MICROBIAL TRANSFORMATION OF 2,5 DIHYDROXYCINNAMIC ACID BY *ASPERGILLUS NIGER* AND *RHIZOPUS ORYZAE*

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ABSTRACT

*2,5-dihydroxy cinnamic acid (**1**), when fermented with fungal culture, Rhizopus oryzae RCMB 014002 gave mainly two metabolites; 2,5-dihydroxy cinnamoyl alcohol (**Cin-RM-1**) and 2-Hydroxy-4,5-dimethoxy cinnamoyl alcohol (**Cin-RM-2**). Aspergillus niger RCMB002007, however, transformed 2,5-dihydroxy cinnamic acid (**1**), into a major metabolite;2-hydroxy-5-methoxy cinnamic acid (**Cin-AM-3**). The structures of the metabolic products were elucidated by means of spectroscopic data. The significance of the metabolites as antioxidants using DPPH radical scavenging assay and lipid peroxidation assay by thiobarbituric acid-reactive substances (TBARS) method using rat tissue homogenates in relation to their structure was discussed.*

***Key words*:** *Microbial transformation, 2,5 dihydroxy-cinnamic acid, Aspergillus niger, Rhizopus oryzae, antioxidant activities.*

INTRODUCTION

Cinnamic acid, the deaminated product of phenyl-alanine in plant tissue, a frequent first step is the elimination of ammonia from the side-chain to generate the appropriate *trans* (*E*) cinnamic acid. Other cinnamic acids are obtained by further hydroxylation and methylation reactions, sequentially building up substitution patterns typical of shikimate pathway metabolites, i.e. an *ortho* oxygenation pattern. Cinnamic acid has a long history of human use as a component of plant-derived scents and flavorings **(Hoskins, 1984**). Cinnamic acid is also an auxin, a class of plant hormones regulating cell growth and differentiation **(Thiman, 1969)**. Several biological activities including antioxidant, anti-inflammatory, cytotoxic, hepatoprotective, immunosuppressive, anti-cholesterolemic, antimicrobial and antiviral activities have been attributed to this class of compounds **(Natella *et al.*, 1999; Kim *et al.*, 2005; Miles *et al.*, 2005; Fernández-Martínez *et al.*, 2007; Gravina *et al.*, 2011; Prateek , 2011; Luana Dalbem *et al.*, 2012)** .

Biotransformations are useful techniques for producing medicinal and agricultural chemicals from both active and inactive materials. These reactions are an important route for introducing chemical functions into inaccessible sites of molecules and thereby to produce rare structures. The biotransformation reactions can involve high degree of *regio-* and *stereo-specificity* and require mild reaction conditions. Many researchers have reported the production of drug metabolites using microbial biotransformations **(Grogan, 2009).**The use of microorganisms may be utilized as models of drug metabolism to predict the fate of xenobiotics in mammalian systems **(Smith and Rosazza, 1982),** since this method often gives sufficient quantities of metabolites, complete chemical structure and pharmacological activities could be determined. In our ongoing research on microbial transformation of phenolic compounds **(Hosny *et al.*, 2001),** 2,5-dihydroxycinnamic acid (**1**), was screened using 11 different microorganisms. Of the organisms which effected transformation, scale up studies was carried out with selected cultures to isolate the maximum number of metabolites

in reasonable yields. Structure elucidation of the isolated metabolites and their possible impact on the antioxidant activity in relation to structure are reported herein.

EXPERIMENTAL SECTION

***General Experimental Procedures*:**

Infra-red spectra were recorded using a Bruker Tensor 27 FT-IR (Bruker OpticsGmbH, Ettlingen, Germany) spectrometer with KBr pellets and UV spectra were determined JASCO V-520 UV/VIS spectrophotometer. JEOL NMR spectrometer operating at 500MHz for ^{1}H-NMR spectra were obtained in CDOD or $CDCl_3$ using TMS as an internal standard with the chemical shifts expressed in δ and coupling constants (*J*) in Hertz. El-MS (VG-ZAB-H F), X-mass (158.64, 800.00) (VGA analytical, Inc.). Silica gel column chromatography (CC) was performed on silica gel 60 (E. Merck, Darmstadt, Germany). TLC was carried out on pre-coated silica gel 60 F_{254} (Merck) plates. Developed chromatograms were visualized by spray with 1% vanillin/H_2SO_4, followed by heating at 100^{o}C for 3 min. TLC plates were developed with solvent systems: A (EtOAc:Hexane, 1:1, v:v) or B ($CHCl_3$:MeOH, 8.5:1.5, v:v). 2,5-dihydroxy cinnamic acid (**1**), used in this study was kindly given as a gift from Prof. Mohammed Hosny, Al-Azhar University, Faculty of Pharmacy, Pharmacognosy Department, Cairo, Egypt. The purity of the substrates was confirmed by TLC and ^{1}H-NMR.

Microorganisms:

Cunninghamella elegans (RCMB 012001), *Cunninghamella echinulata* (RCMB 012002), *Mucorrouxii* (RCMB 015004), *Absidia corymbifera* (RCMB 051002), *Penicillium notatum* (RCMB 001023), *Penicillium aurantiogriseum* (RCMB), *Candida albicans* (RCMB 005004), *Rhodotorula glutins* (RCMB 028001), *Rhizopus oryzae* (RCMB 014002), *Aspergillus niger* (RCMB002007(5)001002(2) and *Aspergillus flavus* RCMB002002(3)were obtained from Mycology and Biotechnology Center, Al-Azhar University, Cairo, Egypt.

