CAFFEIC ACID METABOLISM BY *ABSIDIA CORYMBIFERA*

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ABSTRACT

Biocatalytic processes may offer a cheaper alternative to natural production of valuable compounds. Caffeic acid [3,4-dihydroxycinnamic acid] (1) metabolism was studied using for the first time the fungus *Absidia corymbifera*a cosmopolitan filamentous phytopathogenic fungus as a biocatalyst. Results show that caffeic acid is converted to Ferulic acid [Caff-AM-1], 3,4-dimethoxy-cinnamic acid[Caff-AM-2] and 4-hydroxycinnamyl alcohol [Caff-AM-3]. The structures of the metabolic products were elucidated on the basis of their spectral data. A possible metabolic pathway of the biotransformation and the antioxidant activity using DPPH radical scavenging assay and lipid peroxidation assay by thiobarbituric acid reactive substances (TBARS) method using rat tissue homogenates is also discussed.

Key words: Biotransformation, caffeic acid, Absidia corymbifera, metabolic pathway, antioxidant activity.

INTRODUCTION

Biocatalysis or biotransformation encompasses the use of biological systems to catalyze the conversion of one compound to another. The catalyst part can thereby consist of wholecells, cellular extracts, or isolated enzyme (s). If the conversion is developed by a free and/or immobilized enzyme, it means biocatalysts, but if these transformations take place by the wholecell (with the correct enzyme) we talk about biotransformation **(Leresche and Meyer, 2006).** Although the currentinterest in applying biotransformation in organic synthesis is mainly related to the preparation of enantiopure compounds, these can also use to perform transformations of chiral functional groups. The reason is that biotransformations are 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 **(Luna, 2004).** Therefore, biotransformations attract considerable attention due to its simple, cheap and benign methodologies that combines green chemistry with high efficiency **(Faber and Patel, 2000).** Besides, biotransformation experiments using phytopathogenic fungi provide information on the detoxification mechanism used by these microorganisms and give an indication of the structural modifications that may be necessary if substrates of this type are to be further developed as selective fungal control agents **(Daoubi *et al.*, 2005).**

On the other hand, cinnamic acid derivatives such as caffeic acid (3,4-dihydroxycinnamic acid) can potentially serve as a good source of starting material for the production of value-added compounds. Several studies have demonstrated that valuable aroma, flavoring compounds, and pharmaceutical intermediates, are produced as intermediates in the degradation pathways of such cinnamic acid derivatives **(Velasco *et al.*, 2010).** Thus, biotransformation of these compounds seems to be a reasonable alternative to produce raw materials for different industries. Also, products of such bioconversions are

considered natural **(Shimoni *et al.*, 2000),** which gives them better perspectives of use than synthetic counterparts.

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 **(Miles *et al.*, 2005).** Caffeic acid possesses a wide spectrum of biological activities, e.g. anti-cancer, anti-oxidant, anti-angiogenic, anti-inflammatory antinociceptive, anti-hyperglycemic and hepato-protective properties **(Gulçin, 2006; Mateos *et al.*, 2006; Un-Ju *et al.*, 2006; Jung *et al.*, (2007; Jeong Hun *et al.*, 2009; Soo-Hyun *et al.*, 2011)**. Based on the anti-oxidant and anti-angiogenic effects of caffeic acid, retinal neovascularization of retinopathy of prematurity could be a target for the pharmacological application of caffeic acid. Microbial transformations of caffeic acid by bacteria and fungi conducted so far have focused largely on the identification of metabolic pathways and routes of degradation **(Whiting and Carr, 1959; Samejima *et al.*, 1987; Robert *et al.*, 1988; Defnoun *et al.*, 2000; Toshiki *et al.*, 2012).** Caffeic acid is sequentially degraded to Protocatechuic acid, 4-vinylcatechol, vanillyl alcohol, vanillic acid, dihydro-caffeic acid, 4-ethylcatechol, m-hydroxy-phenylpropionic acid and 3,4-dihydroxyphenylpropionic acid by bacteria and fungi **(Whiting and Carr, 1959; Samejima *et al.*, 1987; Robert *et al.*, 1988; Defnoun *et al.*, 2000; Toshiki *et al.*, 2012).** This paper reports for the first time the capability of the fungus *Absidia corymbifera*, a cosmopolitan filamentous phytopathogenic fungus to biotransform caffeic acid into value-added products. A possible metabolic pathway of the biotransformation and the antioxidant activity is also discussed.

EXPERIMENTAL SECTION

General Experimental Procedures:

Infra-red spectra were recorded using a Bruker Tensor 27 FT-IR (BrukerOpticsGmbH, 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 $CDCl_3$usingTMS 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.).Silicagel column chromatography (CC) was performed on silica gel 60 (E. Merck, Darmstadt, Germany). TLC was carried out on precoated 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). Caffeic acid (**1**), used in this study was 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 Caffeic acid(1):

A two-stage fermentation protocol (Hosny and John (**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 0.5 ml DMF 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, three metabolites were reproducibly formed by *Absidia corymbifera* (RCMB 051002) after 144 h.

