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Biotransformation of oleic acid and antimicrobial and anticancer activities of its biotransformatıon extracts

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Oleic acid is an unsaturated fatty acid found in significant quantities in various edible oils. Scientific studies have shown that oleic acid and its derivatives exhibit a variety of biological activities including antimicrobial and anticancer activities. In the present work, biotransformation of oleic acid was carried out initially using 27 different microbial strains. Extracts obtained from biotransformation with *Alternaria alternata* (clinical isolate) and *Aspergillus terreus* var. *africanus* (clinical isolate) were used in antimicrobial and anticancer activity studies. The *in vitro* antimicrobial activities of the extracts were evaluated against 9 different pathogenic microorganisms. The results indicated that the microbial extracts were more active than oleic acid itself and showed good inhibitory activity against all tested microorganisms. In *in vitro* anticancer activity studies, extract 2 obtained from biotransformation with *Alternaria alternata* exhibited notable anticancer activity against A549 cell line with an IC_{50} value of 62.5 μg/ml whereas positive control cisplatin showed an IC_{50} value of 43.5 μg/ml. The anticancer activity of extract 2 was also found to be selective according to its higher IC_{50} value (122.7 μg/ml) obtained against the healthy cell line, mouse embryonic fibroblasts, NIH3T3. Due to its anticancer effect, extract 2 is considered to participate in further research.

Keywords: Antimicrobial activities, Biotransformation, Cytotoxic activities, Minimum inhibitory concentration, Oleic acid

INTRODUCTION

Oleic acid, also known as 9-octadecenoic acid, is an unsaturated fatty acid present in various vegetable oils such as hazelnut and olive oil as glyceryl esters. It is used commercially as a pharmaceutical solvent and in the preparation of oleates and lotions [1, 2]. Scientific studies have shown that oleic acid and oleic acid-containing extracts exhibit a variety of biological activities including antibacterial activity [3-5], antifungal activity [6, 7], antioxidant activity [8-10], anti-insecticidal activity [11], antiviral activity [12], hemolytic activity against human erythrocyte [13] and anticancer activity against MCF-7 and HT-29 cancer cells [14]. Therefore, molecular modifications of oleic acid have been carried out by biotransformation reactions in recent years in the hope that the addition of functional groups may enhance its activity.

The literature reveals several microbial transformations of oleic acid by using fungi, bacteria and recombinant microorganisms. These include conversion to ricinoleic acid by a soil bacterium [15] and to 10-hydroxyoctadecanoic and 10-oxo-octadecanoic acids by *Rhodococcus rhodochrous* (*Nocardia aurantia*) [16], *Nocardia cholesterolicum* [16, 17] and *Pseudomonas sp*. [16, 18-21]; hydroxylation to 10-hydroxystearic acid by *Pseudomonas* sp. [17, 22, 23], by recombinant *Escherichia coli* [24] and several enterobacteria [25]; hydroxylation to 10-hydroxyoctadecenoic acid by *Pseudomonas aeruginosa* [2, 26-28] and dihydroxylation to 7,10-dihydroxy-8-(E)-octadecenoic acid [29, 30].

The study was aimed to obtain oleic acid extracts through microbial biotransformation using fungi and evaluate their antimicrobial activity and *in vitro* anticancer activity on human lung carcinoma cell line (A549). The cytotoxicity of oleic acid extracts on normal mouse embryonic fibroblast cell line (NIH3T3) was also investigated to determine the selectivity of the extracts.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were used in high purity, obtained from commercial sources. The solvents used for purification were distilled prior to use. Silica gel type 60 (Merck, 230–400 mesh) was used for column chromatography. Thin layer chromatography (TLC) was carried out on a 0.25 mm thick silica gel plate (Merck, 60 G F254) in *n*-hexane (Merck)/ethyl acetate (Merck) (1:1, v/v). Compounds were detected either under UV light (at 254 nm) or by spraying with sulfuric acid (1:1) solution followed by heating on a hot plate.

Microorganisms and media

Fungal cultures used in the present study were obtained from the USA Agriculture Research

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Service Culture Collection (NRRL), American Type Culture Collection (ATCC) and the culture collections at both Faculties of Pharmacy and Science at Anadolu University and the Faculty of Science of Eskisehir Osmangazi University in Turkey.