Analytical-Scale Biotransformation of 2,5dihydroxycinnamic acid (1):

A two-stage fermentation protocol **(Hosny, and Rosazza 1999),** was used for analytical and preparative scale formation of (**1**) metabolites. For screening experiments, solid cultures kept on either potato dextrose agar or sabaraud maltose agar of the following organisms was used: *Cunninghamella elegans* (RCMB 012001), *Cunninghamella echinulata* (RCMB 012002), *Mucorrouxii* (RCMB 015004), *Absidia corymbifera* (RCMB 051002), *Penicillium notatum* (RCMB 001023), *Penicillium aurantiogriseum* (RCMB), *Candida albicans* (RCMB 005004), *Rhodotorula glutins* (RCMB 028001), *Rhizopus oryzae* (RCMB 014002), *Aspergillus niger* (RCMB002007(5)001002(2) and *Aspergillus flavus* RCMB002002(3). Each culture was used separately to inoculate 100 ml flasks containing one fifth of their volume of the following medium: 5% (w/v) soybean meal, 0.5% yeast extract, 0.5% NaCl, 0.5% K_2HPO_4, and 2% dextrose per 1 L of distilled water, adjusted to pH 7.0 with 6 N HCl, was autoclaved at 121^o C for 15 min. Analytical incubations were conducted in 25 mL of sterile medium held in 125 mL stainless steel-capped Delong culture flasks that were incubated for 72 h at 28°C on a rotary shaker operating at 250 rpm. A 10% inoculum derived from 72 h old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h more before receiving 5 mg of **1** in Tween 80-H_2O (0.5 mL; 1:3 V/V), and incubations was continued. Substrate controls consisted of sterile medium and substrate incubated under the same conditions but without microorganism. Samples of 3 mL were withdrawn for analysis at 24, 48, 72, and 144 h after substrate addition, extracted with 1 mL of EtOAc: *n*-BuOH (9:1). The organic layer was separated from aqueous medium by

centrifugation at 1,200 x *g* in a desktop centrifuge and 60 μL samples were spotted onto TLC plate developed with solvent system using CH_2Cl_2 : MeOH : CH_3COOH (15: 0.5: 0.3 ml) as developing solvents. The developed chromatograms were visualized by spraying with vanillin/H_2SO_4, followed by heating with a heating gun until maximum development of the spots color. On the basis of screening experiments, two metabolites were reproducibly formed by *Rhizopus oryzae* (RCMB 014002), and one metabolite formed by *Aspergillus niger* (RCMB002007(5)001002(2) after 72 h.

Preparative Biotransformation of 2,5dihydroxycinnamic acid (1):

Preparative scale transformation of 2,5 dihydroxycinnamic acid *Rhizopus oryzae* (RCMB 014002) and *Aspergillus niger* (RCMB002007(5)001002(2) cultures which gave the best results in screening were incubated separately as before in fifty, 125 mL stainless steel-capped Delong culture flasks, each containing 25 mL of medium,2,5 dihydroxycinnamic acid(**1**) (1.5g, with *Rhizopus oryzae*) and (1g, with *Aspergillus niger*), were dissolved separately in 10 mLDMF,and evenly distributed among the 24h-old stage-II cultures. After 72 h, the contents of 50 flasks were combined and centrifuged at 10,000 x *g* at 4 ˚C for 20 min. The supernatant was extracted with three 500 mL volumes of EtOAc; n-BuOH (9:1 V/V). The organic layer was pooled, dried over anhydrous Na_2SO_4, filtered through sintered glass, and vacuum-concentrated to yield 500 mg and 340 mg of (**1**) with *Rhizopus oryzae* and *Aspergillus niger*, respectively, as viscous brown residues.

Isolation and Purification of the Metabolites

The resulting brown residue from fermentation of 2,5-dihydroxy cinnamic acid (**1**) with *Rhizopus oryzae*(500 mg) was chromatographed over a silica gel TLC first column (1.5 x 100 cm, 100 g) using *n*-hexane gradually enriched with EtOAc (100:0 →0: 100). Three fractions; A (75 mg), B (36 mg) and C (55 mg) were obtained based on TLC analysis; CH_2Cl_2 :MeOH : CH_3COOH (15: 0.5: 0.3 ml) as developing solvents. Fractions A and B were separately re-chromatographed over silica gel column using *n*-hexane: EtOAcgradient solvent system (80:20 → 50:50). Final sample purifications were carried out with Sephadex LH-20 columns eluted with CH_2Cl_2: MeOH (70:30) or (60:40) to afford 2,5-dihydroxy cinnamic acid metabolites **Cin-RM-1** (18 mg) and **Cin-RM-2** (27 mg).

The EtOAc extract from scaling up the reaction of (**1**) with *Aspergillus niger* (340 mg) was chromatographed on a silica gel column (1.5 x 100 cm, 100 g) eluted with mixtures of CH_2Cl_2 and MeOH. Fractions eluted with 3% MeOH in CH_2Cl_2 (52 mg) contained one major spot (R_f 0.55, CH_2Cl_2: MeOH: Acetic acid (15: 0.5: 0.3 ml) and several minors which are more polar. Re-chromatography on silica gel column using the same solvent system afforded metabolite **Cin-AM-3** (16 mg).