Preparative Biotransformation of caffeic acid(1):

Preparative scale transformation of Caffeic acid by *Absidia corymbifera* (RCMB 051002) cultures which gave the best results in screening were incubated as before in fifty, 125 mL stainless steel-capped Delong culture flasks, each containing 25 mL of medium. Caffeic acid (**1**), 870 mg was dissolved in 10 mLDMF, and evenly distributed among the 24h-old stage-II cultures. After 144 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 820 mg of (**1**), as viscous brown residue.

Isolation and Purification of the Metabolites:

The resulting brown residue from fermentation of caffeic acid (**1**) with *Absidia corymbifera*(820 mg) was chromatographed over a silica gel column (1.5 x 100 cm, 100 g) using *n*-hexane gradually enriched with EtOAc (100:0 →0: 100). Fifty millilitre fractions were collected and similar fractions pooled and monitored by TLC, [CH_2Cl_2: MeOH: Acetic acid (15: 0.5: 0.3 ml)], using vanillin/H_2SO_4 as spray reagent to afford three major groups (A–C). Group A (fractions 18–30, 105 mg) was further subjected to CC on silica gel (1.5 x 50 cm, 50 g), isocratically eluted with 5% EtOAc in hexane. Ten millilitre fractions were collected to afford metabolite (**Caff-AM-1**) [24 mg, R_f 0.63, CH_2Cl_2: MeOH: Acetic acid (15: 0.5: 0.3 ml].Group B (fractions 32–42, 80 mg) was similarly treated as above using 8% EtOAc in hexane to afford 20 mg of metabolite **Caff-AM-2** [R_f, 0.56, CH_2Cl_2: MeOH:

Acetic acid (15: 0.5: 0.3 ml].Group C (fractions 44-48, 55 mg) was purified on a silica gel column and eluted with *n*-hexane gradually enriched with EtOAc (90:10 → 80: 20) to afford a one spot (R_f, 0.50, CH_2Cl_2: MeOH: Acetic acid (15: 0.5: 0.3 ml, 26 mg). It was re-chromatographed over Sephadex LH-20 column for final purification eluted with CH_2Cl_2: MeOH: (70:30) to afford caffeic acid metabolite (**Caff-AM-3**, 12 mg).

Metabolite [Caff-AM-1]: was obtained as 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 $(M)^+$, 167 $(M- COOH +H_2O)^+$, 152 $(M- COOH - OH + H_2O +H)^+$, 138 $(M- COOH – OH - CH_3+ H_2O + H)^+$, 123 (M- propenoic acid [$C_3H_4O_2$, side chain] + $H)^+$, 110 (M- propenoic acid [$C_3H_4O_2$, side chain] – CH_3+ $2H)^+$ and 91 (M-propenoic acid [$C_3H_4O_2$, side chain] – $OCH_3)^+$; UV λ_{max} (MeOH): 325, 290 sh, 245 sh, 218 nm, IR (KBr) cm^{-1}: 3435 (OH), 1690 (C=O), 1575 (aromatic ring),1620 (C=C), 1515 (-C-O stretching), 852,802,2875 (CH), 1175-1325 (OCH_3); ^{1}H-NMR (500 MHz, CD_3OD): δ_H 6.43 (1H, d, *J* = 2.8 Hz, H-2), 6.75 (1H, d, *J* = 8.6 Hz, H-5), 6.17 (1H, dd, *J* = 8.6, 2.8 Hz, H-6), 7.26 (1H, d, *J* = 15.6 Hz, H-7), 6.26 (1H, d, *J* = 15.6 Hz, H-8), 3.81 (3H, s, OCH_3-3)

***Metabolite [Caff-AM-2]*:** was obtained as pale yellow amorphous powder. It gave a dark 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.): 209 $(M - H)^+$, 192 $(M + H - H_2O)^+$, 165 $(M + H - COOH)^+$, 152 $(M + H – COOH- CH_3)^+$, 137 (M- propenoic acid $(C_3H_4O_2$, side chain$)^+$, 124 (M +H- propenoic acid [$C_3H_4O_2$, side chain] $–CH_3)^+$, and 109 (M + H- propenoic acid [$C_3H_4O_2$, side chain] – $2CH_3)^+$; UV λ_{max} (MeOH): 285, 250, 325, IR (KBr) cm^{-1}: 3425 (OH), 1680 (C=O), 1570 (aromatic ring),1630(C=C), 1510 (-C-O stretching),1180 (OH), 850,810,2850 (CH), 1170-1345 (OCH_3); ^{1}H NMR (500 MHz, CD_3OD):δ_H6.52 (1H, d, *J* = 2.5 Hz, H-2), 6.94 (1H, d, *J* = 8.6 Hz, H-5), 7.12 (1H, dd, *J* = 8.6, 2.5 Hz, H-6), 7.54 (1H, d, *J* = 16.2 Hz, H-7), 6.39 (1H, d, *J* = 16.2 Hz, H-8), 3.86 (3H, s, OCH_3-3), 3.83 (3H, s, OCH_3-4)

