Biotransformation of oleic acid and antimicrobial and anticancer activities of its biotransformation 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. africanaus (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], antinsecticidal 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-hydroxyoctadecanoic 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 (Merek, 230–400 mesh) was used for column chromatography. Thin layer chromatography (TLC) was carried out on a 0.25 mm thick silica gel plate (Merek, 60 G F254) in n-hexane (Merk)/ethyl acetate (Merk) (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. adamentii NRRL 737, P. chrysogenum NRRL 792, P. primulinum (isolate), P. valentinum (isolate), Fusarium solani ATCC 1284, F. moniliforme NRRL 2374, F. culmorum (isolate), F. heterosporum 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), Trichotomes roseum (isolate) and Botrytis cinerea AHU 9424.

Fungal and bacterial strains used in antimicrobial studies were as follows: Aspergillus niger ATCC 10549, Fusarium heterosporum 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 25°C 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), polypeptide (5 g) (Sigma), NaCl (5 g) (Merck) and Na₂HPO₄ (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 25°C 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 Na₂SO₄ (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 10⁶ 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 37°C 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 × 10³ cells/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 37°C 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 (Hawksley) and spore solutions were prepared (10⁸ 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% NaHCO₃ at 37°C in a humidified atmosphere of 95% air and 5% CO₂. 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% NaHCO₃ at 37°C in a humidified atmosphere of 95% air and 5% CO₂. 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 37°C, 5% CO₂. 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 IC₅₀ values were estimated by plotting a dose response curve of the inhibition % equation (1) versus test compound concentrations.

Inhibition % = 100 - (corrected mean OD sample × 100/corrected mean OD solvent controls)

(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. africanaus (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 25°C. 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.
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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. africana| Ethyl acetate (50): Petroleum ether (50)        | 200                |
| 5          | A. terreus var. africana| Ethyl acetate (55): Petroleum ether (45)        | 100                |
| 6          | A. terreus var. africana| 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           | 125   | 125   | 250   | 125   | 62.5  | 250   | 125        | 128     |
| S. cerevisiae           | 62.5  | 62.5  | 500   | 62.5  | 62.5  | 125   | 125        | 64      |
| H. anomala              | 250   | 250   | 500   | 250   | 125   | 500   | 500        | 64      |
| S. pararosus            | 250   | 250   | 500   | 250   | 125   | 500   | 500        | 64      |
| Bacterial Strains       |       |       |       |       |       |       |            |         |
| 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 | IC₅₀ 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 IC₅₀ 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 IC₅₀ value is comparable with that of the positive control, cisplatin (IC₅₀ = 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 IC₅₀ 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\textsubscript{50} value of 280.6 µg/ml against NHI3T3 cell line than its IC\textsubscript{50} value of 117 µg/ml against A549 cell line. The IC\textsubscript{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. *africanaus* (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\textsubscript{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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