Metabolite [Cin-RM-1]: Was obtained as a colorless oily product. It gave a bluish green color with vanillin/sulfuric acid. The molecular formula was determined to be $C_9H_{10}O_3$ by EI-MS *m/z* (rel. int.): 166 $(M)^+$, 150 $(M- OH)^+$, 137 $(M- CH_2OH + H)^+$, 122 (M- 44 $[C_2H_4O$ from side chain$)^+$, 109 (M- 58 $[C_3H_6O$, prop-2-en-1-ol$] + H)^+$; UV λ_{max} (MeOH): 267 and 292 nm, IR (KBr) cm^{-1}: 3350 (-OH), 3030 (=C-H), 1515, 1590 (aromatic C=C), 2870 (CH), 2980, 1550 (C=C), 1430, 1390, 1100 (C-O); ^{1}H-NMR (500 MHz, CD_3OD):δ_H6.85(1H, d, *J* = 8.5 Hz, H-3),7.21 (1H, d, *J* = 2.5 Hz, H-4),7.60(1H, dd, *J* = 8.5, 2.5 Hz, H-6), 6.83(1H, d, *J* = 16.2 Hz, H-7),6.78(1H, ddd, *J* = 16.2, 7.8, 6.7 Hz, H-8), 4.25 (1H, dd, *J* = 11.5, 6.7 Hz, H-9a),3.75 (1H, dd, *J* = 11.5, 6.7 Hz, H-9b).

Metabolite [Cin-RM-2]: Was obtained as a colorless amorphous solid. It gave a blue color with vanillin/sulfuric acid.The molecular formula was determined to be $C_{11}H_{14}O_4$ by EI-MS *m/z* (rel. int.): 210 $(M)^+$, 195 $(M- OH + H)^+$, 175 $(M- 2H_2O + H)^+$, 151 (M- 58 $[C_3H_6O$, prop-2-en-1-ol, side chain$]$ - $H)^+$, 122 (M- 58 $[C_3H_6O$, prop-2-en-1-ol, side chain$]$ – $OCH_3 + H)^+$, 110 $(M- 2 OCH_3 - 2H_2O)^+$; UV λ_{max} (MeOH): 265 and 298 nm, IR (KBr) cm^{-1}:

3330 (-OH), 3020 (=C-H), 1595(aromatic C=C), 2875 (CH), 2980,1560 (C=C), 2820 (OCH_3),1410, 1100 (C-O); ^{1}H NMR (500 MHz, CD_3OD):δ_H6.74 (1H, s, H-3),6.98 (1H, s, H-6), 6.32 (1H, d, *J* = 16.5 Hz, H-7), 6.83 (1H, ddd, *J* = 16.5, 7.8, 5.8 Hz, H-8), 4.19(1H, dd, *J* = 14.2, 4.7 Hz, H-9a), 3.70 (1H, dd, *J* = 14.2, 4.7 Hz, H-9b), 3.77 (3H, s, 4-OCH_3), 3.73 (3H, s, 5-OCH_3).

Metabolite [Cin-AM-3]: Was obtained as a pale yellow amorphous powder. It gave a blue color with vanillin/sulfuric acid.The molecular formula was determined to be $C_{10}H_{10}O_4$ by EI-MS *m/z* (rel. int.): 194 $(M)^+$, 177 $(M- H_2O + H)^+$, 163 $(M- OCH_3 + H)^+$, 148 $(M- OCH_3 - OH)^+$, 134 (M- Carboxylic acid - $OH)^+$, 121 $(M- OCH_3$- Carboxylic acid + $H)^+$, 107 $(M- OCH_3$ – OH - Carboxylic acid$)^+$; UV λ_{max} (MeOH): 325, 290sh, 245 sh, 218 nm, IR (KBr) cm-1: 3450 (carboxylic acid O-H stretching), 1690 (carboxylic acid C=O stretching), 1275 cm-1 (carboxylic acid C-O stretching) 1510; 1605 (aromatic C=C) 2875 (CH), 2820 (OCH_3); ^{1}H-NMR (500 MHz, CD_3OD):δ_H6.85 (1H, d, *J* = 9.0 Hz, H-3),6.85 (1H, d, *J* = 9.0 Hz, H-4), 6.97 (1H, dd, *J* = 2.8, 9.0 Hz, H-6), 7.92 (1H, d, *J* = 16.2 Hz, H-7),6.41 (1H, d, *J* = 16.2 Hz, H-8), 3.85 (3H, s, 5-OCH_3); ^{13}C NMR (100 MHz, CD_3OD): δ_C123.56 (C-1), 150.95 (C-2), 117.98 (C-3), 113.72 (C-4), 152.09 (C-5), 112.60 (C-6), 140.07 (C-7), 118.29 (C-8), 56.50 (OCH^3-5).

In-Vitro Biological Evaluation:

Antioxidant activity: The antioxidant activity of 2,5dihydroxycinnamic acid (**1**) and its metabolites[**Cin-M-1-M-3]**, were analyzed using two different techniques; DPPH radical scavenging activity and $FeSO_4/H_2O_2$-stimulated lipid peroxidation in rat tissue homogenate.

