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Antibacterial activity of vanillic acid and catechol produced by microbial biotransformation of caffiec acid

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Abstract

Biotransformation is a process by which organic compounds are transformed from one form to another to reduce the persistence and toxicity of the chemical compounds. This process is aided by major range of microorganisms and their products such as bacteria, fungi and enzymes. Caffeic acid [3, 4-dihydroxycinnamic acid] metabolism was studied and screened using eleven fungal cultures. Vanillic acid (**1**) was the major metabolite after 10 days incubation with *Aspergillus awamori* AUMC58 while catechol (**2**) was the major one with *Aspergillus versicolor* AUMC480 cultures after same incubation period. Different chromatographic techniques were applied for purification of the products. The structures of the metabolic products were elucidated on the basis of their spectral data. Antibacterial activity of caffeic acid and its metabolites were evaluated against five pathogenic bacterial strains using disc diffusion technique and determination of minimum inhibitory concentration (MIC). Caffeic acid displayed highest activity against *B. cereus* while **1** recorded highest antibacterial activity against *S. typhi* with inhibition zones 8.7 and 8.8 mm, respectively. While **2** showed strong antibacterial activity against *E.coli and P. aeruginosa* with inhibition zone value 8.8 mm. In contrast, all of them showed the lowest antibacterial activity against *Staph. aureus* and *E.coli*. Caffeic acid, **1** and **2** had higher antibacterial activity than ampicillin against *P. aeruginosa*.

Keywords: *Antibacterial, Microbial Biotransformation, Caffiec, Vanillic, Catechol.*

INTRODUCTION

Microbial biotransformation is a combination of biochemical reactions to transform the structures of the phytochemicals and organic compounds, by exploiting microorganisms and their isolated enzymes, to develop a variety of useful constituents, through regio/stereo-selectivity reactions [1]. These microorganisms become the functional part of new chemical processes for organic synthesis, extensively used commercially and industrially. The recognition of biotransformation as important manufacturing tool has increased within chemical and pharmaceutical industries in recent years [2]. Biotransformation is carried out usually at room temperature and atmospheric pressure, avoiding the use of extreme reaction conditions, and minimizing problems of isomerization, racemization, epimerization or transposition [3]. Therefore, biotransformation attracts considerable attention due to its simple, cheap and benign methodologies that combines green chemistry with high efficiency [4]. Caffeic acid (3,4-dihydroxycinnamic acid) is a phenolic compound widely distributed in medicinal plants, including fruits, vegetables, wine, coffee and olive oil, among others, and is therefore present in human plasma in a diet dependent concentration [5]. Caffeic acid displays a wide spectrum of biological activities, e.g. anti-cancer, anti-oxidant, anti-inflammatory, antimicrobial, anti-hyperglycemic and hepatoprotective properties. Caffeic acid is sequentially degraded to protocatechuic acid, 4-vinylcatechol, vanillyl alcohol, vanillic acid, dihydro-caffeic acid, 4-ethylcatechol, m-hydroxy-phenyl propionic acid and 3,4-dihydroxyphenylpropionic acid by bacteria and fungi[6-8] .The increased resistance of pathogenic microorganisms is ascribed to the extreme use of antibiotics. To contend with onset of resistant microbes, scientists have been seeking in finding of new antimicrobials[9].This paper reports for the capability of the *Aspergillus versicolor* AUMC480 and *Aspergillus awamori* AUMC58 cultures to bio transform caffeic acid into new products and evaluation of the antibacterial activity of these metabolites using agar diffusion method.

MATERIAL AND METHODS

Instruments and Materials for microbial biotransformation

For microbial biotransformation; Sabouraud-dextrose agar, SDA (Becton Dickinson and Co., Cockeysville, Med 21030). Yeast extract microbiological grade (Oxoid LTD, England). Peptone microbiological grade (Sigma chemical Co., USA). Potato dextrose agar (DIFCO, USA). Dextrose AR-grade (Sigma chemical Co., USA). Sodium chloride, analytical grade, Di Potassium hydrogen phosphate and Glycerol (ADWIC, Egypt). Nutrient agar (Oxoid LTD, England). Caffeic acid was from (sigma Aldrich, St. Louis, USA). Digital balance, (Scout^{TM} Pro (OHAUS) model, USA). AutoclavePb1, (Germany). Sonicated water bath, (Branson3510E-MTH, Mexico).Gyratory shaker incubator, (Lab line instrument, USA). Thermostatic oven WT-binder 7200 (Germany).