Fungi used in biotransformation studies were as follows: *Aspergillus parasiticus* NRRL 2999, *A. niger* NRRL 326, *A. niger* ATCC 10549, *A. alliaceus* NRRL 317, *A. terreus* var. *africanus* (isolate), *A. nidulans* (isolate), *Penicillium claviforme* MR 376, *P. adametzii* NRRL 737, *P. chrysogenum* NRRL 792, *P. primulinum* (isolate), *P. valentinum* (isolate), *Fusarium solani* ATCC 1284, *F. moniliforme* NRRL 2374, *F. culmorum* (isolate), *F. heterosporium* DSM 62719, *Saccharomyces cerevisiae* ATCC 9763, *Hansenula anomala* ATCC 20170, *Sporobolomyces pararoseus* ATCC 11385, *Mucor ramannianus* ATCC 1839, *Neurospora crassa* (isolate), *Corynespora cassiicola* DSM 62475, *Alternaria alternata* (isolate), *Trametes versicolor* ATCC 200801, *Phanerachaete chrysosporium* ME 446, *Pycnoporus cinnabarinus* (isolate), *Trichotesium roseum* (isolate) and *Botrytis cinerea* AHU 9424.

Fungal and bacterial strains used in antimicrobial studies were as follows: *Aspergilllus niger* ATCC 10549, *Fusarium heterosporium* DSM 62719, *Penicillium valentinum* (isolate), *Saccharomyces cerevisiae* ATCC 9763, *Hansenula anomala* ATCC20170, *Sporobolomyces pararoseus* ATCC 11385, *Bacillus subtilis* NRRL B-4378, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739.

All microorganisms were stored at -85°C (Ultrafreezer, New Brunswick) in 15% glycerol and maintained on nutrient agar (Merck) and malt extract agar (Merck) slants at 4°C, respectively. Prior to testing, the purity of microorganisms was checked by subculturing twice on nutrient agar and malt extract agar.

Biotransformation

The cultures were precultured on potato dextrose agar (PDA) (Merck) slants at 25ºC for 7 days prior to biotransformation experiments. A medium for growing fungi used in biotransformation experiments was prepared by mixing glucose (20 g) (Merck), yeast extract (5 g) (Merck), polypeptone (5 g) (Sigma), NaCl (5 g) (Merck) and Na_2HPO_4 (5 g) (Merck) in distilled water (1 l). Spores were aseptically transferred into 20 Erlenmeyer flasks (250 ml) containing 100 ml of freshly prepared and autoclaved medium described above and left on an orbital shaker at 25ºC for 2 days for the full growth. A solution of oleic acid (500 mg) (Roth) in ethanol (20 ml) was then evenly distributed between these Erlenmeyer flasks containing the fungi. The fermentations were continued for a further 7 days.

Extraction and purification

After 7 days, the mycelium was filtered off and the broth was extracted with ethyl acetate. The extract was dried over Na_2SO_4 (Merck) and the solvent was evaporated to give an oily residue which was dissolved in acetone. Silica powder was then added to this solution and excess acetone was removed on an evaporator. The resulting powder containing biotransformation mixture was loaded into the glass column (3 cm wide and 60 cm long) followed by purification *via* column chromatography. Approximately 120 g of powdery silica gel (Merck) was used as stationary phase and glass tubes with a capacity of 10 ml of eluent solution (1.5 cm wide and 12 cm long) were used to collect the fraction. Increasing concentrations of ethyl acetate (Merck) in light petroleum (Merck) were used as an eluent system.

In vitro antimicrobial activity

The broth microdilution method recommended by the Clinical Laboratory Standards Institute (CLSI) was used for assessing *in vitro* antibacterial and antifungal activities of extracts [31]. Ampicillin and amphotericin B were used as standard antibacterial and antifungal agents, respectively. They were purchased from Sigma. All tests were assayed in duplicate in two independent experiments.

Broth microdilution test for bacteria

Broth microdilution testing was performed in accordance with the guidelines of CLSI M100-S16 [32]. The minimum inhibitory concentrations (MIC) of oleic acid and its metabolites were studied by broth micro dilution method using 96-well microtiter plates (Sigma). Overnight grown microbial suspensions in double strength Mueller-Hinton broth (MHB) (Merck) were standardized turbidometrically to approximately 10^8 CFU 1/ml (using Mac Farland No: 0.5). Test extracts were dissolved in DMSO (50%) and diluted in MHB to get a concentration range of 0.98–2000 µg/ml. The solution was then two-fold diluted in MHB (100 µl), inoculated with bacterial strains and then incubated at 37ºC for 24 h. Resazurin solution (Sigma) was added to confirm the MICs. The MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition. The results of antimicrobial testing were compared with those of standard ampicillin (Sigma) as antibacterial agent. The final concentrations were between 0.04 and 40 μg/ml and DMSO was assayed as the negative control.