Chemicals: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylatedhydroxytoluene (BHT), 2-thiobarbituric acid, ferrous sulphate, hydrogen peroxide, were purchased from Sigma Chemical Company (St. louis, Mo, USA).

Animals: Male Westar rats (250–300 g) were handled according to international regulations. They were allowed to take standard laboratory diet and water ad libitum, and the animals were maintained at 24 °C with 12 h light period.

A. DPPH radical scavenging activity

The ability of the extracts to scavenge free radicals was determined according to the method of De La Torre Boronat and Lopez Tamames (1997). In a 96-well plate, 10 µL of each sample or standard dissolved in ethanol (100 µg/mL) was added to 190 µL of 316 µM/mL DPPH solution. A blank was prepared using ethanol. After incubation at 30 °C for 30 min, the absorbance of each solution was measured at 517 nm. DL-α-tocopherol and BHT were used as positive controls. The scavenging activity of the samples was calculated as a percentage of free radical inhibition according to the formula:

$$\% \text{ inhibition} = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100$$

Where ^{A}blank is the absorbance of the blank at zero time and ^{A}sample is the absorbance of the sample after 30 min. All experiments were carried out in triplicate.

B. $FeSO_4/H_2O_2$-stimulated lipid peroxidation in rat tissue homogenate (Cesar *et al.*, 1988; Taha *et al.*, 2011).

Male Westar rats (250–300 g) were sacrificed, and the rat tissues (brain, heart and liver: 0.3–0.5 g) were rapidly removed and homogenized in 10 volumes of 15 mM Krebs buffer. Homogenates were centrifuged at 3000 x for 10 minutes at 4 °C to give supernatants

containing (1.2 mg of protein/ ml; brain), (1.7 mg of protein/ ml; heart) and (2.5 mg of protein/ ml; liver) using Coomassie plus protein assay reagent and albumin standard as determined by the **Bradford method** (**1976**). During aerobic incubation of the tissue homogenates, MDA released reacts with thiobarbituric acid (TBA) to give a pink color. The capability of the samples to inhibit MDA formation is used as a measure of their antioxidant activity. The pink color complex of thiobarbituric acid reacting substance (TBARS) is measured at 532 nm for the test samples and positive standards (DL-α- tocopherol and BHT) (200 μg/mL), as well as, 2,5dihydroxycinnamic acid (**1**) and their metabolites (**Cin-M-1-M-3**, 100 μg/mL). The results were expressed as nano-moles of MDA equivalents per milligram of protein of rat (brain, heart and liver) homogenates. All measurements were done in triplicate. The capability to inhibit MDA formation was calculated using the following equation:

$$\textbf{Inhibition effects (\%)} = 1 - \frac{\textbf{MDA in tissue homogenate with test extracts}}{\textbf{MDA in tissue homogenate without test extracts}} \times 100$$

Statistical Analysis

All data were expressed as mean ± SE. Student's t-test [33] was applied for detecting the significance of difference between each sample; $P < 0.05$ was taken as the level of significance.

RESULTS AND DISCUSSION

A- Structure Elucidation of isolated metabolites

Of 11 microorganisms screened for their abilities to catalyze the bioconversion of 2,5-dihydroxy cinnamic acid (**1**), *Rhizopus oryzae* (RCMB 014002) and *Aspergillus niger* (RCMB002007 (5)001002(2) reproducibly formed after 72 h of incubation two major metabolites [**Cin-RM-1** and **Cin-RM-2**] from *Rhizopus oryzae* and [**Cin-AM-3**] from *Aspergillus niger* in good yield. None of the observed metabolites were formed in control cultures or in media containing no microorganisms but incubated under the same conditions. Following solvent extraction and column chromatographic purification, samples of metabolites were subjected to spectral analysis. Spectra (UV, IR, NMR, and mass spectrometry) for isolated metabolites were established by comparing their spectral data to those given in the literature.

Metabolite (Cin-RM-1): was obtained as a colorless oily product. It gave a bluish green color with vanillin/sulfuric acid. The UV spectrum recorded in MeOH showed absorption maxima attributable to a conjugated aromatic ring at λ_{max} 267 and 292 nm. The IR spectrum showed characteristic bands accounting for a phenolic group at 3350cm^{-1} (-OH), 3030cm^{-1} (=C-H), 1515, 1590cm^{-1} (aromatic C=C), 2870cm^{-1} (CH), 2980,1550cm^{-1} (C=C), 1430, 1390, 1100cm^{-1} (C-O). Addetionally, in IR spectrum, peak at 1680 cm^{-1}(C=O) in (**1**) was disappeared and a broad peak appeared at 3350 cm^{-1}(OH). The empirical formula was determined by accurate mass measurement as $C_9H_{10}O_3$based on the molecular ion base peak atm/z 166 $(M)^+$ with 14 mass unit lower than of parent molecule (substrate, 180 mass unit), It also showed fragment ions at m/z 150 $(M- OH)^+$, 137 $(M- CH_2OH + H)^+$, 122 (M- 44 [C_2H_4O from side chain)$^+$ and 109 (M- 58 [C_3H_6O, prop-2-en-1-ol] + H)$^+$by EI-MS.

