Archives of Pharmaceutical Sciences Ain Shams University 2018; Vol. 2(1):37-46

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Research Article

Biotransformation of caffeic acid into a promising biologically active metabolite by *Candida albicans* isolate CI-24

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ABSTRACT

In this study, a total number of 92 clinical isolates of *Candida species* was used to test their ability to transform caffeic acid using the two-stage fermentation protocol. The success of the biotransformation process was confirmed by TLC autography method and nuclear magnetic resonance analysis. The obtained chromatograms showed that 7 isolates could perform caffeic acid biotransformation. The biological activities (antibacterial, antifungal, antiviral, and cytotoxic activities) of the extracts of the selected isolates were determined. According to the obtained results, the *Candida* isolate CI-24 had the most promising biotransformation ability. The methanolic extract of the respective isolate showed a promising anticancer activity against *Caco-2* cell line and a potential antibacterial activity against *Staphylococcus aureus* ATCC 25923. The isolate was genetically identified as *Candida albicans (Accession number* MH356583) using 28S rRNA sequencing. As determined by NMR and LC-MS analysis, caffeic acid was transformed by *Candida albicans* strain CI-24 into para-hydroxybenzoic acid.

Keywords: *Biotransformation; Caffeic acid; Candida albicans; Antibacterial; Cytotoxicity*

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Citation | Raghda AS, Ahmed ME, Walid FE, Mahmoud AY, Nadia AH. 2018. Biotransformation of caffeic acid into a promising biologically active metabolite by *Candida albicans* isolate CI-24. Arch Pharm Sci ASU 2(1): 37-46
DOI: 10.21608/aps.2018.18733
Online ISSN: 2356-8380
Print ISSN: 2356-8399
Journal no. 1

Published by: Ain Shams University, Faculty of Pharmacy

1. INTRODUCTION

Natural products with industrial applications can be produced via primary or secondary metabolism of living organisms (plants, animals or microorganisms). The number of discovered natural compounds exceeds one million due to the continuous improvement of screening and isolation techniques **[1]**. Recently, attention has turned to phenolic compounds with biological activity due to their influence on human metabolism ability to prevent some chronic diseases such as neurodegenerative and cardiovascular diseases as well as proving to have antioxidant, anti-mutagenic, anti-allergic, anti-inflammatory, and antimicrobial activities **[2-4]**.

Chemical modifications of the structure of the compounds are usually carried out using chemical pathways of synthesis. However, it often generates harmful residues or waste

products which may be harmful to the environment and other living creatures. Moreover, the transformation of some chemical entities into new compounds may be difficult as chemical reactions lack some enzymes required in various cases. Biotransformation is, therefore, an appropriate way to produce biologically active compounds for different industries. In addition, the obtained bioactive products are considered natural which gives them better perspectives of use than their synthetic counterparts **[5]**.

Caffeic acid (hydroxycinnamic acid) is a natural secondary metabolite. It was reported that many valuable aroma, flavoring compounds, and pharmaceutical intermediate are obtained during its degradation pathway. Recently, there has been interesting in the microbial biotransformation of caffeic acid to produce bioactive products **[6]**. The present study focused on the screening of caffeic acid biotransformation abilities by *Candida spp.* clinical isolates and assessment of the biological activity of the biotransformed metabolites.

2. MATERIALS AND METHODS

2.1. Microorganisms

A total number of 92 *Candida* isolates was obtained from the Microbiology Laboratories of Al-Demerdash Hospital (n= 88) and Al-Azhar Mycology laboratory (n= 4). Microorganisms were maintained on Sabouraud's dextrose agar (SDA) slants and stored in a refrigerator at 4 °C prior to use. Glycerol stock 50% was prepared for long-term preservation at -80 °C **[7]**.

