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Microbial Transformation of Curcumin and Evaluation of the Biological Activities of the Isolated Metabolites

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Abstract

Natural products are structurally and biologically interesting metabolites, but usually isolated in minute amounts. Microbial transformation system is a tool to introduce new biologically active derivatives from natural products. Based on the diverse pharmacological effects of curcumin, and the great importance of biotransformation in the field of natural products, this study was undertaken to produce more biologically active metabolites, which could exceed the activity of curcumin. Eight metabolites were isolated from curcumin by microbial transformation. The chemical structures of the metabolites were established using physical and spectroscopic techniques. The metabolites were identified as: tetrahydrocurcumin (**1**), hexahydrocurcumin (**2**), octahydrocurcumin (**3**), demethoxyhexahydrocurcumin (**4**), demethoxyoctahydrocurcumin (**5**), vanillin (**6**), didesmethylcurcumin (**7**), and curcumin-4`-*O*-*β*-D-glucoside (**8**). Metabolite **7** was obtained for the first time from natural sources. The antioxidant, cytotoxic, and antimicrobial activities of the substrate and the isolated metabolites were evaluated using DPPH, SRB, and agar well diffusion methods. Metabolite **7** exhibited more potent antioxidant activity (IC_{50} 8.62 µg/mL) than curcumin (IC_{50} 33.91 µg/mL) and quercetin (IC_{50} 17.64 µg/mL). Metabolites **8**, **7**, **1**, **3**, then **2**, exhibited more potent cytotoxic effect (IC_{50} 9.47, 11.12, 16.80, 19.46 and 28.00 µg/mL, respectively) than curcumin (IC_{50} 33.80 µg/mL). Metabolites **7**, **8**, **1**, **2**, and **3**, respectively showed the highest antimicrobial activity.

Keywords: Biotransformation, Reduction, Demethoxylation, Glucosylation, DPPH assay, SRB assay, Agar well diffusion method.

1. INTRODUCTION

Natural products are structurally and biologically interesting metabolites, but they may be isolated in minute amounts. The syntheses of such natural products help in obtaining such rare metabolites in substantial amounts. In recent years, microbial transformation is progressing significantly from limited interest into highly active area in green chemistry including preparation of pharmaceutical products due to its minimal waste production and energy consumption [1-2]. Biotransformation can be clarified as the specific modification of a definite compound to a distinct product with structural similarity, by the use of biological catalysts including microorganisms like fungi, bacteria and even cell-free systems [3].

Diarylheptanoids, as a class of structurally distinctive compounds with notable biological activities, have been of increasing interest in the past decades. Diarylheptanoid encompasses compounds which bears the 1,7-diphenylheptane skeleton as a special characteristic in natural product estate due to its potential therapeutic action for several diseases [4-9]. This group of natural products is mainly present in family Zingiberaceae [10].

Curcumin, as a member of the diarylheptanoid compounds, is a major constituent of the turmeric plant *Curcuma longa* L. Curcumin has been shown to afford a wide range of pharmacological activities encompassing antioxidative, antiinflammatory, antibacterial, antifungal, antiviral, antiproliferative, proapoptotic and antiatherosclerotic effects as well as medicinal benefits against neurodegenerative diseases, arthritis, allergy, inflammatory bowel disease, nephrotoxicity, AIDS, psoriasis, diabetes, multiple sclerosis, cardiovascular disease and lung fibrosis. Moreover, curcumin can suppress inflammatory cytokines as well as various target proteins in cancer cell lines.

Owing to its multi-faceted health benefits, curcumin has been used as health supplements as well as natural remedy, while several clinical trials are under way to investigate its potential therapeutic usage [11]. Based on the aforementioned information regarding biotransformations and curcumin, microbial transformation of curcumin was selected to undertake this study to highlight the values of this foremost subject.