The^{1}H-NMR spectrum of metabolite **(Cin-RM-1)** displayed a typical signals of resonances that was associated with a 2,5-disubstituted (*E*)-cinnamyl alcoholunit; three aromatic proton signals as an ABX spin-system at δ_H 6.85 (1H, d, J = 8.5 Hz, H-3), 7.21 (1H,

d, *J* = 2.5 Hz, H-4), 7.60 (1H, dd, *J* = 8.5, 2.5 Hz, H-6),indicating the presence of a tri-substituted aromatic ring in the molecule. Two olefinic protons as an AB spin-system at δ_H6.83 (1H, d, *J* =16.2 Hz, H-7) and 6.78(1H, ddd, *J* = 16.2, 7.8, 6.7 Hz, H-8), coupling constant 16.2 Hz between olefinic protons H-7 and H-8 suggests clearly them being in*trans* geometry to each other's. Additionally, signals for a methylene group bearing a secondary hydroxyl as doublet of doublet at δ_H4.25 (1H, dd, *J* = 11.5, 6.7 Hz, H9a) and 3.75 (1H, dd, *J* = 11.5, 6.7 Hz, H9b) were also observed. On the basis of these spectral data it was suggested that the carbonyl group of 2,5-dihydroxy cinnamic acid (substrate) was reduced to alcohol functional group. Therefore, the structure of metabolite **[Cin-RM-1]** was identified as 2,5-dihydroxy cinnamoyl alcohol.

Metabolites (Cin-RM-2): was obtained as a colorless amorphous solid. It gave a blue color with vanillin/sulfuric acid. The UV spectrum recorded in MeOH showed absorption maxima attributable to a conjugated aromatic ring at λ_{max}265 and 298 nm. The IRspectrum displayed characteristic absorption bands at 3330 $cm^{-1}$for hydroxyl group. The absorption band near 1595, 1504, 1465 $cm^{-1}$is for aromatic C=C stretching vibration and the band at 2875 $cm^{-1}$is for C-H bending vibration of aromatic compound. The absorption band at 2980, 1560 $cm^{-1}$for -C=C- stretching. The bands at 2820 and 1176-1323 cm^{-1}showed presence of methoxy group (–C-O stretching frequency) in the molecule. The empirical formula was determined by accurate mass measurement as $C_{11}H_{14}O_4$based on the molecular ion base peak atm/z 210 $(M)^+$ with 30 mass unit over that of substrate (180 mass unit) and 44 mass unit over that of metabolite **(Cin-RM-1)** (166 mass unit). It also showed fragment ion peaks at m/z 195 $(M- OH + H)^+$, 175 $(M- 2H_2O + H)^+$, 151 (M- 58 [C_3H_6O, prop-2-en-1-ol, side chain] - $H)^+$, 122 (M- 58 [C_3H_6O, prop-2-en-1-ol, side chain] – $OCH_3 + H)^+$, 110 $(M- 2 OCH_3 - 2H_2O)^+$, by EI-MS.

The^{1}H-NMR spectrum of [**Metabolite-RM-2**], displayed two *meta*-coupled singlets each for 1H, at δ_H6.74 (H-3) and δ_H6.98 (H6)in the aromatic region indicating the presence of a tetra-substituted aromatic ring in the molecule. It also displayed a typical signals of resonances atδ_H6.32 (1H, d, *J* =16.5 Hz, H-7), 6.83 (1H, ddd, *J* = 16.5, 7.8, 5.8 Hz, H-8), 4.19 (1H, dd, *J* = 14.2, 4.7 Hz, H9a) and 3.70 (1H, dd, *J* = 14.2, 4.7 Hz, H9b). Coupling constant 16.5 Hz between olefinic protons H-7 and H-8 suggests clearly them being in*trans* geometry to each other's.Additionally, two sharp proton signals for two OCH_3groups at δ_H3.77 (3H, s, 4-OCH_3) and 3.72(3H, s, 5-OCH_3) were also observed.

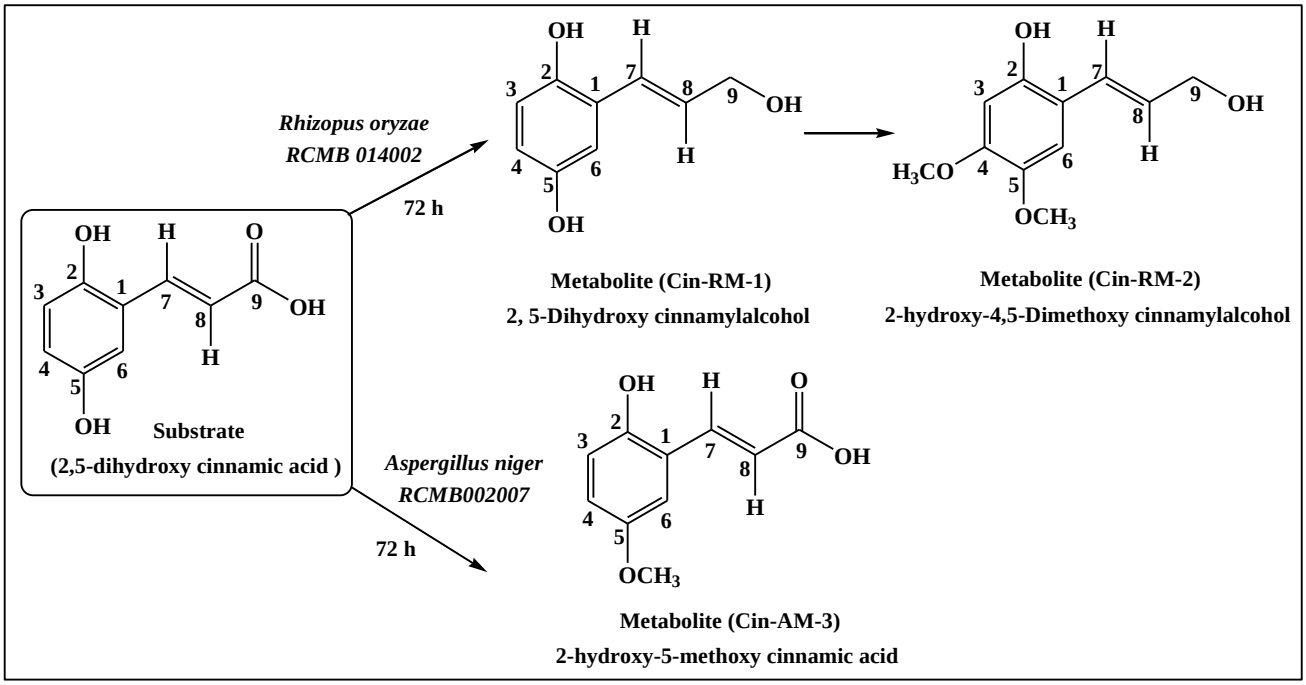
Therefore, the structure of metabolite **[Cin-RM-2]** was identified as **2-Hydroxy-4,5-Dimethoxy cinnamoyl alcohol**.