Microbial reference strains used for antimicrobial activity assessment were obtained from the American Type Culture Collection (ATCC) which included: *Escherichia (E.) coli* ATCC, 25922, *Klebsiella pneumoniae*, ATCC 700603, *Salmonella typhimurium*, ATCC, 14028, *Proteus mirabilis* ATCC, 14153, *Shigella sonnei*, ATCC 25931, *S. aureus* ATCC, 25923, *S. epidermidis*, ATCC 1228, Methicillin-resistant *S. aureus*, MRSA ATCC 4330, *Pseudomonas (P.) aeruginosa* clinical isolate, *Clostridium difficil*e ATCC 43255, *C. albicans* ATCC, 10231, *Aspergillus niger*, ATCC 16888, *Penicillium chrysogenum*, ATCC 10106, and *Fusariumoxysporum* ATCC 62506.

2.2. Cell lines

Four cell lines; kidney epithelial cells derived from the African green monkey (*Vero* cell line, ATCC No. CCL-81), colorectal adenocarcinoma derived from human colon (*Caco-2* cell line), human liver carcinoma cells (*HepG2* cell line), and a breast cancer cell line (*MCF-7* cell line) were obtained from VACSERA (Cairo, Egypt). *Caco-2, HepG2*, and *MCF-7* cell lines were used to evaluate the potential cytotoxic activities of the extracts; stock cultures of these cell lines were grown in T-75 tissue culture flasks containing 20 mL of RPMI-1640 medium (VACSERA, Cairo, Egypt) with 1% antibiotic-antimycotic solution (10.000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B/mL) and 10% v/v fetal bovine serum (FBS). The medium was changed at 48 h intervals and cells were detached by trypsin solution (0.25% in phosphate buffered saline). *Vero* cell line is a continuous non-tumourigenic when a cell passage was not prolonged **[8]**. It was used to detect the cytotoxic effect of compounds, showed promising cytotoxic activity on the normal cell line. It was propagated in Eagle minimum essential medium (EMEM) (VACSERA, Cairo, Egypt) with Hank's balanced salt solution (HBSS) supplemented with 10% v/v FBS and antibiotics solution (100 IU penicillin and 100 IU streptomycin/mL) and maintained in EMEM with Earl’s balanced salt solution (EBSS) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 2% v/v FBS and antibiotics solution.

2.3. Viruses

Rapidly growing virus strains producing cytopathic effects (CPE) in *Vero* cell cultures within 3 days were used during this study. The used viruses were: Hepatitis A virus (HAV-H10) which causes subtotal destruction of cells **[9]**; Herpes Simplex Virus type 1 which causes cell fusion polykaryon formation and focal degeneration (HSV-1) **[10]**; Coxsackie B4 (COX-B4) which produces CPE resemble those of poliovirus but develop more slowly commencing with foci of rounded refractile cells which then lyse and detach **[11]**.

2.4. Screening of *Candida* isolates for transforming ability

The screening process was carried out by a two-stage fermentation protocol as described by Hosny *et al*. **[12]**. In brief, a loopful of microbial growth was withdrawn from fresh Sabouraud Dextrose Agar **(**SDA) slants and transferred to 250 mL flasks containing 50 mL soybean-yeast extract medium supplemented with 2% w/v glucose. The inoculated flask was incubated at 28 °C with shaking at 250 rpm for 24 h. After incubation, inocula of 10% v/v from the culture ($83X10^{10}$- $95X10^{10}$ CFU/mL) were transferred to 250 mL flasks containing 50 mL soybean-yeast extract medium supplemented with 2% w/v glucose and incubated under the same conditions for 24 h. An aliquot (about 500 µL) of caffeic acid (Sigma Aldrich®, St. Louis, MO, USA) solution in dimethylformamide (10 mg/mL) was added to each flask and then kept under the same conditions for 72 h.

2.5. Extraction

Flasks contents were centrifuged at 6000 rpm for 5 min. The obtained cell-free supernatants were extracted using ethyl acetate: n-butanol mixture (9:1), repeated three times with 20 mL solvent mixture. The solvent mixtures were evaporated at 60 °C using rotary evaporator (Heidolph instruments GmbH and Co., Schwabach, Germany) to obtain dried extracts which were dissolved in methanol and kept at 4 °C to be used in further studies.