Microorganisms for biotransformation

Aspergillus alliaceous NRRL315, *Aspergillus niger* NRRl3, *Penicillium chrysogenum* ATCC 948, *Aspergillus flavus* AUMC 4787, *Aspergillus awamori* AUMC58, *Aspergillus ochraceous* AUMC11328, *Penicillium chermesinum* AUMC275, *Cunningamella blackeseleeana* AUMC5618, *Paecilomyces variotii* AUMC480 and *Aspergillus versicolor* NRRL 1306 were obtained from American Type culture collection (ATCC), Northern

Regional Research Laboratories (NRRL) and from Assiut University Mycological center (AUMC).

Instruments and materials for chromatographic techniques

The NMR spectra were recorded using, Bruker a 400 MHz for ^{1}H NMR and 100.40 MHz for ^{13}C NMR. The spectra were run in DMSO and chemical shifts were given as δ ppm relative to tetramethylsilane (TMS) as an internal standard. Rotary evaporator (BUECHI, Germany). Laminar flow hood (EACT 8613, USA). UV lamp-Espectroline (R) Model M-10 fluorescence Analysis cabinet (USA). For column chromatography, Silica gel G 60, 70-230 mesh (Merck, Germany) was used. TLC was carried out on aluminum sheets precoated silica gel G F_{254} (Merck, KGaA Darmstadt, Germany). Developed chromatograms were visualized by spray with Anisaldehyde/sulfuric acid reagent. TLC plates were developed using solvent systems: (**S_1**) Methylene chloride: Methanol (9:1 V/V) and (**S_2**) Methylene chloride%.

Materials for the antibacterial activity

Two Gram-positive bacteria *Bacillus cereus* EMCC 1080, *Staphylococcus aureus* ATCC 13565 and three Gram-negative bacteria *Salmonella typhi* ATCC 25566, *Escherichia coli* 0157 H7 ATCC 51659 and *Pseudomonas aeruginosa* NRRL B-272 were obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt. Nutrient agar media (Fluka, BioChemika, Spain). Tryptic soya broth, TSB (BD, Sparks, USA).

Small Scale biotransformation of Caffeic acid

A two-stage fermentation protocol was employed [10]. For screening experiments, solid cultures kept on either potato dextrose agar or Sabouraud dextrose agar. At first, the fungi were grown on PDA slants. Slants were incubated for 3 days at 25±2°C, before storage in a refrigerator till use. **Stage I** fermentation liquid culture was initiated by transferring a few drops of suspended cells into 250 ml Erlenmeyer flask, containing 50 ml of sterile liquid media of the following composition: 20g dextrose, 5g NaCl, 5 g peptone, 5 g K_2HPO_4, 5g yeast and 10 ml glycerol, all dissolved in 1 L distilled water present in 250 ml Erlenmeyer flask and allowed to grow for 72 hr at 25±2°C on a gyratory shaker operating at 100 rpm. **Stage II** cultures were obtained by transferring 5ml of stage I culture to each of 250 ml flasks containing 50 ml of fresh liquid media. Both substrates and organism controls were treated in the same way. Cultures were allowed to grow for 24 h, before the addition of the substrate solution. The solutions were added in each flask separately in the form of (10 mg caffeic acid dissolved in 0.25 ml DMSO), 5ml was periodically withdrawn from each culture (3, 5, 7, 10 and 14 days) and extracted successively with ethyl acetate. Each extract was separately evaporated to dryness under reduced pressure at 40°C.The metabolites were detected using TLC using solvent system methylene chloride: methanol (9:1 V/V) and visualized by spraying with Anisaldehyde/sulfuric acid reagent.

Large scale biotransformation of caffeic acid

Large scale biotransformation of caffeic acid by *Aspergillus awamori* AUMC58 and *Aspergillus versicolor* AUMC480 cultures, gave the best results in screening. Caffeiec acid 1 g for each experiment was dissolved in 10 ml DMSO, and then equally divided among 10 flasks (1000 ml) containing 200 ml of stage II culture and allowed to grow at 25±2°C on gyratory shaker operating at 100 rpm. After elapse of the suitable time (10 days) of fermentation the experiments were terminated .The filtered liquid media obtained from the biotransformation of each experiment was exhaustively extracted with ethyl acetate. The organic phases were combined and evaporated to dryness to give 2.15 g (**FI**) residue for *Aspergillus awamori* AUMC58 experiment and 2 g (**FII**) for *Aspergillus versicolor* AUMC480 experiment.