2. MATERIALS AND METHODS

2.1. General experimental procedures:

UV spectra were measured on a Shimadzu UV- 1800 spectrophotometer. IR spectra were recorded with a Jasco FT/IR-6100 spectrophotometer. Electron spray ionization mass spectra were performed with a Thermo Scientific ISQ Single Quadrupole Mass Spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with Bruker Avance III High Performance Digital FT-NMR Spectrometer operating at 400 MHz for proton and 100 MHz for carbon. Chemical shifts were given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. Melting point determination was carried out using Gallenkamp melting point apparatus. TLC was performed using precoated TLC sheets silica gel G 254 F (E. Merck, Germany). For column chromatography, silica gel (E. Merck, 70-230 mesh) and sephadex LH-20 (Sigma- Aldrich

chemical Co.) were used. Curcumin (≥ 98% purity) was obtained from Sigma Aldrich Chemical Company Inc, USA. Microorganisms used for transformation were obtained from American Type Culture Collection (ATCC), from Northern Regional Research Laboratories (NRRL) and from Assiut University Mycological Center (AUMC). Sabouraud-dextrose agar (Becton Dickinson and Co., Cockeysville, Med 21030), yeast extract microbiological grade (Oxoid LTD, 329185, England), peptone microbiological grade (Sigma Chemical Co., St. Louise, Mo., 63118, USA), potato- dextrose agar PDA (DIFCO, Detroit, 48201, USA), dextrose AR-grade (Sigma Chemical Co., St. Louise, Mo., 63118, USA), and nutrient agar (Oxoid LTD, 329185, England) were used for preparation of microbial transformation media.1,1-Diphenyl-2-picrylhydrazyl (Sigma Aldrich, Inc., USA) and quercetin (Fluka AG, Germany) were used for evaluation of the antioxidant activity of curcumin and the isolated metabolites. Cytotoxic activity was performed in collaboration with Egyptian National Cancer Institute (El-Kasr Al-Ainy, Cairo- Egypt). Antimicrobial activity was assessed using Muller Hinton Agar (DIFCO, USA) for bacteria and Sabouraud dextrose agar media for fungi (DIFCO, USA). Microorganisms used for determination of antimicrobial activity were as follows: *Staphylococcus aureus* ATCC 6538, a standard strain representative for Gm (+ve) bacteria; *Escherichia coli* ATCC 10536, a standard strain representative for Gm (-ve) bacteria; *Candida albicans* ATCC 10231, a standard strain representative for yeast, and *Aspergillus niger* ATCC 11709, a standard strain representative for mold. Gentamycin and Fluconazole were used as reference drugs for antimicrobial activity. All microorganisms for antimicrobial activity were kindly supplied by department of microbiology, faculty of pharmacy, Tanta University. All chemicals and solvents used in this study were of the analytical grades.

2.2. Microbial transformation of curcumin:

More than forty strains of fungi of different classes were used in the initial screening of curcumin biotransformation. Standard two-stage fermentation technique was used. Stage I cultures were initiated from two-week old slants (under aseptic conditions) by transferring microbial cells into 250 mL Erlenmeyer flask, containing 50 mL of sterile liquid medium and allowed to grow for 72 hours at 27^{o}C on a gyratory shaker operating at 100 rpm. Stage II cultures were obtained by transferring 5 mL of stage I culture to 250 ml flask containing 50 mL of fresh liquid medium and allowed to grow for 24 hours before the addition of the substrate solution [11]. The metabolites formation was tested periodically to confirm the suitable time for the maximum production. The preliminary screening indicated that *Alternaria alternata* AUMC 4685, *Cunninghamella blackesleeana* NRRL 1369, *Cunninghamella elegans* NRRL 2310 and *Penicillium brevicompactum* AUMC 2751 were the most efficient microorganisms for maximum biotransformation of curcumin into several metabolites. Therefore, they were used for large scale fermentation of curcumin.