***Metabolite [Caff-AM-2]*:** was obtained as a colorless powder. It gave a dark blue color with vanillin/sulfuric acid. The molecular formula was determined to be $C_9H_{10}O_2$ by EI-MS *m/z* (rel. int.): 150 $(M)^+$,112 $(M -2H-2H_2O)^+$, 90 (M - prop-2-en-1-ol [C_3H_6O, side chain] - $2H)^+$,90 $(M - CH_2OH - OH)^+$.; UV λ_{max} (MeOH): 265, 315 nm, IR (KBr) cm_{-1}: 3510 (OH), 1680 (C=O), 1545 (aromatic ring), 1640(C=C), 1520 (-C-O stretching), 1180 (OH), 855,815,2875 (CH); ^{1}H-NMR (500 MHz, CD_3OD): δ_H 7.18 (2H, d, *J* = 8.6 Hz, H-2, 6), 6.94 (2H, d, *J* = 8.6 Hz, H-3, 5), 6.58 (1H, d, *J* = 16.5 Hz, H-7), 7.57 (1H, dt, *J* = 16.5, 6.5 Hz, H-8), 4.73 (1H, dd, *J* = 13.5, 6.5 Hz, H-9a), 4.81 (1H, dd,*J* = 13.5,6.5 Hz, H-9b)

In-Vitro Biological Evaluation:

***Antioxidant activity*:** The antioxidant activity of 2,5dihydroxycinnamic acid(1) and its metabolites[Caff-AM-1-AM-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 LopezTamames** (**1997**). In a 96-well plate, 10 µL of

each sample or standard dissolved in ethanol (100μg/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 (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 **(Bradford, 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, caffeic acid (1) and their metabolites (Caff-AM-1-AM-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:

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

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 caffeic acid (1), *Absidia corymbifera* (RCMB 051002) reproducibly formed after 144 h of incubation three major metabolites [Caff-AM-1-AM-3]. 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 [Caff-AM-1]

Was obtained as pale yellow amorphous powder. It gave a blue color with vanillin/sulfuric acid. [Caff-AM-1] exhibited UV absorptions confirming its phenolic nature at (325, 290sh, 245 sh, 218 nm). The IRspectrum exhibited sharp absorption band at 3450 $cm^{-1}$for OH group of carboxylic acid, an absorption band at 1690 $cm^{-1}$for –C=O of carboxylic acid and an absorption band near 1620 $cm^{-1}$for -C=C- stretching. The band at 1175-1325 cm^{-1} showed presence of methoxy group (–C-O stretching frequency).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 $(M)^+$, 167 $(M- COOH + H_2O)^+$, 152 $(M- COOH - OH + H_2O + H)^+$, 138 $(M- COOH – OH - CH_3+ H_2O + H)^+$, 123 (M- propenoic acid [$C_3H_4O_2$, side chain] + $H)^+$, 110 (M- propenoic acid [$C_3H_4O_2$, side chain] – CH_3 + $2H)^+$ and 91 (M- propenoic acid [$C_3H_4O_2$, side chain] – $OCH_3)^+$by EI-MS.

The ^{1}H-NMR spectrum of [Caff-AM-1], exhibited three aromatic proton signals as an ABX Spin-system at δ_H6.43 (1H, d, *J* = 2.8 Hz, H-2), 6.75 (1H, d, *J* = 8.6 Hz, H-5) and6.17 (1H, dd, *J* = 8.6, 2.8 Hz, H-6), indicating the presence of a tri-substituted aromatic ring in the molecule. The^{1}H-NMR spectrum also displayed two doublet protons as an AB spin-system (*J* = 15.6 Hz), each for 1H, at δ_H7.26 (H-7) and 6.26 (H-8).The large value of coupling constant indicated the presence of *trans*-disubstituted ethylene moiety in the molecule. The downfield signal for three hydrogens at δ_H 3.81 indicates that methyl group is attached to electron withdrawing oxygen atom of OCH_3 group. The molecular ion peak atm/z 194 $(M)^+$ with 14 mass unit over that of substrate and the ^{1}H-NMR spectrum of [Caff-AM-1] revealed that this metabolite had the same carbon skeleton as [substrate] with one methoxyl group versus a hydroxyl group in [substrate].

On the basis of these spectral data [Caff-AM-1] was characterized as Ferulic acid; (3-methoxy-4-hydroxy cinnamic acid) **(Rosazza *et al.*, 1995)**.