Broth microdilution test for yeasts

CLSI broth microdilution testing was also performed exactly as outlined in document M27-A2 by using 96-well microtiter plates in RPMI-1640 medium (Sigma) and inocula of 0.5–2.5 × 10^3cells /ml (Mac Farland 0.5). The final concentrations of oleic acid and its extracts were between 0.98–2000 μg/ml. MIC values were determined for 24 h at 37ºC incubation. Resazurin solution was added to confirm the MICs. The MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition [33].

Broth microdilution test for fungi

Similarly, *in vitro* susceptibility testing of fungal strains was also performed with broth microdilution methods as described in CLSI document M38-A2 [34]. Spore counts were made in a Thoma counting chamber (Hawskley) and spore solutions were prepared (10^8 spore 1/ml) by using sterile 0.02% Tween-80 (Merck) applied for collecting spores from plate.

Cytotoxicity test

NIH3T3 and A549 cells line were used for cytotoxicity tests. NIH3T3 cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Thermo Scientific, USA) supplemented with fetal calf serum (Hyclone, Thermo Scientific, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (Hyclone, Thermo Scientific, USA) and 7.5% $NaHCO_3$ at 37°C in a humidified atmosphere of 95% air and 5% CO_2. A549 cells were incubated in RPMI medium (Hyclone, Thermo Scientific, USA) supplemented with fetal calf serum, 100 IU/ml penicillin and 100 mg/ml streptomycin and 7.5% $NaHCO_3$ at 37°C in a humidified atmosphere of 95% air and 5% CO_2. NIH3T3 and A549 cells were seeded at 10000 cells into each well of 96-well plates. After 24 h of incubating period, the culture media were removed and extracts were added to culture media at 8 concentrations (500; 250; 125; 62.5; 31.2; 15.6; 7.8; 3.9 μg/ml). After 24 h of incubation, cytotoxicity test was performed using the In Cytotox-XTT 1 Parameter Cytotoxicity Kit (Xenometrix AG, Switzerland), which measures mitochondrial activity (tetrazolium hydroxide (XTT)) in NIH3T3 and A549 cells. The cells were washed with phosphate buffer saline (PBS) and then added to 200 μl/well of fresh culture medium. XTTI and XTTII solution were mixed at 1:100 ratio. Then, 50 μl of this mixture was added to all wells. The plate was incubated for 3 h at 37ºC, 5% CO_2. Then, the content of the well was mixed by pipetting up and down. OD of the plate was read at 480 nm with a reference wavelength at 680 nm. Inhibition % was calculated for each concentration of the biotransformation extracts and IC_{50} values were estimated by plotting a dose response curve of the inhibition % equation (1) *versus* test compound concentrations.

$$\text{Inhibition } \% = 100 - (\text{corrected mean OD sample} \times 100/\text{corrected mean OD solvent controls}) \quad (1)$$

Cisplatin was used as positive control. Stock solutions of extracts were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The final DMSO concentration was under 0.1%. All experiments were performed in triplicate [35].

Biotransformation reactions

Pre-biotransformation screening experiments with oleic acid were carried out with 27 different microorganisms. Among the evaluated, the fungi *Alternaria alternata* (isolate) and *Aspergillus terreus* var. *africanus* (isolate) showed the presence of polar compounds when compared with oleic acid used as a reference compound according to chromatographic analyses which urged further preparative scale biotransformation studies for 7 days at 25ºC. From these biotransformation reactions, a pure single metabolite of oleic acid could not be obtained and metabolites were collected from the column as a mixture of at least two or more by eluting the column with increasing concentrations of ethyl acetate in petroleum ether. These fractions were named as 1, 2, 3, 4, 5 and 6 and used for antimicrobial and cytotoxic activity studies (Table 1).

Antimicrobial activities

The MIC values of oleic acid biotransformation extracts (1-6) are presented in Table 2. According to the MICs results, the most effective antifungal activity was shown by extract 5 at 62.5-125 μg/ml concentration for both mold and yeasts. Extracts 1, 2 and 4 gave better results than oleic acid itself against *Hansenula anomala* ATCC 20170 and *Sporobolomyces pararoseus* ATCC 11385. Extracts 1, 2 and 4 at a concentration of 250 μg/ml were more effective than oleic acid (500 μg/ml concentration) against all fungi. Among all extracts, the most effective antibacterial activity was shown by extract 5 at 125 μg/ml concentration.