2.2.1. Curcumin biotransformation by *Alternaria alternate* AUMC 4685

Curcumin (400 mg) was dissolved in 20 mL acetone, and then equally divided among ten flasks, each containing 200 ml of stage II culture and incubated at 27°C on a gyratory shaker operating at 100 rpm. After elapse of two weeks of fermentation the experiments were terminated. The fermentation broth was filtered and extracted with ethyl acetate till exhaustion. The combined extracts were evaporated to dryness under reduced pressure to give 1.1 g residue. The residue was loaded on the top of silica gel column (44 g, 50 × 2 cm) then eluted with methylene chloride and gradually increasing polarity by ethyl acetate (2% each) up to 20%. Ninety six fractions (20 mL each) were collected and examined on TLC using methylene chloride-methanol (19:1, v/v) as a mobile phase. Similar fractions were pooled and evaporated to dryness. Fractions 13-18 were combined and evaporated to dryness to give 40 mg residue. Further purification was achieved through rechromatography on silica gel column (2 g, 15 × 0.5 cm) using isocratic elution with methylene chloride-methanol (97:3, v/v). Twenty two sub-fractions (3 mL each) were collected and examined on silica gel G sheet using methylene chloride-methanol (19:1, v/v) as a mobile phase. Subfractions 6-11(3 mL each) were combined and evaporated to give a pure compound designated as metabolite **1** (20 mg).

Fractions 32-40 were combined and evaporated to dryness to yield 44 mg residue. Further purification was carried out on silica gel column (2 g, 15 × 0.5cm) using isocratic elution and methylene chloride-methanol (97:3, v/v) as a mobile phase. Twenty five sub-fractions (3 mL each) were collected and examined on silica gel G sheet using methylene chloride:methanol (19:1, v/v) as a mobile phase. Subfractions 10-17 were combined and evaporated to give a pure compound designated as metabolite **2** (22 mg). Fractions 71-81 were combined and evaporated to give a pure compound designated as metabolite **3** (24 mg).

2.2.2. Curcumin transformation by *Penicillium brevicompactum* AUMC 2751

The same procedures for fermentation (ended after 12 days) and extraction were carried out as previously mentioned for transformation of curcumin by *Alternaria alternata* AUMC 4685. The residue (900 mg) obtained from the fermentation reaction was loaded on the top of silica gel column (36 g, 25 × 1 cm) using wet method. Elution method was adopted starting with 100 % methylene chloride and gradually increasing polarity by ethyl acetate (2%) with increasing percentage of ethyl acetate up to 20%. Fifty four fractions (30 mL each) were collected and examined on TLC using methylene chloride-methanol (19:1, v/v) as a mobile phase. Fractions 15-19 were combined and evaporated to dryness to give residue (39 mg). Further purification was carried out using silica gel column (2 g, 15 × 0.5 cm) and isocratic elution with methylene chloride-methanol (97:3, v/v). Twenty two subfractions (3 mL each) were collected and examined on silica gel G sheet using methylene chloride-methanol (19:1, v/v). Subfractions 8-14 were combined and evaporated to give a pure compound designated as

metabolite **4** (16 mg). Fractions 28-32 were combined and evaporated to give a pure compound designated as metabolite **5** (17 mg)**.**

2.2.3. Curcumin transformation by *Cunninghamella blackesleeana* NRRL 1369

The residue (800 mg) obtained from the fermentation reaction (ended after 14 days), as previously described, was loaded on the top of silica gel column (32 g, 25×1cm). Elution was adopted starting with 100 % hexane and gradually increasing polarity by 5% ethyl acetate and up to 45%. Fifty fractions (25 mL each) were collected and examined on TLC, using methylene chloride-ethyl acetate (9:1, v/v) as a mobile phase. Fractions 9-15 were combined and evaporated to dryness to give 30 mg residue. Purification was carried out using sephadex LH-20 column (10 g, 25×1 cm), and methanol for HPLC as a mobile phase. Twenty sub-fractions (2 mL each) were collected and examined on TLC using solvent system: methylene chloride-ethyl acetate (9:1, v/v). Sub-fractions 3-9 were combined and evaporated to give a pure compound designated as metabolite **6** (26 mg). Fractions 31-36 were combined and evaporated to dryness to give a 20 mg residue. Further purification was carried out using sephadex LH-20 column (10 g, 25×1cm), and methanol for HPLC as a mobile phase. Twenty sub-fractions (3 mL each) were collected and examined on silica gel G sheets using methylene chloride-ethyl acetate (9:1, v/v) as solvent system. Subfractions 12-16 were collected and evaporated to yield a pure bright yellow amorphous powder designated as metabolite **7** (15 mg).