Isolation and Purification of the Metabolites

Both **FI** (2.15 g) and **FII** (2g) were chromatographed separately on silica gel column (100 g 3 cm×60 cm) using dry packing technique. Gradient elution method was adopted starting with hexane then hexane/DCM mixtures with increasing polarity up to 100% DCM then DCM/MeOH mixtures with increasing polarity up to 50% MeOH. In case of **FI,** (60 subfractions 15 ml each) were collected, examined on pre coated silica gel G plates, using solvent system methylene chloride: methanol (9:1 V/V), the similar fractions were combined together into 5 collective fractions (FI a - FI e). Fraction FI b (350 mg) was finally purified on sephadex column, eluted with methanol to give pure metabolite **1** (165 mg). **F2** (75 subfractions 15 ml each) were collected, examined on pre coated silica gel G plates, using solvent system methylene chloride: methanol (9:1 V/V) and similar fractions pooled together into 6 collective fractions (FI a - FI f). Fraction FI c (300 mg) was finally purified on sephadex column, eluted with methanol to give pure metabolite **2** (110 mg).

Antibacterial activity

The sensitivity test of Caffeic acid and its metabolites (Vanillic acid and Catechol) were determined with different bacterial cultures using disc diffusion method by Kirby-Bauer technique [11]. Petri dishes were prepared with 20 ml nutrient agar and the bacterial cultures were uniformly from tryptic soy broth using cotton swabs. Each extract and fraction was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to give 10 mg ml^{-1} and 1 mg ml^{-1} for extract and fraction, respectively. Sterilized discs (6 mm) from Whatman No. 1 filter paper were loaded by extracts and dried completely under sterile conditions. The discs were placed on the seeded plates by using a sterile forceps. DMSO represented as negative control and Ampicillin (500 µg ml^{-1}) and Ceftriaxone (500 µg ml^{-1}) were used as positive control. After that, inoculated plates were incubated at 37°C for 24h. At the end of the incubation period, inhibition zones were measured and expressed as the diameter of clear zone including the diameter of the paper disc.

Determination of minimum inhibitory concentration (MIC)

The determination of MIC was conducted using tube dilution method [12]. A 24 h culture of the tested bacterial species was diluted in 10 ml of tryptic soy broth (TSB) with reference to the 0.5 McFarland standards to achieve inocula of 10^8 $cfuml^{-1}$. In culture tube containing nine

different concentrations of caffeic acid and its metabolites (Vanillic acid and Catechol) (4.0, 2.0, 1.75, 1.5, 1.0, 0.75, 0.50, 0.25, 0.1 mg ml^{-1} in DMSO) were prepared. Each tube was inoculated with 100µl of bacterial cell suspension and incubated at 37^0C for 24h. The growth of the inoculum in broth is indicated by turbidity of the broth and the lowest concentration of the extract which inhibited the growth of the test organism was taken as the minimum inhibitory concentration (MIC).

RESULTS

Structure Elucidation of isolated metabolites

Metabolite 1 was isolated as off-white amorphous powder (165mg). R_f - value 0.65 (**S_2**) it gave dark spot under short UV-light and shiny violet fluorescent spot under long UV-light. **^{1}H NMR** (400 MHz, **CD_3OD**), at δ ppm 7.575 (H, m, H-6), 7.56 (1 H, m, H-2), 6.86 (1 H, d, *J*= 8.73, H-5), 3.90 (3 H, s, O-CH_3). **^{13}C NMR** (100 MHz, **DMSO-*d_6***) at δ ppm 167.61 (C-7), 150.98 (C-4), 147.27 (C-3), 123.57 (C-1), 122.24 (C-6), 115.08 (C-2), 112.85 (C-5) and 55.64 (OCH_3). All proton and carbon signals are consistent with that of vanillic acid by comparing data with that reported before [13].

Metabolite 2 was isolated as off-white amorphous powder (110 mg). R_f- value 0.70 (**S_2**) it gave dark spot under short UV-light and shiny violet fluorescent spot under long UV-light.**^{1}H NMR** (400 MHz, CD_3OD), at δ ppm 6.75 (2 H, m, H-3, 6), 6.65 (2 H, m, H-4, 5). **^{13}C NMR** (100 MHz, DMSO-*d_6*), at δ ppm 146.40 (C-1, 2), 119.45 (C-4, 5), 117.83 (C-3, 6). All proton and carbon signals are consistent with that of Catechol.

Results of Antibacterial activity

The antibacterial activity of caffeic acid and its metabolites **1** and **2** against five pathogenic bacterial strains are illustrated in table **1**, reported the MIC values against the tested pathogenic bacteria are illustrated in table **2**.