Metabolite [Cin-AM-3]: was obtained as pale yellow amorphous powder. It gave a blue color with vanillin/sulfuric acid. Compound **[Cin-AM-3]** exhibited UV and IR absorptions confirming its phenolic nature (325, 290sh, 245 sh, 218 nm), [3450cm^{-1} (carboxylic acid O-H stretching), 1690cm^{-1} (carboxylic acid C=O stretching), 1275 cm-1 (carboxylic acid C-O stretching) 1510; 1605cm^{-1} (aromatic C=C),2875cm^{-1} (CH) and 2820cm^{-1} (OCH_3).

The molecular formula was determined as $C_{10}H_{10}O_4$on the basis of the molecular ion peaks observed at m/z 194 $(M)^+$, 177 $(M- H_2O + H)^+$, 163 $(M- OCH_3 + H)^+$, 148 $(M- OCH_3 - OH)^+$, 134 (M- carboxylic acid - $OH)^+$, 121 $(M- OCH_3$- carboxylic acid + $H)^+$, 107 $(M- OCH_3$ – OH -carboxylic acid$)^+$by EI-MS. This formula was in good agreement with the ^{13}C-NMR spectrum, which showed 10 signals containing five methines, one methoxyl and four quaternary carbons.The molecular ion peak atm/z 194 $(M)^+$ with 14 mass unit over that of substrate and the ^{1}H- and ^{13}C-NMR spectra of **[Cin-AM-3]**revealed that this metabolite had the same carbon skeleton as **[substrate]**, the only difference **[Cin-AM-3]** possessed one methoxyl group versus a hydroxyl group in **[substrate]**.

The ^{1}H- and ^{13}C-NMR spectra of **[Cin-AM-3]**, exhibited three aromatic proton and carbonsignals as an ABX spin-systemat δ_H6.85 (1H, d, *J* = 9.0 Hz, H-3, δ_C117.98), 6.81 (1H, d, *J* = 2.8 Hz, H-4, δ_C113.72) and 6.97 (1H, dd, *J* = 2.8, 9.0 Hz, H-6, δ_C 112.60), indicating the presence of a trisubstituted aromatic ring. Two olefinic protons and carbons as an AB spin-system at δ_H7.92 (1H, d, *J* = 16.2 Hz, H-7, δ_C140.07) and 6.41 (1H, d, *J* = 16.2 Hz, H-8, δ_C118.29), the large value of coupling constant (16.2 Hz) indicated the presence of *trans*-disubstituted ethylene moiety in the molecule. The downfield signal for three hydrogens at δ_H 3.85,δ_C 56.50) indicates that methyl group is attached to electron withdrawing oxygen atom of OCH_3 group at C-5. The^{13}C chemical shifts of a carbon at δ_C 169.78 indicated the presence of carboxylic functional group in the molecule. The upfield chemical shifts of one of the ethyleniccarbon (C-8) and a quaternary carbon at δ_C 123.56 (C-1) indicated that the carboxylic group is located at C-8 position. The^{13}C-chemical shifts of carbon atoms at δ_C152.09 (C-5) versus 150.39 (C-5) in2,5-dihydroxy cinnamic acid, indicated that the methoxyl group was attached at C-5 position.

Therefore, the structure of metabolite **[Cin-AM-3]** was identified as **2-hydroxy-5-methoxy cinnamic acid**.