2.2.4. Curcumin transformation by *Cunninghamella elegans* NRRL 2310:

The same procedures for fermentation (ended after 10 days) and extraction were carried out as previously mentioned. The residue (750 mg) was loaded on the top of silica gel column (30 g, 25×1 cm). Elution was adopted starting with methylene chloride and gradually increasing polarity by methanol by 5% increment up to 40%. Forty five fractions (25 mL each) were collected and examined on precoated silica gel G sheet, using solvent system: methylene chloride-methanol (17:1, v/v). Fractions 19-24 eluted with methylene chloride-methanol (80:20, v/v) showed one major spot on TLC. They were combined and evaporated to give brownish residue (21 mg) which further purified using sephadex LH-20 column (10 g, 25×1 cm) and eluted with methanol for HPLC. Thirty sub-fractions were collected (5 mL each) and examined on silica gel G sheet using solvent system: methylene chloride-methanol (17:1, v/v). Sub-fractions 21-25 were combined and evaporated to give a pure compound designated as metabolite **8** (18 mg).

2.3. Biological activity

2.3.1. Antioxidant activity

The antioxidant activity of the substrate (curcumin) and its metabolites were investigated using DPPH radical scavenging method [12]. All measurements were performed in triplicate. IC_{50} values were calculated by four parameter logistic (4PL) nonlinear regression analysis and expressed as mean ± SE (n=3). The results are compiled in table (1).

2.3.2. Cytotoxic activity

Cytotoxic activity of curcumin and its metabolites against Caco-2 colorectal cancer cell line was investigated using SRB assay [13]. The results are illustrated in table (2).

2.3.3. Antimicrobial Activity

The antibacterial and antifungal activities of curcumin and its isolated metabolites were assessed using agar well diffusion method [14]. Test solutions were prepared for curcumin and its metabolites in DMSO at concentrations of 0.5 and 1 mg/mL. Experiments were run in triplicates, both (+ve) and (-ve) controls were run simultaneously and results were recorded by measuring the diameter of inhibition zones in millimeter. The results are recorded in tables (3 and 4).

3. RESULTS

3.1. Identification of the isolated metabolites

Metabolite **1** was obtained as white crystals (20 mg, 5% yield), m.p. 91°C. It exhibited a blue fluorescence at 365 nm, and a dark violet color when sprayed with anisaldehyde/sulfuric acid reagent. R_f value=0.76 (solvent system: methylene chloride-methanol, 19:1, v/v). (+) ESI-Mass spectrum showed the presence of a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 373. The UV spectrum (MeOH) of metabolite **1** showed λ_{max}at 217, 246 and 280 nm. IR (KBr) cm^{-1}: 3446, 3046, 3015, 2956, 2919, 1629, 1507, 1225 and 1153. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.57 (2H, t, *J*=7.2 Hz, H-1), 2.87 (2H, t, *J*=7.2 Hz, H-2), 3.50 (2H, s, H-4), 2.87 (2H, t, *J*=7.2 Hz, H-6), 2.57 (2H, t, *J*=7.2 Hz, H-7), 6.70 (1H, s, H-2`), 6.83 (1H, d, *J*=8 Hz, H-5`), 6.67 (1H, d, *J*=8 Hz, H-6`), 3.87 (3H, s, $OCH_3$`), 6.70 (1H, s, H-2``), 6.83 (1H, d, *J*=8 Hz, H-5``), 6.67 (1H, d, *J=8 Hz*, H-6``), 3.87 (3H, s, $OCH_3$``).^{13}C-NMR (100 MHz, $CDCl_3$): δ 29.1 (C-1), 45.4 (C-2), 203.5 (C-3), 45.7 (C-4), 203.5 (C-5), 45.4 (C-6), 29.1(C-7), 120.8 (C-1`), 114.6 (C-2`), 147.0 (C-3`), 143.9 (C-4`), 115.7 (C-5`), 132.8 (C-6`), 56.0 (OCH_3`), 120.8 (C-1``), 114.6 (C-2``), 147.0 (C-3``), 143.9 (C-4``), 115.7 (C-5``), 132.8 (C-6``), 56.0 (OCH_3``). The results were compared with the previously published data [15-17] and this metabolite was identified as tetrahydrocurcumin.