Table 1. The biotransformation extracts

Extract No	Fungus	Eluent (%)	Eluent volume (ml)
1	*A. alternata*	Ethyl acetate (25): Petroleum ether (75)	200
2	*A. alternata*	Ethyl acetate (45): Petroleum ether (55)	100
3	*A. alternata*	Ethyl acetate (55): Petroleum ether (45)	200
4	*A. terreus* var. *africanus*	Ethyl acetate (50): Petroleum ether (50)	200
5	*A. terreus* var. *africanus*	Ethyl acetate (55): Petroleum ether (45)	100
6	*A. terreus* var. *africanus*	Ethyl acetate (60): Petroleum ether (40)	100

Table 2. Antimicrobial activity of the biotransformation extracts (1–6) as mic (μg/ml)

Fungal Strains	1	2	3	4	5	6	Oleic Acid	Amf. B.
A. niger ATCC	250	250	500	250	125	500	500	128
F. heterosporium	250	250	500	250	125	500	500	64
P. valentinum	250	250	500	250	125	500	500	128
S. cerevisiae	125	125	250	125	62.5	250	125	128
H. anomala	62.5	62.5	500	62.5	62.5	125	125	64
S. pararoseus	250	250	500	250	125	500	500	64
Bacterial Strains								Ampicillin
B. subtilis	250	250	500	250	125	250	500	1
S. aureus	250	250	500	250	125	500	500	4
E. coli	250	250	500	250	125	500	500	2

Table 3. Cytotoxic activity of the biotransformation extracts against cell lines

Extract No	IC_{50} values for cell lines (μg/ml)	
	NIH3T3	A549
1	>500	463.2
2	122.7	62.5
3	>500	500
4	90.5	>500
5	46.3	89.6
6	280.6	117
Cisplatin	ND	43.5

Extracts 1 and 2 were more effective than oleic acid at 250 μg/ml concentration against all bacteria. On the other hand, extract 6 was effective against *B. subtilis* only. As a result, the microbial extracts of oleic acid were more active than oleic acid itself and showed good inhibitory activity against all tested microorganisms.

Cytotoxic activities

It is reported in the last world cancer report that lung cancer is the first cause of newly estimated cancer-related deaths. The most commonly diagnosed cancer types are prostate, lung and colorectal in males; breast, colorectal and lung in females in developed countries whereas they are lung, liver and stomach among males and breast, cervix uteri and lung among females in developing countries [36]. Therefore the anticancer activities of the biotransformation extracts were determined against human lung adenocarcinoma cell line, A549, by XTT assay, which were not studied before. Extracts 1, 2, 3, 4, 5 and 6 showed anticancer activity against A549 with IC_{50} values of 463.2, 62.5, 500, >500, 89.6 and 117 μg/ml, respectively. According to these results, extract 2 exhibited the highest cytotoxic activity and its IC_{50} value is comparable with that of the positive control, cisplatin (IC_{50} = 43.5 μg/ml).

Selectivity is the major problem of chemotherapy limiting drug discovery process. Primarily, an effective anticancer agent should selectively kill cancer cells without causing unacceptable toxicity on healthy cells [37]. Therefore, the cytotoxicity of these extracts was evaluated in a normal mouse embryonic fibroblast cell line, NIH3T3, by performing XTT test for determining selectivity. Based on the results of the XTT test against NIH3T3 cell line, extracts 2 and 6 were found to be selectively cytotoxic against A549 cell line. Extract 2 showed higher IC_{50} value against NIH3T3 cell line than A549 cell line which were

122.7 and 62.5 μg/ml, respectively whereas extract 6 showed higher IC_{50} value of 280.6 μg/ml against NIH3T3 cell line than its IC_{50} value of 117 μg/ml against A549 cell line. The IC_{50} values of the extracts against cell lines are presented in Table 3.

CONCLUSIONS

Microbial biotransformation of oleic acid with *Alternaria alternate* (clinical isolate) and *Aspergillus terreus* var. *africanus* (clinical isolate) produced extracts which exhibited anticancer and antimicrobial activities. The *in vitro* antimicrobial activities of the extracts were evaluated against 9 different pathogenic microorganisms. The microbial extracts of oleic acid were more active than oleic acid itself and showed good inhibitory activity against all tested microorganisms. *In vitro* anticancer evaluation studies revealed that extract 2 showed notable anticancer activity against A549 cells with an IC_{50} value of 62.5 μg/ml, similar to our positive control, cisplatin, and further studies are required to evaluate the anticancer activities of extract 2.

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