(Figure 1) Structure of 2,5-dihydroxy cinnamic acid (1) and its metabolites**.**

CONCLUSION

The first step of the 2,5-dihydroxycinnamic acid pathway by *Rhizopus oryzae* is the conversion of 2,5-dihydroxycinnamic acid to 2,5-dihydroxycinnamic acid-CoA, which is catalyzed by cinnamoyl-CoA reductase **(Kato *et al.*, 1991).** The subsequent reduction by cinnamyl alcohol dehydrogenase leads to the intermediate 2,5-dihydroxycinnamaldehyde followed by 2, 5-dihydroxy cinnamyl-alcohol [**Cin-RM-1**], (**Figure 2**). The biosynthetic pathway of 2-hydroxy-4,5-dimethoxy cinnamyl-alcohol [**Cin-RM-2**], involves a methylation of the hydroxyl group at C-5 position of metabolite (**Cin-RM-1**), by cinnamoyl-*O*-methyltransferase (COMT), followed by hydroxylation of the aromatic C-4 position of the intermediate 2-hydroxy-5-methoxy cinnamylalcohol by cinnamyl-4-hydroxylase. Further

Methylation of this hydroxyl group by *O*-methyltransferase (COMT) **(Maria -Joao *et al.*, 2007),** leads to 2-hydroxy-4,5-Dimethoxy cinnamylalcohol [**Cin-RM-2**].

For the isolated metabolite (**Cin-AM-3**) by *Aspergillus niger*, 2,5-dihydroxycinnamic acid*O*-methyltransferase (COMT)catalyzes preferentially the methylation of the hydroxyl group at C-5 position of the substrate leads to 2-hydroxy-5-methoxy cinnamic acid (**Cin-AM-3**), (**Figure 2**).

B- Results of Biological evaluation (Antioxidant Activities)

B.1- Assay for DPPH free radical scavenging activity

2,2-Diphenyl-1-picryl hydrazyl (DPPH) is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Kuo *et al.* (1999)). The DPPH radical is considered to be a model of a lipophilic radical. A chain reaction in lipophilic radicals was initiated by the lipid autoxidation. The purpose of this study was to evaluate the antioxidant activites of the substrate; 2,5-dihydroxy cinnamic acid and their isolated metabolites obtained by *Rhizopus oryzae* and *Aspergillus niger* cultures fungi as new potential sources of natural antioxidants. Well known antioxidant D, L-α-tocopherol and butylated-hydroxyl toluene(BHT) were used for comparison.The scavenging effects of substrate, metabolites and positive controls D, L, α-tocopherol and BHT on DPPH radical are compared and shown in (**Table 1**). It was observed that 2,5-dihydroxycinnamic acid showed DPPH quenching with IC_{50} values at 51.3%. Metabolite; **Cin-RM-2** (2-hydroxy-4,5-Dimethoxy cinnamylalcohol), showed the highest activity in DPPHradica quenching (63.4%), than those of such typical antioxidants D, L, α-tocopherol (62.8%) and BHT (50.2%) respectively. **Cin-AM-3**(2-hydroxy-5-methoxy cinnamic acid) [54.2%], **Cin-RM-1** (2, 5-Dihydroxy cinnamylalcohol) [53.5%], showed slightly less DPPH radical quenching than D,L, α-tocopherol but its more than BHT.

In conclusion, the results obtained with isolated metabolites have indicated that scavenging effects is dependent on their chemical structure and thought to be due to their hydrogen donating activity. In general phenolic OH is known as scavenger of free radicals and it consequently exhibits antioxidative activity **(Hosny, and Rosazza, 2002).** Especially, in regards to substitution on the phenyl ring several studies have reported that the existence of an electron donating group such as methoxyl substitution as with several metabolites obtained in this study enhances antioxidant effectiveness **(Hosny, and Rosazza, 2002),** claimed that the phenolic group is essential for the free-radical-scavenging activity and that the presence of the methoxy group further increased the activity.

Table (1) Effects of substrates, metabolites and positive controls on the *in vitro* Free Radical Generation.

Bioassay	**DPPH % decoloration**
Substrate	
2,5-dihydroxy-cinnamic acid	**51.3 ± 1.30**

Metabolite	
Cin-RM-1	**53.5± 1.40**
Cin-RM-2	**63.4± 1.50**
Cin-AM-3	**54.2± 1.40**
Positive control	
D,L, α-tocopherol	**62.8± 1.50**
BHT	**50.2± 1.30**

Values are presented as mean ± SE of 3-test sample observation.$P< 0.05$for all values.

B.2- Ferrous sulphate-H2O2-stimulated lipid peroxidation in rat tissue homogenate.

Lipid peroxidation is a free radical mediated process which has been implicated in a variety of disease states. It involves the formation and propagation of lipid radicals, uptake of oxygen, a re-arrangement of the double and unsaturated lipids that results in a variety of degraded products (e.g., alkenes, malondialdehyde (MDA), lipid hydroperoxides and conjugated dienes) that eventually causes destruction of membrane lipids. Thus lipid peroxidation and conjugated diene measurement plays important role along with MDA assay **(Halliwell and Chirico, 1993)**. The increased peroxidation can result in changes in cellular metabolism of the hepatic and extra-hepatic tissues. Increase in accumulation of MDA, conjugated diene and hydro-peroxides in cells can result in cellular dehydration and whole cell deformity and death **(Halliwell and Chirico, 1993).** Itis well known that defense mechanism in liver, kidney, heart, brain and lungs are prone to oxidative damage. Alteration of fatty acid composition by increased lipid levels may contribute for lowering the resistance of tissues and higher rate of oxidative stress.

B.2- Ferrous sulphate-H2O2-stimulated lipid peroxidation in rat tissue homogenate.