Metabolite [Caff-AM-2]

Was obtained as pale yellow amorphous powder. It gave a dark blue color with vanillin/sulfuric acid. [Caff-AM-2] exhibited UV absorptions confirming its phenolic nature atλ_{max}285, 250, 325 nm. The IRspectrum exhibited sharp absorption band at 3425 $cm^{-1}$for OH group of carboxylic acid, an absorption band at 1680 $cm^{-1}$for –C=O of carboxylic acid, an absorption band at 1570 $cm^{-1}$for aromatic ring and an absorption band near 1630 $cm^{-1}$for -C=C- stretching. The band at 1170-1345 cm^{-1} showed presence of methoxy group (–C-O stretching frequency).The molecular formula was determined as $C_{11}H_{12}O_4$ on the basis of the molecular ion peaks observed at m/z 209 $(M - H)^+$,192 $(M + H - H_2O)^+$, 165 $(M + H - COOH)^+$,152 $(M + H - COOH – CH_3)^+$,137 (M- propenoic acid ($C_3H_4O_2$, side chain)$^+$,124 (M + H- propenoic acid [$C_3H_4O_2$, side chain] – $CH_3)^+$ and 109 (M + H- propenoic acid [$C_3H_4O_2$, side chain] – $2CH_3)^+$by EI-MS.

The ^{1}H-NMR spectrum of [Caff-AM-2], exhibited three aromatic proton signals as an ABX Spin-system at δ_H6.52 (1H, d, *J* = 2.5 Hz, H-2), 6.94 (1H, d, *J* = 8.6 Hz, H-5) and7.12 (1H, dd, *J* = 8.6, 2.5 Hz, H-6),indicating the presence of a tri-substituted aromatic ring in the molecule. The^{1}H-NMR spectrum also displayed two doublet protons as an AB spin-system (*J* = 16.2 Hz), each for 1H, at δ_H7.54(H-7) and 6.39 (H-8).The large value of coupling constant indicated the presence of *trans*-disubstituted ethylene moiety in the molecule. On the other hand the presence of signals for two singlet methoxyl groups at δ_H 3.86 (3H, s, OCH_3-3) and 3.83 (3H, s, OCH_3-4) indicates that two methyl groups were attached to electron withdrawing oxygen atom of OCH_3 groups. These NMR data revealed that metabolite[Caff-AM-2]had the

same proton skeleton as in metabolite [Caff-AM-1]with additional signals arising from one more methoxyl group that was also confirmed from ion peak at m/z its 209 $(M-H)^+$versus ion peak at m/z 194$(M)^+$ in metabolite [Caff-AM-1].

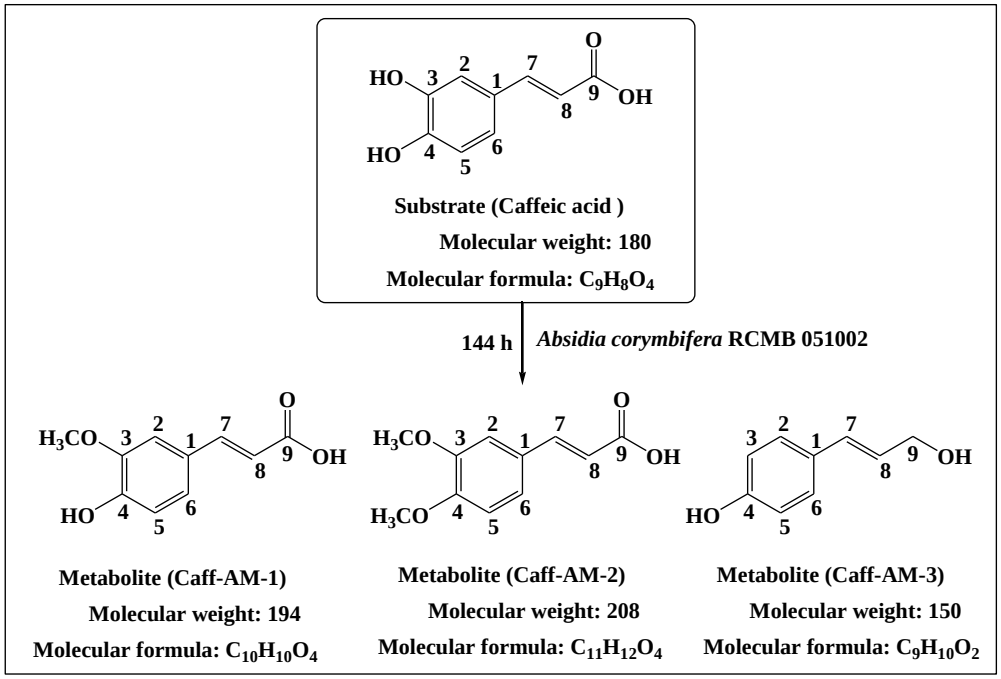
From these results, the structure of metabolite [Caff-AM-2] was concluded to be 3,4-dimethoxy-cinnamic acid[(*E*)-3-(3,4-dimethoxy-phenyl)-propenoic acid].