2.6. Chromatographic assessment

This was carried out by thin layer chromatography (TLC) autography method in which different extracts were spotted on silica-gel plates (Merck®, Burlington, MA, USA) and developed the chromatogram in methylene chloride:methanol (9:1) solvent system. Plates were sprayed with 1-1-Diphenyl-2-picrylhydrazyl (DPPH; 2 mg/mL) **[13]**. Extracts of standard caffeic acid and organisms were used as controls. For confirmation of the TLC results, nuclear magnetic resonance spectroscopy (NMR) (400 Mhz, Bruker Avance HD III, Fällanden, Switzerland) was performed at Center for Drug Discovery Research and Development (Faculty of Pharmacy, Ain Shams University, Cairo, Egypt).

2.7. Assessment of biological activities of the extracts

The obtained extracts were screened for antibacterial, antiviral, antifungal, and anticancer activities.

2.7.1. Evaluation of antibacterial activity

The antibacterial activity of the extracts was assessed by agar well diffusion method **[14]**. Bacterial suspensions were prepared by picking 4-5 colonies from a fresh culture of each organism and introduced into a tube containing 2 mL sterile saline and turbidity was adjusted to 0.5 McFarland's standard (1.5×10^8 CFU/mL). By a sterile cotton swab, each bacterial suspension was transferred and spread on Mueller Hinton's agar medium and then it was left 10 min for drying. Thereafter, 10 mm wells were punched in the agar medium and filled with 100 µL of each extract and incubated at 37 °C for 24 h.

For the evaluation of the antimicrobial activity of the extracts against *C. difficile* ATCC 43255, a cup diffusion assay was performed as described previously **[15,16]**. Corresponding molten agar selective medium for *C. difficile* containing approximately 10^5 CFU/mL of the indicator strains was poured in plates. After solidification, cups (10 mm) were made by sterile cork borer and 100 µL of the extracts was transferred into the well. Plates were incubated anaerobically for 48 h at 37 °C. After incubation, inhibition zones were determined by measuring the diameter of the inhibition zones in mm around the wells. In both experiments, organisms' extracts, media extract with and without caffeic acid were used as controls.

2.7.2. Assessment of antifungal activity

Assessment of antifungal activity of the tested extract was carried out by disk diffusion testing according to the Clinical and Laboratory Standard Institute guidelines **[17]**. Organisms' extracts, media extract with and without caffeic acid were used as controls. Nystatin discs (100 IU) were used as positive control.

2.7.3. Assessment of antiviral activity

Determination of the antiviral activity of the prepared extracts was based on cytopathic effect inhibition assay by using MTT assay **[18]**. Organisms' extracts, media extract with and without caffeic acid were used as controls.

2.7.4. Assessment of anticancer activity (cytotoxicity)

Cytotoxicity of different extracts was determined through determination of cell viability of *Caco-2*, *MCF-7* and *HepG2* cells treated with the extracts in comparison with untreated control using MTT assay. Cells were grown as a monolayer (10^4 cells/well) in EMEM growth medium (VACSERA, Cairo, Egypt) supplemented with 10% v/v inactivated fetal bovine serum before treatment with the extracts. Different concentrations of the extracts were added to the cell monolayer and incubated for 24 h into a CO_2 incubator at 37 °C with 5% CO_2. Media extracts with and without caffeic acid and cells without extract were used as negative controls. After incubation, the cells were observed under an inverted microscope before completing the assay to observe the difference in morphology between cell controls and treated ones at different concentrations of tested substances. The percentage of cytotoxicity was determined by MTT assay as described by Meerloo *et al.* **[19]**.