There is good evidence that superoxidedismutase (SOD) and catalase are enzymes that scavenge free radicals during lipid peroxidation. The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reaction and improve the quality and stability of food products **(Halliwell and Chirico, 1993).**

For rat tissue homogenate (brain, heart and liver), the unstimulated control experiments the amount of thiobarbituric reactive substance (TBARS) [MDA levels without $FeSO_4$-H_2O_2] formed in rat tissue homogenate (brain, heart and liver) were (0.36 ± 0.15 nmol, MDA/mg protein), (0.22 ± 0.10 nmol, MDA/mg protein) and (0.16 ± 0.05 nmol, MDA/mg protein), respectively. After induction with 200 μm Fe^{2+}-H_2O_2, The amount of TBARS increased to (0.72 ± 1.30 nmol, MDA/mg protein), 0.65 ± 1.25 nmol, MDA/mg protein) and (0.44 ±1.15 nmol, MDA/mg protein) of brain, heart and liver, respectively (**Table 2**).

A control experiment indicated that substrates and isolated fungal metabolites did not effect the measurement of TBARS because the absorbance at 532 nm was not effected by adding different substrates and isolated fungal metabolites to the rat tissue homogenate that already have been oxidatively modified because omission of rat homogenate from the reaction mixture abolished chromogen formation. D, L-α-tochopherol and BHT also inhibited this Fe^{2+}-induced lipid peroxidation with IC_{50} values in the range of (28.23 – 35.10%), (22.05 – 38.70%) and (33.15 – 46.18%) in heart, brain and liver rat tissue homogenates, respectively. However, as shown in (**Table 2**), adding 200-500 μg/mL of substrateson rat tissue homogenates (brain, heart and liver), reduce MDA formation in the presence of Fe^{2+}-$H_2O_2$with IC_{50} values (22.10%,10.20% and 32.25%)in heart, brain and liver rat tissue homogenatesrespectively, indicating lower anti-lipid peroxidation activities of substrates. The results obtained with the fungal metabolites; **Cin-RM-1, Cin-RM-2 and Cin-AM-3** showedlow radical quenching for all tissue homogenate than those of such typical antioxidants D, L, α-tocopherolbut its more than BHT (**Table 2**).

Our results clearly showed that lipid peroxidation in rat tissue homogenates (brain, heart and liver) induced by ferrous ion/H_2O_2 as measured by MDA formation, was slightly inhibited by substrate and their metabolites. Since D,L,α-tocopherol is thought to be associated with lipid-rich membranes, it is anti-oxidative is highly effective in protecting membranes against lipid peroxidation, as peroxyl and alkoxyl radicals. The data obtained from the present study indicates that the isolated metabolites hasanti-lipid peroxidative character with similar reaction mechanisms to those of D,L,α-tocopherol and BHT for rat liver tissue homogenates.

Table (2) Inhibition Effect of substrates, metabolites and positive controls on $FeSO_4$-H_2O_2 Induced Lipid Peroxidation (MDA production)in Rat Tissue Homogenate.

Bioassay	**Inhibition effect (%)***		
	Brain	**Heart**	**Liver**
Normal control without $FeSO_4$-H_2O_2 (MDA level)	**0.36± 0.15**	**0.22± 0.10**	**0.16± 0.05**
Induction by $FeSO_4$-H_2O_2 (MDA level)	**0.72±1.30**	**0.65±1.25**	**0.44 ±1.15**
Substrates			
2,5-dihydroxy-cinnamicacid	22.10 ± 1.15	10.20±0.08	32.25 ± 1.20
Metabolites			
Cin-RM-1	19.75±1.15	21.40±1.15	30.50 ± 1.20
Cin-RM-2	27.55±1.20	16.95±1.15	**42.50 ± 1.40**
Cin-AM-3	29.15 ± 1.25	14.70± 1.10	**44.28 ± 1.40**
Positive controls			
D,L, α-tocopherol	**35.10 ± 1.30**	**38.70 ± 1.30**	**46.18± 1.50**
BHT	**28.23± 1. 25**	**22.05 ± 1.20**	**33.15 ± 1.30**

* Values are presented as mean ± SE of 3-test sample observation. $P< 0.05$ for all values.

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التحول الميكروبي لحمض 2و5 ثنائي هيدروكسي السينناميك بواسطة فطر *الأسبيراجيللس نيجر* و *الريزوبس اوريزا*

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عند تخمر حمض 2و5 ثنائي هيدروكسي السينناميك بالمزرعة الفطرية *ريزوبس اوريزا*، أعطى أثنان من المواد الأيضية 2و5 ثنائي هيدروكسي سيناموبل الكحول ، 2-هيدروكسي-4و5–ثنائي ميثوكسي سيناموبل الكحول. علاوة على ذلك، فطر *الأسبيراجيللس* نيجر قام بتحويل هذه المادة الى مادة أيضية رئيسية، 2-هيدروكسي-5-ميثوكسي حمض السينناميك. تم توضيح تراكيب المواد الأيضية التي أنتجت بواسطة هذه الفطريات عن طريق وسائل البيانات الطيفية. تم فحص أهمية هذه المواد الأيضية كمضدات للأكسدة بواسطة الماسح الأشعاعي دي بي بي اتش وتم فحص تأكسد الدهون بواسطة طريقة مواد التفاعل الحمضية ثيوباربيتيويرك باستخدام أنسجة الفئران المطحونة بالنسبة لتركيبها.