Metabolite [Caff-AM-3]

Was obtained as white powder. It gave a dark blue color with vanillin/sulfuric acid and exhibited UV absorptions confirming its phenolic nature at (λ_{max} 265 and 315 nm). The IR spectrum of [Caff-AM-3] showed absorption bands at 3510, 1680, 1640 and 1545 cm^{-1} ascribable to hydroxyl, carbonyl, olefin and aromatic ring functionalities, respectively. The molecular formula $C_9H_{10}O_2$ of[Caff-AM-3]was determined from the molecular ion base peak at *m/z* 150 $(M)^+$ together with fragment ion peaks at *m/z* 112 $(M - 2H - 2H_2O)^+$, *m/z* 90 for (M - prop-2-en-1-ol [C_3H_6O, side chain] - 2H)$^+$ or $(M - CH_2OH - OH)^+$ observed in the electron impact (EI)-MS.

The ^{1}H-NMR resonances of [Caff-AM-3], clearly showed signals typically found in a *p*-coumaryl alcohol(204). It showed, four aromatic protons at δ_H7.18 (2H, d, *J* = 8.6 Hz, H-2, H-6) and δ_H 6.94 (2H, d, *J* = 8.6 Hz, H-3, H-5) represented AA' BB' spin-pattern of 4'-oxygenated B-ring. In the^{1}H-NMR spectrum of [Caff-M-3], also showed signals due to a hydroxy-methylene at δ_H 4.73(1H, dd, *J* = 13.5, 6.5 Hz, H-9a), and δ_H 4.81 (1H, dd, *J* = 13.5,6.5 Hz, H-9b) and two olefinic protons as an AB spin-system at δ_H6.58 (1H, d, *J* = 16.5 Hz, H-7) and 7.57 (1H, dt, *J* = 16.5, 6.5 Hz, H-8), which could be assigned to an *trans*-cinnamoyl moiety.

Onthe basis of these findings, the structure of metabolite **[Caff-AM-3]**,was determined to be***p*-coumaryl alcohol**[4-hydroxycinnamyl alcohol] **(Hosoya *et al.*, 2008)**.



(Figure 1) Structure of Caffeic acid(1) and its metabolites.

CONCLUSION

Caffeic acid is structurally a relatively simple compound however it contains several metabolically active sites, a property that leads to the formation of a large number of metabolites. The *O*-methylation of caffeic acid through the catalytic activity of Catechol-*O*-methylt-ransferase (COMT) has ken surmised to be a major route of metabolism of caffeic acid though significant amounts of ferulic acid were found in the urine following the oral administration of caffeic acid to human and rats **(Nardini *et al.*, 1997**). Catechol-*O*-methyltransferase is an *S*-adenosyl-L-methionine-dependent methyl-transferase enzyme that catalyzes the methylation of catechol substrates. Physiologically, it is responsible for the elimination of biologically active or toxic catechols, making it a protein with great clinical relevance as therapeutic target in serious disorders, like schizophrenia and Parkinson´s disease **(Maria-Joao *et al.*, 2007**).The active site of COMT suggested that the methyl building unit is supplied from L-methionine which activates one of the substrate catecholic hydroxylic groups and is introduced by a nucleophilic substitution reaction. In nature, the leaving group is enhanced by converting L-methionine into *S*-adenosylmethionine (SAM) (Figure 2a). This gave positively charged sulfur and facilitates the SN^2-type nucleophilic substitution mechanism (Figure 2b). Thus, *O*-methyl linkage may be obtained using hydroxyl and amino functions as nucleophiles. Methionine is subsequently regenerated by the methylation of homocysteine, using N^5-methyl-tetrahydrofolate as methyl donor. The active site of COMT suggested that the methylation of catechols involved a lysine as a general base, which activates one of the substrate catecholic hydroxylic groups for a nucleophilic attack on the active methyl group of the coenzyme SAM **(Maria-Joao *et al.*, 2007**).

Our results suggest that the preferred route of caffeic acid metabolism by *Absidia corymbifera* may involve many enzymes (Figure 3). Caffeic acid can be metabolized sequentially by COMT to form their *O*-methylated products, ferulic acid (Caff-AM-1) and (*E*)-3-(3,4-dimethoxy-phenyl) propenoic acid(Caff-AM-2). We have speculated that*p*-coumarylalcohol(Caff-AM-3), was formed from caffeic acid (substrate) first by dehydroxylation yielding the intermediate 4-coumaric acid (*p*-coumaric acid) following by reduction of *p*-coumaric acid via coenzyme A ester and aldehyde leads to the corresponding alcohol through oxidation reaction with the β-oxidation enzyme acyl CoA dehydrogenase to form their unsanirated product *p*-coumaric acid. Formation of the coenzyme A ester facilitates the first reduction step by introducing a better leaving group ($CoAS^-$) for the NADPH-dependent reaction. The second reduction step, aldehyde to alcohol, utilizes a further molecule of NADPH (Figure 3). Therefore, it has been well established that enzyme systems capable of performing metabolic processes of caffeic acid involves methylation, dehydroxylation and hydrogenation are functioning continuously.