2.8. Genetic identification of the most promising isolate(s)

A pure culture of the promising isolate was sent to Sigma Scientific Co. (Cairo, Egypt), where DNA extraction, PCR amplification of 28S ribosomal RNA gene and sequencing were carried out. The sequences obtained were assembled using Bioedit® software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) to obtain the final consensus sequence. The sequence was aligned against sequences in the GenBank database using the NCBI BLAST® (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/) **[20]**. The final consensus sequence was annotated and submitted into the NCBI GenBank database using BankIt software (https://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank).

2.9. Structure elucidation of the bioactive metabolite

Samples of the crude extracts, organism extracts, and caffeic acid were analyzed using liquid chromatography coupled with electrospray ionization mass spectroscopy (LC/ESI-MS).

2.9.1. Ultra performance liquid chromatography (UPLC) analysis

The analysis was performed by applying 1 mg/mL of the sample on a Waters Xevo TQD mass spectrometer with UPLC Acquity mode (Milford, MA, USA). Gradient elution was adopted beginning with methanol:H_2O (10:90) with 0.1% formic acid till 100% methanol as a mobile phase with a flow rate 1 mL/min, the run took 26 min.

2.9.2. Mass spectrometry

Waters Xevo TQD mass spectrometer was used to accomplish mass spectrometric analysis. The sample was dissolved in MeOH as a mixture and injected directly into the UPLC/ESI-MS system. The negative ESI ionization ion mode was applied under the following conditions: drying and nebulizing gas, N_2; capillary temperature, 250 °C; spray voltage, 4.48 kV; capillary voltage, 39.6 V; tube lens voltage, 10.00 V; and full scan mode in mass range *m/z* 100-2000. The data were processed using Mass Lynx 4.1 software.

3. RESULTS

3.1. Screening of Candida isolates for transforming ability

The obtained TLC chromatograms showed that seven isolates coded; CI-2, CI-9, CI-4, CI-14, CI-24, CI-61, and CI-107 could perform caffeic acid biotransformation which is denoted by the disappearance of the caffeic acid spot in each extract **(Fig. 1)** and the obtained results were confirmed by NMR.

NMR analysis was performed on standard caffeic acid, the used medium, and the extracts of the seven tested isolates. NMR spectrum of caffeic acid revealed the presence of the trans olefinic signals at δ6.24 (d, 15.9, 1H) and δ 7.45 (d, 15.9, 1H) together with the aromatic protons at δ 6.78 (d, 8, 1H), δ 6.91 (dd, 8, 2, 1H) and δ 7.02 (d, 2, 1H) as shown in **Fig. 2**.

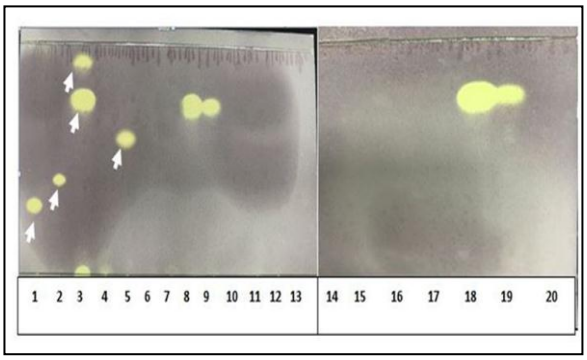


Fig. 1. TLC Chromatograms of seven *Candida* isolates showed their abilities of caffeic acid biotransformation.

The samples: 1, CI-14; 2, CI-24; 3, CI-9; 4, CI- 2; 5, CI-61; 6, CI-4; 7, CI-107; 8, caf_s; 9, caf_m; 10, med; 11,CI- 2_{org}; 12, CI-24_{org}; 13, CI-9_{org}; 14, CI-14_{org}; 15, CI-61_{org}; 16, CI-4_{org}; 17, CI-107_{org}; 18, caf_s; 19, caf_m; 20, med (Caf_s, standard caffeic acid extract; Caf_m, medium with caffeic acid extract; Med, medium extract; Org, organism extract).