Metabolite **2** was obtained in the form of white crystals (22 mg, 5.5% yield), m.p. 80°C with R_f value of 0.6 (solvent system: methylene chloride-methanol, 19:1, v/v). It exhibited blue fluorescence under UV at 365 nm, and gave violet spot when sprayed with anisaldehyde/sulfuric acid reagent. (+) ESI-Mass spectrum showed the presence of a pseudo- molecular ion peak $[M+H]^+$ at m/z 375. The UV spectrum (MeOH) of metabolite **2** showed λ_{max} at 216, 237, 279 and 368 nm. IR (KBr) cm^{-1}: 3396, 3046, 3013, 2956, 2928, 1706, 1603, 1516, 1223 and 1096.^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.82 (2H, t, *J*=8.0 Hz, H-1), 2.72 (2H, t, *J*=8.0 Hz, H-2), 2.54 (2H, m, H-4), 4.03 (1H, m, H-5), 1.69 (2H, m, H-6), 2.66 (2H, dd, *J*=9.6, 7.2 Hz, H-7), 6.69 (1H, s, H-2`), 6.80 (1H, d, *J*=8.0 Hz, H-5`), 6.64 (1H, d, *J*=8.0 Hz, H-6`), 3.86 (3H, s, $OCH_3$`), 6.69 (1H, s, H-2``), 6.80 (1H, d, *J*=8.0 Hz, H-5``), 6.64 (1H, d, *J*=8.0 Hz, H-6``), 3.86 (3H, s, $OCH_3$``). ^{13}C-NMR (100 MHz, $CDCl_3$): δ29.5 (C-1), 45.6 (C-2), 211.7 (C-3), 49.9 (C-4), 67.2 (C-5), 38.6 (C-6), 31.6 (C-7), 133.9 (C-1`), 111.4 (C-2`), 146.7 (C-3`),

144.1(C-4`),114.9 (C-5`), 121.1 (C-6`), 56.1($OCH_3$`), 132.8 (C-1``), 111.2 (C-2``), 146.6 (C-3``), 143.9 (C-4``), 114.7 (C-5``), 120.9 (C-6``), 56.0 (OCH_3``). Metabolite **2** was identified as hexahydrocurcumin after comparing the results with the previously published data [17-18].

Metabolite **3** was obtained in the form of off-white crystals (24 mg, 6.0% yield), m.p. 98°C with R_f value 0.35 (solvent system: methylene chloride-methanol, 19:1, v/v). The metabolite exhibited a bright blue fluorescence under UV at 365 nm, and gave violet color when sprayed with anisaldehyde/sulfuric acid reagent. (+) ESI-Mass spectrum showed the presence of a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 377. UV spectrum (MeOH) of metabolite **3** showed λ_{max} at 217, 234 and 284 nm. IR (KBr) cm^{-1}: 3398, 3033, 2949, 2889 1604, 1514, 1221 and 1094. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.65 (2H, t, *J*=7.2 Hz, H-1), 1.75 (2H, m, H-2), 3.88 (1H, m, H-3), 1.60 (2H, t, *J*=8.0 Hz, H-4), 3.88 (1H, m, H-5), 1.75 (2H, m, H-6), 2.65 (2H, t, *J*=7.2 Hz, H-7), 6.69 (1H, s, H-2`), 6.82 (1H, d, *J*=8.0 Hz, H-5`), 6.78 (1H, d, *J*=8 Hz, H-6`), 3.84 (3H, s, $OCH_3$`), 6.69 (1H, s, H-2``), 6.82 (1H, d, *J*=8.0 Hz, H-5``), 6.78 (1H, d, *J*=8 Hz, H-6``), 3.84 (3H, s, $OCH_3$``). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 31.6 (C-1), 40.2 (C-2), 72.7 (C-3), 43.3 (C-4), 72.7 (C-5), 40.2 (C-6), 31.6 (C-7), 133.9 (C-1`), 111.4 (C-2`), 146.7 (C-3`), 144.0 (C-4`), 114.7 (C-5`), 121.1 (C-6`), 56.1 (OCH_3`), 133.9 (C-1``), 111.4 (C-2``), 146.7 (C-3``), 144.0 (C-4``), 114.7 (C-5``), 121.1 (C-6``), 56.1 (OCH_3``). The metabolite was identified as octahydrocurcumin [17-18].