Table 1. Antibacterial activity of caffeic acid and its metabolites

Bacteria	Phenolic compounds (Inhibition Zone mm (Mean±S.E)					
	DMSO	Ampicillin	Ceftriaxone	Caffeic acid	1	2
B. cereus	0	12.5±0.50	9.0±1.00	8.7±0.58	7.8±0.29	7.3±0.28
Staph. aureus	0	10.2±0.76	8.3±0.58	7.3±0.58	7.3±0.28	7.2±0.28
E.coli	0	13.8±1.25	19.2±2.35	7.5±0.50	8.3±0.58	8.8±0.76
P. aeruginosa	0	7.3±0.28	9.7±1.04	8.3±0.58	7.8±0.28	8.8±1.04
S. typhi	0	21.0±1.00	13.2±1.76	8.2±1.25	8.8±0.76	8.5±1.32

Table 2. Minimum inhibitory concentration (MIC as µg/ml) of caffeic acid and its metabolites

Compounds	MIC (µg/ml) (Mean ± S.E)				
	B. cereus	***Staph. aureus***	***E.coli***	***P. aeruginosa***	***S. typhi***
Ampicillin	51.2±2.86	33.3±4.43	41.7±3.86	41.7±2.58	33.3±2.48
Ceftriaxone	266.7±20.78	166.7±18.14	33.3±4.78	133±15.48	41.2±2.58
Caffeic acid	1833.3±86.28	1666.7±58.21	666.7±36.14	1333.3±48.58	583±28.58
1	1333.3±28.86	2000±50.0	916.7±36.14	583.3±28.36	1833.3±78.21
2	1833.3±82.58	1666.7±78.48	666.7±36.82	1333.3±48.78	666.7±36.28

DISCUSSION

Eleven fungi were screened for their abilities to catalyze the bioconversion of caffeic acid. *Aspergillus awamori* AUMC58 culture reproducibly formed after 10 days of incubation one major metabolite **1** while *Aspergillus versicolor*AUMC480 culture formed after same incubation time one major metabolite **2**. None of the observed metabolites were formed in the control cultures. Following solvent extraction and column chromatographic purification, samples of metabolites were subjected to spectral analysis. Spectra (NMR) for isolated metabolites were established by comparing their spectral data to those given in the literature. Results of the antibacterial activity showed that caffeic acid displayed highest activity against *Bacillus cereus* and metabolite **1** recorded highest antibacterial activity against *Salmonella typhi* with inhibition zones 8.7 and 8.8 mm, respectively. While highest antibacterial activity against *E.coli* and *Pseudomonas aeruginosa* was observed by metabolite **2** with inhibition zone value 8.8 mm. In contrast, the lowest antibacterial activity was observed by catechol, vanillic acid and caffeic acid against *Staphylococcus aureus* followed by catechol and caffeic acid against *Bacillus cereus* and *E.coli* respectively. Caffeic acid, vanillic acid and catechol had higher antibacterial activity than ampicillin against *Pseudomonas aeruginosa*. As shown in Table 2, the MIC values of caffeic acid, vanillic acid and catechol against tested pathogenic bacteria were ranged between 0.58 and 2.0 (mg/ml).The lowest MIC value 0.58 (mg/ml) was recorded against *Salmonella typhi* and *Pseudomonas aeruginosa* by caffeic acid and vanillic acid respectively. While the lowest MIC value 0.67 (mg/ml) was recorded by catechol against *Salmonella typhi*. The highest MIC values 2.0 (mg/ml) was observed against

Staphylococcus aureus by vanillic acid followed by caffeic acid and catechol against *Bacillus cereus and* vanillic acid against *Salmonella typhi* with MIC value 1.83 (mg/ml). Recently antibiotic resistance by human pathogenic bacteria has been reported extensively [14]. Therefore several efforts are being done to discover antibacterial molecule's with wide range activity against pathogenic bacteria. Many phenolic compounds are known to possess antibacterial activity against wide range of pathogenic bacteria and mycotoxigenic fungi [15]. [16] indicated that antibacterial activity of caffeic acid were due to the inhibition of the bacterial RNA polymerase enzyme also[17] reported that caffeic acid esters were effective against Gram -ve and Gram +ve bacteria . [18] found that caffeic acid had antibacterial activity against different *Staphylococcus aureus* strains with MIC values varied from 256 to 1024 Mg/ml.[19]revealed that vanillic acid showed antibacterial activity against food borne pathogens like *Staphylococcus aureus* and *E.coli* .[20]reported that vanillic acid isolated from the *cacoris annobonae* had antibacterial activity against *Bacillus cereus*, *E.coli* and *Pseudomonas aeruginosa* with MIC values ranged between 156.3 and 312.5 1024Mg/ml. While [21] found that vanillic acid had antibacterial activity against antibiotic resistance *Staphylococcus aureus* MRSA and *Staphylococcus aureus* MSSA strains and the MIC was found to be 2.5mg/ml. Catechol showed antibacterial activity against *Pseudomonas putida*, *Pseudomonas pyocyanea* and *Corynebacterium xerosis* [22]. It was reported that catechol derivative had antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *E.coli* with MIC values varied from 5 to 55 mg/ml [23].

Conflict of interest- The authors declare no conflict of interest.

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