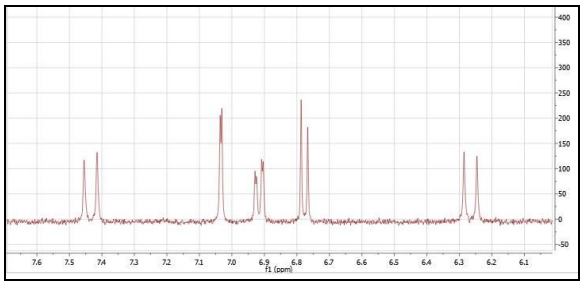


Fig. 2. ^{1}H NMR spectrum of the extract of standard caffeic acid

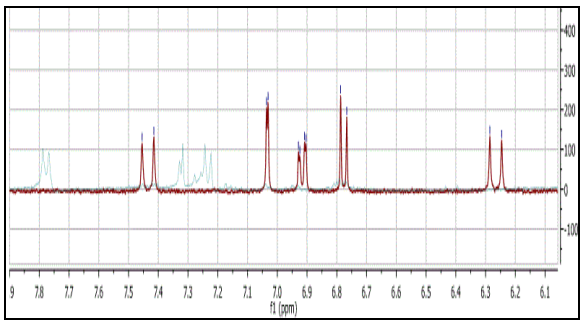


Fig. 3. ^{1}H NMR spectrum of the extract of the isolate coded CI-24 versus caffeic acid (Red, caffeic acid; Blue, extract)

NMR spectrum of the tested isolates showed the disappearance of caffeic acid signals and

presence of different signals in the spectrum of the extract of the isolate coded CI-24 **(Fig. 3)**, while the presence of caffeic acid signals with minor shifting in the chemical shift values was observed in the NMR spectrum of the extract of the isolate coded CI-9 **(Fig. 4)**. On the other hand, NMR spectrum of other extracts (coded CI-2, CI-61, CI-4, CI-14, and CI-107) revealed the disappearance of caffeic acid signals without observing any other signals **(Fig. 5)**.

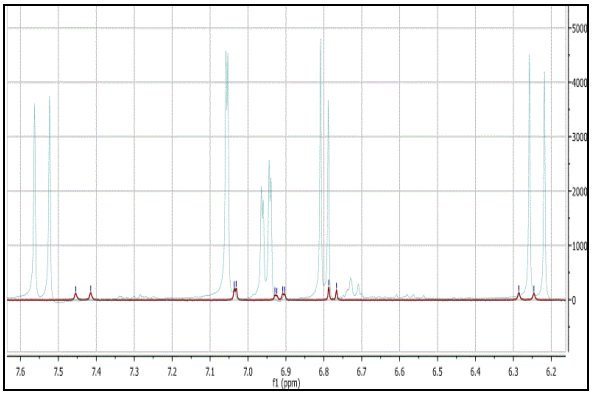


Fig. 4. ^{1}H NMR spectrum of the extract of the isolate coded CI-9 versus caffeic acid (Red, caffeic acid; Blue, extract)

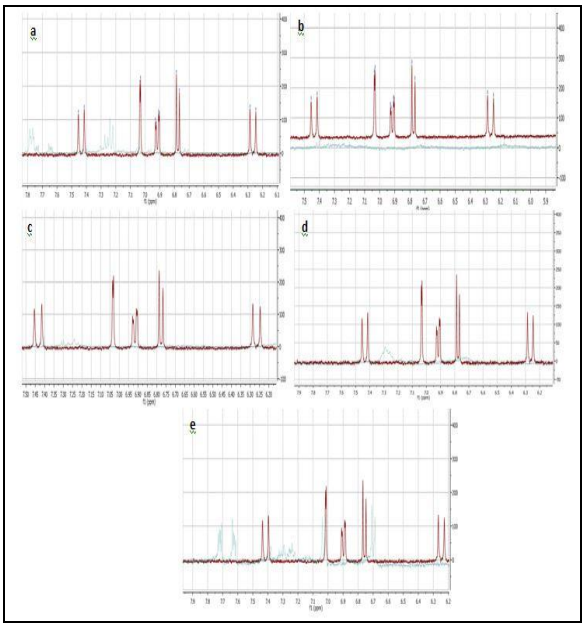


Fig. 5. ^{1}H NMR spectrum of different extracts (a) CI-2; (b) CI-61; (c) CI-4; (d) CI-14; and (e) CI-107 (Red, caffeic acid; Blue, extract)