Metabolite **4** was obtained in the form of light yellow amorphous powder (16 mg, 4.0% yield), R_f value 0.41 (solvent system-methylene chloride:methanol,19:1, v/v). It exhibited a blue fluorescence under UV at 365 nm and gave purple color when sprayed with anisaldehyde/sulfuric acid reagent. (+) ESI-Mass spectrum showed the presence of pseudo-molecular peak $[M+H]^+$ at *m/z* 345. UV spectrum (MeOH) of metabolite **4** showed λ_{max} at 218, 238 and 281 nm. IR (KBr) cm^{-1}:3429, 3020, 2951, 2924, 1707, 1604, 1515, 1225 and 1100. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 2.81 (2H, t, *J*=7.6 *Hz*, H-1), 2.71 (2H, m, H-2), 2.53 (2H, m, H-4), 4.02 (1H, m, H-5), 1.69 (2H, m, H-6), 2.71 (1H, m, H-7a), 2.60 (1H, dd, *J*=9.6*,* 7.2 Hz, H-7b), 7.02 (1H, d, *J*=8.0 Hz, H-2`), 6.73 (1H, d, *J=8.0 Hz*, H-3`), 6.73 (1H, d, *J*=8.0 Hz, H-5`), 7.02 (1H, d, *J=8.0 Hz*, H-6`), 6.70 (1H, s, H-2``), 6.82 (1H, d, *J*=8.0 Hz, H-5``), 6.65 (1H, d, *J*=8.0 Hz, H-6``), 3.85 (3H, s, $OCH_3$``). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 28.9 (C-1), 45.4 (C-2), 211.6 (C-3), 49.5 (C-4), 67.2 (C-5), 38.4 (C-6), 31.0 (C-7), 132.8 (C-1`), 129.6 (C-2`), 115.5 (C-3`), 154.0 (C-4`), 115.5 (C-5`), 129.6 (C-6`), 134.1 (C-1``), 111.2 (C-2``), 146.6 (C-3``), 144.1 (C-4``), 115.1 (C-5``), 121.1 (C-6``), 56.1 (OCH_3``). This metabolite was identified as demethoxyhexahydrocurcumin [17-18].