B- Results of Biological evaluation (Antioxidant Activities)

Free radicals are a major cause of oxidative stress that may lead to DNA strand breakage, gene mutation and DNA-DNA and DNA-protein cross links. Free radicals are known to be a product of normal metabolism. When oxygen is supplied in excess or its reduction is insufficient, reactive oxygen species (ROS) such as hydroxyle (OH^-), superoxide ($O2^-$), nitric oxide (NO), lipid peroxyle (Loo^-), radical and non-free radical species such as lipid peroxide (LOOH-) and different forms of activated oxygen **(Ibrahim *et al.*, 2011).** ROS are involved in an organism's vital activities including phagocytosis, regulation of cell proliferation, intracellular signaling and synthesis of biologically active compounds **(Ibrahim *et al.*, 2011).** ROS have been implicated in several diseases including carcinogenesis, malaria, heart diseases, arteriosclerosis, diabetes and many other health problems, **(Ibrahim *et al.*,2011**). The role of ROS in the a etiology and progression of several clinical manifestations

has led to the suggestion that the antioxidants can be beneficial as prophylactic agents. Nevertheless, all aerobic organisms, including humans, have antioxidant defenses that protect against oxidative harm and repair damaged molecules. However, the natural antioxidant mechanisms can be insufficient, the supply of antioxidants through dietary ingredients, is of great interest for a healthy life **(Ibrahim *et al.*, 2011).** In this study caffeic acid (1) and its metabolites were investigated for their antioxidant properties using1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and measurement of $FeSO_4/H_2O_2$-stimulated lipid peroxidation in rat tissue homogenates.

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; caffeic acid and its isolated metabolites obtained by *Absidia corymbifera* cultures 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 Caffeic acid (1) showed DPPH quenching with IC_{50} values at (62.2 %).Metabolites; **Caff-AM-1** (ferulic acid) highest activity among all the tested samples (69.3%), followed by

Caff-AM-2 (3,4-dimethoxy-cinnamic acid) (66.5%),in DPPHradical quenching, than those of such typical antioxidants D, L, α-tocopherol (62.8%) and BHT (50.2%) respectively.

Caff-AM-3 (*p*-coumaryl alcohol) [51.6%], 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 anti-oxidative activity **(Hosny *et al.*, 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 *et al.*, 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.

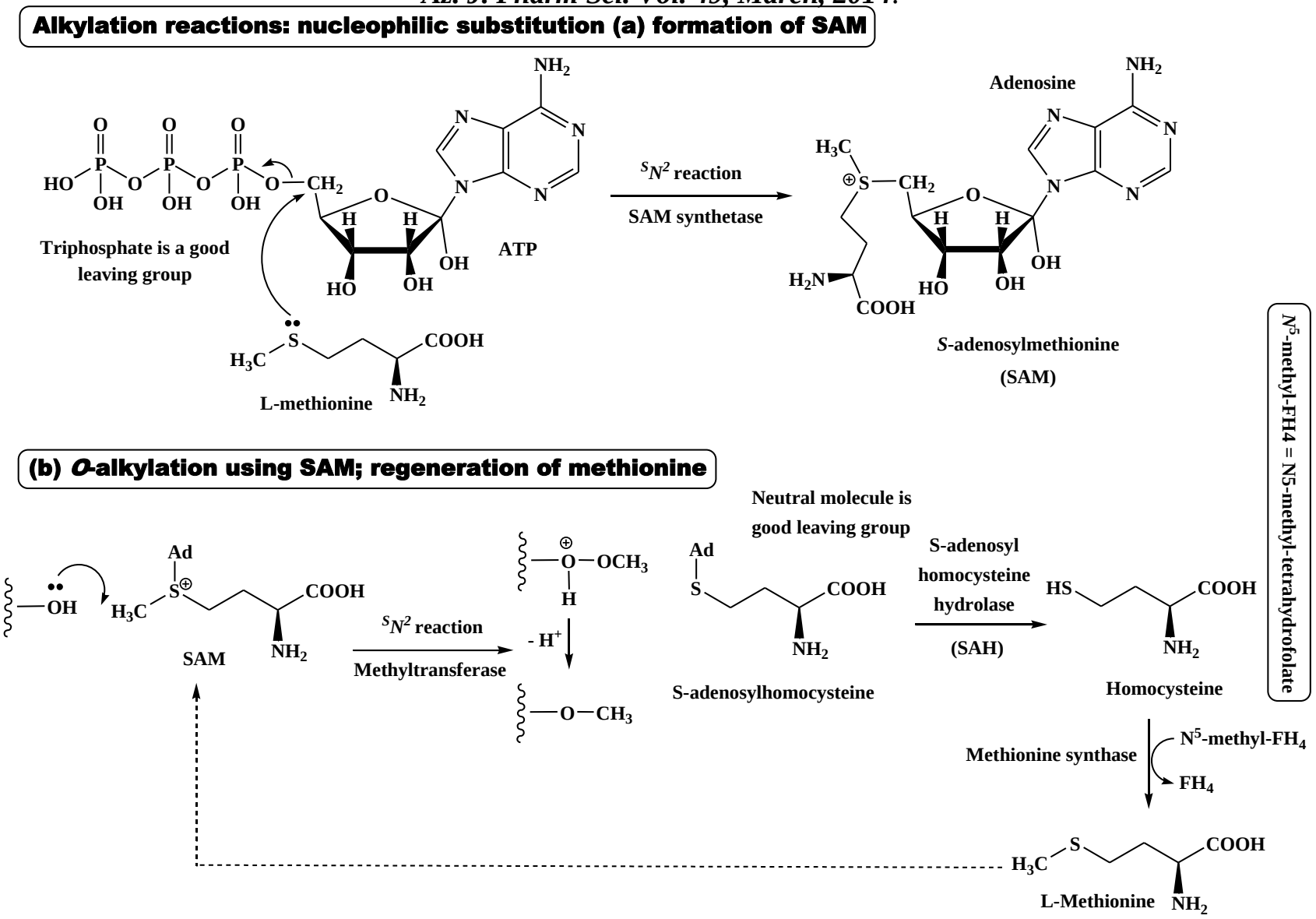
Table (1) Effects of caffeic acid, its metabolites and positive controls on the *in vitro* Free Radical Generation.