3.2. Assessment of antimicrobial activity

According to the obtained results, the extracts CI-61, CI-4, CI-9, and CI-24 showed observed inhibition zones against *S. aureus*, *E. coli*, *S. Typhimurium, Proteus mirabilis, Shigella sonnei, and P. aeruginosa* **(Table 1)**. None of the tested isolates showed activity against *S. epidermidis*, *MRSA, K. pneumoniae*, and *Clostridium difficile.* Regarding the antifungal and antiviral activities, none of the tested extracts showed any promising activity.

Table 1. Antibacterial activities of crude extracts recovered from caffeic acid biotransformation against some standard bacterial strains

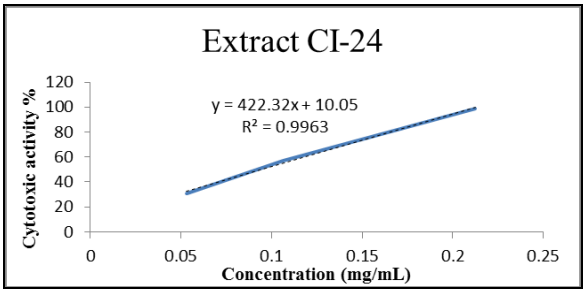
Extract	The diameter of inhibition Zone (mm)								
	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*
CI-4	-	-	-	-	-	-	-	-	-
CI-107	-	-	-	-	-	-	-	-	-
CI-2	-	-	-	-	-	-	-	-	-
CI-61	13	-	-	-	-	-	-	-	-
CI-4	19	-	-	25	-	28	16	25	13
CI-9	-	-	-	15	-	-	-	-	12
CI-24	19	-	-	-	-	-	-	-	-
Gentamicin	20	20	-	20	15	20	25	15	15
Cephalothin	35	28	20	15	-	25	30	-	-
Trimethoprim/ Sulfamethoxazole	30	30	35	25	25	30	35	20	-

Note: A, *S. aureus*; B, *S. epidermidis*; C, *MRSA*; D, *E. coli;* E, *K. pneumoniae;* F, *S.typhimurium;* G, *P.mirabilis;* H, *S.sonnei;* I, *P.aeruginosa*

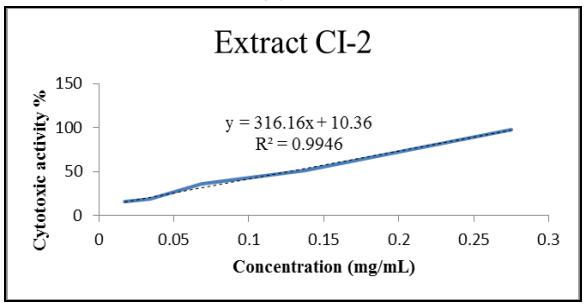
3.3. Assessment of Cytotoxic activity

Results revealed that the cell-free extracts of the *Candida* isolates coded CI-24, CI-2, and CI-107 showed promising cytotoxic activities against *Caco-2* cell line, while the other extracts showed no activity. In addition, no activity was observed with caffeic acid (Data not shown). For the isolates with cytotoxic activities, dose-

response curves were plotted and the CD_{50} values were calculated from the obtained equation, where CD_{50} is the concentration of the drug that is required for 50% cytotoxic activity. The CD_{50} of CI-24, CI-2, and CI-107 are 94.5, 125.37, and 214.8, respectively **(Fig. 6 and Fig. 7)**. However, no cytotoxic activity for all the tested extracts against *MCF-7* and *HepG2* cell lines was observed (Data not showed).