Metabolite **5** was obtained in the form of pale yellow amorphous powder (17 mg, 4.25% yield) with R_f value 0.20 (solvent system: methylene chloride-methanol,19:1, v/v).The compound showed a blue florescence under UV at 365 nm and gave faint purple color when sprayed with anisaldehyde/sulfuric acid reagent. (+) ESI-Mass spectrum showed the presence of a pseudo-molecular ion peak $[M+H]^+$ at m/z 347. UV spectrum (MeOH) of metabolite **5** showed λ_{max} at 216, 233, 258 and 317 nm. IR (KBr) cm^{-1}: 3428, 3031, 2923, 2863, 1605, 1512, 1225, and 1096. ^{1}H-NMR (400 MHz, $CDCl_3$): δ3.31 (2H, t, *J*= 7.2 Hz, H-1), 2.66 (2H, m, H-2), 4.05 (1H, m, H-3), 1.58 (2H, t, *J*=7.6 Hz, H-4), 4.05 (1H, m, H-5), 1.67 (2H, m, H-6), 2.66 (2H, m, H-7), 7.04 (1H, d, *J*=8.0 Hz, H-2`), 6.74 (1H, d, *J*=8.0 Hz, H-3`), 6.74 (1H, d, *J*=8.0 Hz, H-5`), 7.04 (1H, d, *J*=8.0 Hz, H-6`), 6.69 (1H, s, H-2``), 6.81 (1H, d, *J*=8.0 Hz, H-5``), 6.66 (1H, d, *J*=8.0 Hz, H-6``), 3.86 (3H, s, $OCH_3$``). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 28.0 (C-1), 43.3 (C-2), 67.3 (C-3), 42.2 (C-4), 65.2 (C-5), 37.5 (C-6), 32.1 (C-7), 133.8 (C-1`), 129.7 (C-2`), 115.4 (C-3`), 154.3 (C-4`), 115.4 (C-5`), 129.7 (C-6`), 135.0 (C-1``), 110.1 (C-2``), 146.9 (C-3``), 144.3 (C-4``), 115.2 (C-5``), 121.2 (C-6``), 56.2 (OCH_3``). Metabolite **5** was identified as demethoxyoctahydrocurcumin [17-18].

Metabolite **6** was obtained in the form of white crystals (26 mg, 6.5% yield), m.p. 82°C, R_f value 0.64 (solvent system: methylene chloride-ethyl acetate, 9:1, v/v). The metabolite exhibited a gray color (UV) at 254 nm and gave yellowish orange color when sprayed with 2,4-Dinitrophenylhydrazine reagent. (+) ESI-Mass spectrum of metabolite **6** revealed the presence of a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 153. UV spectrum (MeOH) of the compound showed λ_{max} at 314 nm. IR (KBr) cm^{-1}: 3179, 3025, 2980, 2947, 2856, 2741, 1668, and 1264. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.43 (1H, d, *J*=2.0 Hz, H-2), 7.04 (1H, d, *J*=7.6 Hz, H-5), 7.40 (1H, dd, *J*=7.6, 2.0 Hz, H-6), 3.96 (3H, s, OCH_3), 9.81 (1H, s, CHO).^{13}C-NMR (100 MHz, $CDCl_3$): δ 130.0 (C-1), 114.4 (C-2), 151.7 (C-3), 147.2 (C-4), 108.9 (C-5), 127.5 (C-6), 56.2 (OCH_3), 190.8 (CHO). The compound was identified as vanillin [19-20].

Metabolite **7** was obtained as a pure bright yellow amorphous powder 15 mg, 3.75% yield, R_f 0.43 (solvent system: methylene chloride-ethyl acetate, 9:1, v/v). It has a yellow fluorescence under UV at 365 nm and showed dark yellow spot when sprayed with anisaldehyde/sulfuric acid reagent. ESI-Mass spectrum showed a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 341. UV spectrum (MeOH) of metabolite **7** showed λ_{max} at 250 and 419 nm. IR spectrum cm^{-1}: 3412, 3065, 3013, 2947, 1629, 1599, 1508, 1233, 1157 and 1027. ^{1}H-NMR (400 MHz, acetone-d_6): δ 7.61 (1H, d, *J*=16.0 Hz, H-1), 6.71 (1H, d, *J*=16.0 Hz, H-2), 5.99 (1H, s, H-4), 6.71 (1H, d, *J*=16.0 Hz, H-6), 7.61 (1H, d, *J*=16.0 Hz, H-7), 7.32 (1H, s, H-2`), 6.90 (1H, d, *J*=8.0 Hz, H-5`), 7.18 (1H, d, *J*= 8.0 Hz, H-6`), 7.32 (1H, s, H-2``), 6.90 (1H, d, *J*=8.0 Hz, H5``), 7.18 (1H, d, *J*=8.0 Hz, H6``).$^{13}$C-NMR (100 MHz, acetone-$d_6$): δ141.8 (C-1), 122.8 (C-2), 184.9 (C-3), 102.1 (C-4), 184.9 (C-5), 122.8 (C-6), 141.8 (C-7), 128.7 (C-1`), 115.6 (C-2`), 146.8 (C-3`), 149.1 (C-4`), 116.7 (C-5`), 123.1 (C-6`), 128.7 (C-1``), 115.6 (C-2``), 146.8 (C-3``), 149.1 (C-4``), 116.7 (C-5``), 123.1 (C-6``). The metabolite was identified as didesmethylcurcumin [21].