Bioassay	**DPPH % decoloration**
Substrate	
Caffeic acid	62.2± 1.50
Metabolite	
Caff-AM-1	**69.3 ± 1.60**
Caff-AM-2	**66.5 ± 1.55**
Caff-AM-3	51.6± 1.30
Positive control	
D,L, α-tocopherol	**62.8± 1.50**
BHT	**50.2± 1.30**

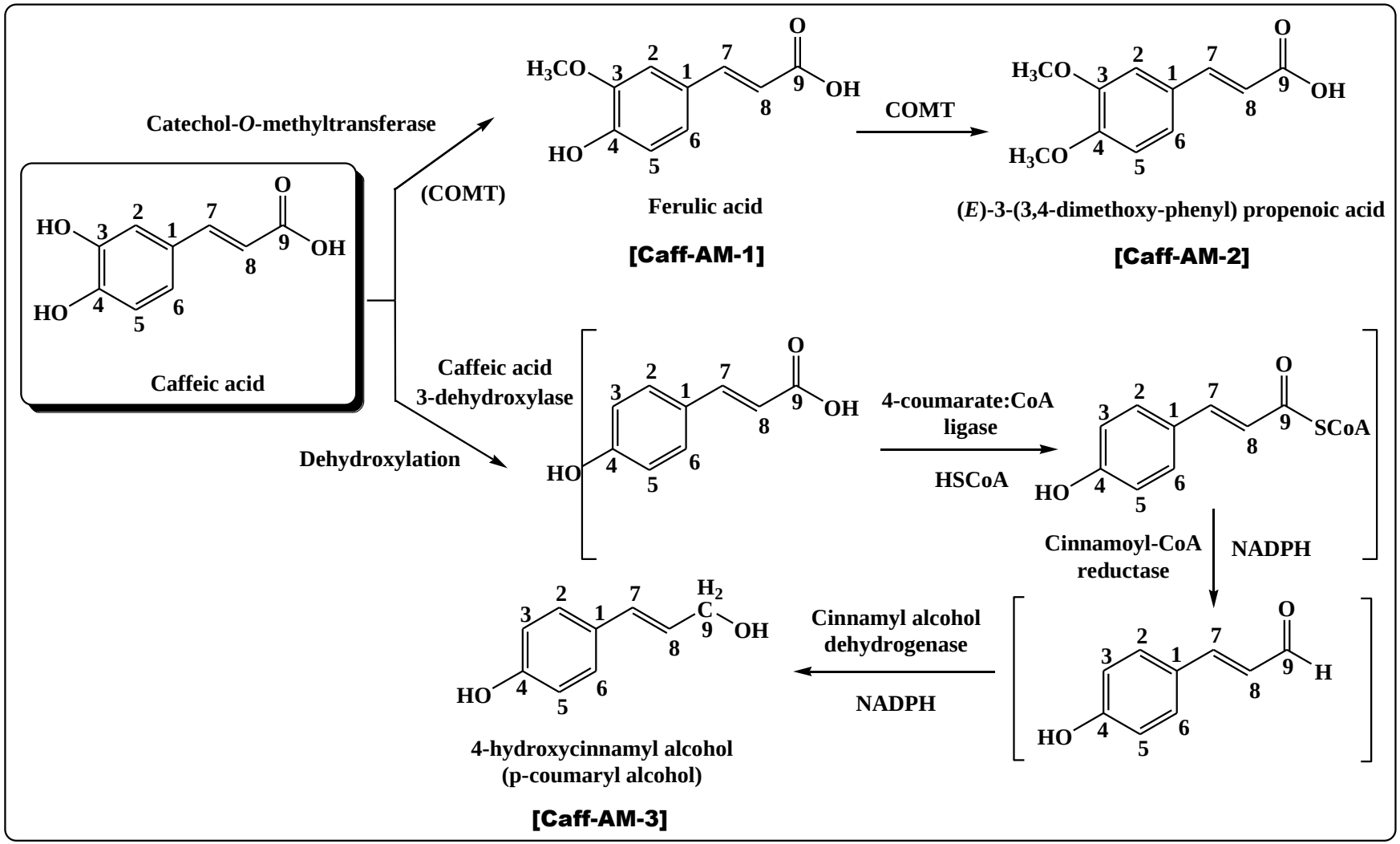
Values are presented as mean ± SE of 3-test sample observation. $P< 0.05$for all values.