(a)



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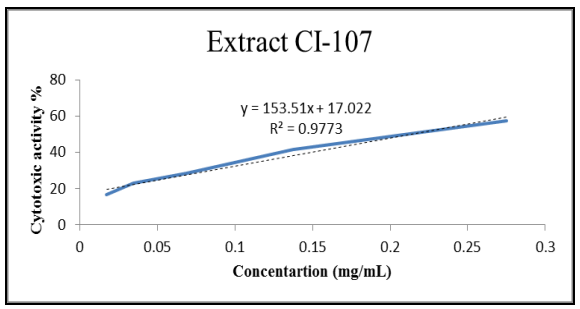

(c)

Fig. 6. Dose-response curve of extract from (a) *Candida* isolate coded CI-24, (b) *Candida* isolate coded CI-2, and (c) *Candida* isolate coded CI-107

According to the above-mentioned results, *Candida* isolate coded CI-24 that showed promising antibacterial and cytotoxic activities against *S. aureus* and *Caco-2* cell line, respectively. It was identified using the 28S ribosomal RNA (NCBI nucleotide accession code, MH356583) as *Candida albicans*.

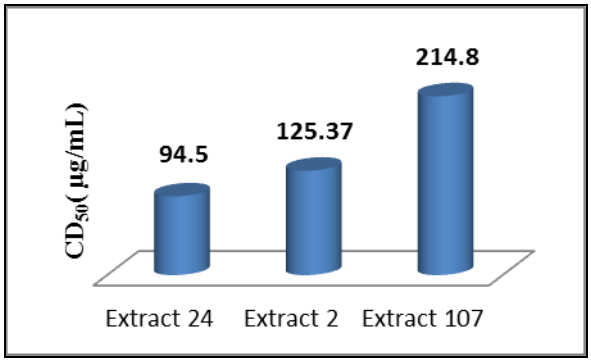

Fig. 7. Cytotoxic activities expressed as CD_{50} of extracts of *Candida* isolates coded CI-24, CI-2, and CI-107 against *Caco-2* cell line.

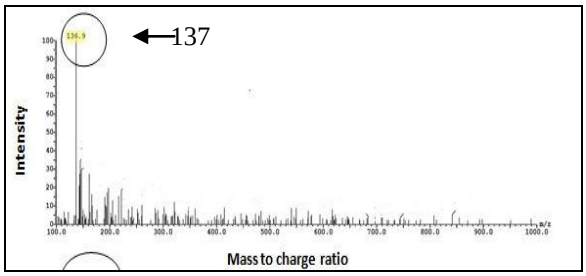

Fig. 8. MS profile of para-hydroxybenzoic acid under the ESI negative scan mode

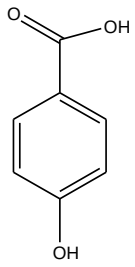

Fig. 9. Para-hydroxy benzoic acid

3.4. Structure elucidation of the bioactive metabolite of CI-24 crude extract

LC-ESI-MS analysis of the methanolic extract of isolate CI-24 revealed complete consumption

of caffeic acid. The MS data in the negative ionization mode is illustrated in **Fig. 8**.

Collectively, the NMR and LC-ESI-MS analysis of the methanolic extract of strain CI-24 after treatment with caffeic acid revealed the transformation of caffeic acid into para-hydroxybenzoic acid $C_7H_6O_3$ **(Fig. 9)** and Molecular Weight of 138.

4. DISCUSSION

Microbial biotransformation is considered the use of microorganisms to catalyze chemical reactions such as reduction, oxidation, hydroxylation, and esterification to obtain valuable and recoverable end-products. Such naturally evolved diversity renders their biochemistry so flexible and amenable to the catalysis of useful reactions and their applications in chemistry **[21]**.

Caffeic acid is a natural secondary metabolite produced by many plant species **[6]**. Due to the high chemical versatility and modifiability of caffeic acid, it can potentially serve as a good starting material for the production of valuable compounds **[5]**.

