacteria but MIC of *cunninghamella* oil was varied in all of the pathogens. The MIC of oleic and linoleic acid determined 0.5 mg/ml in the majority of strains (Table 3).

cillin-resistant *S. aureus* (MRSA) (7). For instance, linoleic and oleic acids were reported as potent antibacterial (17). Some plant-derived antimicrobial oils are most effective against Gram-positive organisms, though some are more antagonistic towards Gram-negative species. Linoleic acid was also stated as a model compound of unsaturated fatty acids, which selectively inhibits the FabI enzyme in *S. aureus* and *E. coli*, catalyzing the final and rate-limiting step of the chain elongation process of type II fatty acid synthesis (FAS-II) in bacteria. The result of this study confirmed these data and free oleic and linoleic acid were effected antibacterial activity against 5 food-borne pathogens. These data are important for the treatment of infections caused by these bacteria; *S. aureus* is described as one of the main agents responsible for infection, as its virulence and ability to acquire antimicrobial resistance results in a seri-

ous problem throughout the world for hospitals and health professionals (7).

Differences of MIC could be due to the nature and level of the antimicrobial agents present in the extracts and their mode of action on different test microorganisms. Structural differences in chemical compounds result in variances in mechanisms of action against bacterial species (19). It has been mentioned, however, that combinations or total oils may be beneficial as a means of preventing the development of microbial resistance.

CONCLUSION

Results indicate that microbial essential oils may be suitable for their antimicrobial properties in food. However, that combination or whole oils may be beneficial as a means of preventing the development of microbial resistance than each fatty acid alone.

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