B.2- Ferrous sulphate-H_2O2-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, malon-dialdehyde (MDA), lipid hydro-peroxides 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).** It is 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.



(Figure 2) Formation of SAM and Alkylation reactions.



(Figure 3) Possible metabolic pathways of caffeic acid by *Absidia corymbifera* RCMB 051002.

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 $\pm$ 0.15 nmol, MDA/mg protein), (0.22 $\pm$ 0.10 nmol, MDA/mg protein) and (0.16 $\pm$ 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 $\pm$ 1.30 nmol, MDA/mg protein), 0.65 $\pm$ 1.25 nmol, MDA/mg protein) and (0.44 $\pm$1.15 nmol, MDA/mg protein) of brain, heart and liver, respectively (**Table 2**).A control experiment indicated that substrates and isolated fungal metabolites did not affect the measurement of TBARS because the absorbance at 532 nm was not affected by adding different substrates and isolated fungal metabolites to the rat tissue homogenate that already have been oxidatively modified because omission of rat homogenate from the reaction mixture abolished chromogen formation. D, L-α-tochopherol and BHT also inhibited this Fe^{2+}-induced lipid peroxidation with IC_{50} values in the range of (28.23-35.10%), (22.05-38.70%) and (33.15-46.18%) in heart, brain and liver rat tissue homogenates, respectively. However, as shown in (Table 2), adding 200-500 μg/mL of caffeic acid on rat tissue homogenates, reduce MDA formation in the presence of Fe^{2+}-$H_2O_2$with IC_{50} values (38.15%), (37.50%) and (55.40%),in heart, brain and liver rat tissue homogenates, respectively, indicating lower anti-lipid peroxidation activities of substrate. The results obtained with the tested metabolites significantly reduced malon-dialdehyde (MDA) formation in the presence of$FeSO_4$-H_2O_2 in tissue homogenates indicating anti-lipid peroxidation activities. The inhibition percentages were in the range of (23.65–48.12%), (25.10–30.85%) and (36.55–58.15%) in heart, brain and liver rat tissue homogenates, for metabolites (Caff-AM-1, Caff-AM-2 and Caff-AM-3),respectively, as recorded in (Table2).

It was interesting to note that the inhibition effects produced by the tested samples were more pronounced for liver tissue homogenates than heart and brain tissue homogenates, which could be especially beneficial in treatment of liver disease in cases with oxidative stress due to elevated levels of TBARS. Caff-AM-1 and Caff-AM-2, showed the highest inhibition activity against $FeSO_4/H_2O_2$-stimulated lipid peroxidation in liver rat tissue homogenate (58.15 and 49.10 %), respectively, which was higher than both reference standards. Since DL-α-tocopherol is thought to be associated with lipid-rich membranes; its anti-oxidative ability is highly effective in protecting membranes against lipid peroxidation, as peroxyl and alkoxylradicals. The data obtained from the present study indicates that the tested metabolites have an anti-lipid peroxidative character with similar reaction mechanisms to those of DL-α-tocopherol.

Table (2) Inhibition effect of caffeic acid, its metabolites and positive controls on $FeSO_4$-H_2O_2induced 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			
Caffeic acid	38.15±1.25	37.50 ±1.25	55.40±1.45
Metabolites			
Caff-AM-1	**48.12±1.45**	30.85±1.20	**58.15±1.50**
Caff-AM-2	25.05± 0.15	29.40±1.20	**49.10±1.45**
Caff-AM-3	23.65±1.15	25.10±1.20	36.55±1.30
Positive controls			
DL, 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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أيض حمض الكافيك بواسطة فطر *الأبسيديا كورمبيفيرا*

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عمليات التحول الحيوي ربما تقدم بديل أرخص للمنتج الطبيعي كإضافة للمركبات القيمة. تم دراسة أيض حمض الكافيك (3و4 – ثنائي هيدروكسي حمض السيناميك) بإستخدان الفطر المغزلي المسبب لأمراض النبات (*الأبسيديا كورمبيفيرا*) كمحول حيوي. أظهرت النتائج تحول حمض الكافيك إلى حمض الفيروليك، 3و4 ثنائي ميثوكسي حمض السيناميك و 4-هيدروكسي سيناميل الكحول. *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 ثيوباربيتويرك باستخدام أنسجة الفئران المطحونة بالنسبة لتركيبها.