In the present study, several clinical *Candida* isolates were tested for their abilities to transform caffeic acid. The obtained results revealed that seven *Candida* isolates (coded CI- 24, CI-4, CI-61, CI-107, CI-2, CI-14, and CI-9) were able to transform caffeic acid. Based on the TLC analysis that later confirmed by NMR analysis thereafter, where caffeic acid, medium, and microorganisms methanolic extracts were used as controls. The NMR analysis was performed on standard caffeic acid to confirm its purity and to use the data in the comparison with the biotransformation products of different fungal isolates treated with caffeic acid to demonstrate possible biotransformation. The obtained spectrum of caffeic acid revealed typical spectrum to the published data **[22]**. Additionally, the NMR spectrum of the used medium (soybean-yeast extract medium supplemented with 2% w/v glucose) and the methanolic extract of the isolates without treatment with caffeic were performed to compare their spectra with those obtained from the isolates after addition of caffeic acid to eliminate any possible contribution of the medium and the fungal natural products.

Regarding the NMR spectrum of the tested extracts, the obtained spectra showed complete biotransformation of caffeic acid in four extracts (CI-4, CI-2, CI-14, and CI-61). While one extract (CI-9) showed the shifting of caffeic signals which may be due to the formation of dimmers. The obtained NMR spectra of extracts CI-24 and CI-107 revealed the appearance of new signals.

Further studies were carried out on the prepared crude extracts (n= 7) to evaluate their antimicrobial and anticancer activities. The results of antibacterial activity revealed that only four extracts coded CI-61, CI-4, CI-9, and CI-24 have different spectra of activities against the tested reference strains. Regarding the strain CI-24, a promising antibacterial activity against the tested reference strain of *S. aureus* was observed. Pure caffeic acid showed no antibacterial activity, however, a slight activity at high concentration (> 250 µg/mL) against *E. coli and Proteus vulgaris* was reported previously **[23]**.

Regarding antifungal and antiviral activities, none of the tested extracts showed any significant activity. However, it has been reported that caffeic acid and some of its derivatives possess promising antifungal and/or antiviral activity **[24-26]**. Therefore, biotransformation of caffeic acid by all the tested isolates was associated with a loss of the activity of the original caffeic acid compound.

The cytotoxic activity of the obtained extracts was evaluated against *Caco-2*, *MCF-7*, and

HepG2 cell lines by using MTT assay. A promising activity was observed against *Caco-2* cell line by the extracts CI-24, CI-2, and CI-107. Caffeic acid and its derivatives showed promising cytotoxicity against some types of cell lines such as *J45.01* human acute lymphoblastic leukemia T cells, human leukemia *HL-60* cells and human multiple myeloma cell line **[27-29]**. In the present study, the transformed compound by the isolates coded CI-24, CI-2, and CI-107 have promising cytotoxic activity against *Caco-2* cell line. The obtained results revealed that the isolate coded CI-24 showed the highest cytotoxic activity (CD_{50}= 94.5) in addition to its potential antibacterial activity against *S. aureus*. This isolate (CI-24) was genetically identified as *C. albicans* (NCBI *accession number*, MH356583). The NMR and LC/MS analysis of the methanolic extract of strain coded CI-24 after treatment with caffeic acid revealed the transformation of caffeic acid into para-hydroxybenzoic acid. The obtained MS data in the negative ionization mode was compared to those previously reported in the literature **[30, 31]**.

5. CONCLUSION

A number of 92 Clinical *Candida* isolates was tested for caffeic acid biotransformation using two-stage fermentation protocol. The obtained results revealed that seven isolates showed biotransformation ability of caffeic acid. Further studies were carried out on the obtained cell-free extracts. The extract obtained from isolate CI-24 showed promising cytotoxic activity against *Caco-2* cell line and antibacterial activity against *S. aureus* reference strain ATCC 25923. This isolate was genetically identified as *Candida albicans* (Accession number MH356583). Finally, NMR and LC-MS analysis revealed that caffeic was transformed into para-hydroxybenzoic acid.

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