Metabolite **8** was obtained as an orange amorphous powder (18 mg, 4.5% yield), R_f value 0.28 (solvent system: methylene chloride-methanol, 17:1, v/v). It exhibited a yellowish orange fluorescence under UV at 365 nm and showed orange spot when sprayed with anisaldehyde/sulfuric acid reagent. It gave a dark brown color when treated with 10% H_2SO_4 and heated, and gave positive Molisch's test. The acid hydrolysis of metabolite **8** yielded glucose (Co-TLC) and curcumin aglycone (Co-TLC). (+) ESI-Mass spectrum of metabolite **8** displayed a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 531. UV spectrum (MeOH) of metabolite **8** showed λ_{max}at 266 and 428nm. IR (KBr) cm^{-1}: 3396, 2966, 2835, 1629, 1600, 1507, 1278, 1154 and 1023. ^{1}H-NMR (400 MHz, acetone-d_6): δ 7.65 (1H, d, *J*=16.0 Hz, H-1), 6.78 (1H, d, *J*=16.0 Hz, H-2), 6.01 (1H, s, H-4), 6.74 (1H, d, *J*=16.0 Hz, H-6), 7.61 (1H, d, *J*=16.0 Hz, H-7), 6.96 –7.23 (6H, m, H-2`, 5`, 6`, 2``, 5``, 6``), 3.93 (3H, s, $OCH_3$`), 3.91 (3H, s, $OCH_3$``), 5.05 (1H, d, *J*=7.2 Hz, Glc-H-1), 3.99 – 4.26 (6H, m, Glc-H). ^{13}C-NMR (100 MHz, acetone-d_6): δ 140.9 (C-1), 123.0 (C-2), 184.2 (C-3), 101.1 (C-4), 183.7 (C-5), 122.7 (C-6), 139.9 (C-7), 129.7 (C-1`), 111.5 (C-2`), 149.2 (C-3`), 150.0 (C-4`), 116.3 (C-5`), 122.1 (C-6`), 55.6 (OCH_3`), 127.3 (C-1``), 110.7 (C-2``), 147.9 (C-3``), 149.6 (C-4``), 115.4 (C-5``), 121.4 (C-6``), 55.4 (OCH_3``), 101.0 (C- 1` Glu), 77.1 (C- 2` Glu), 77.0 (C- 3` Glu), 73.8 (C- 4` Glu), 70.5 (C- 5` Glu), 61.7 (C- 6`Glu).The compound was identified as curcumin-4`-*O*-β-D-glucoside [22].

3.2. Biological activity

3.2.1. Antioxidant activity

The antioxidant activity of curcumin and its isolated metabolites was evaluated using DPPH radical scavenging method. The results are presented in table 1. The results showed that all the tested metabolites and the substrate had radical scavenging activities and their activities were dose dependent. Metabolite **7** presented the most potent activity, followed by metabolite **8**, then, metabolites **1**, **2**, **3**, **4**, **5** and **6**, respectively. Vanillin (metabolite **6**) has an IC_{50} almost three fold to that of curcumin which indicate its lower activity comparing to the other tested compounds.

3.2.2. Cytotoxic activity

The cytotoxic activity of curcumin and the isolated metabolites were assessed using SRB assay. The results showed that all tested compounds had cytotoxic activity and they were dose dependent. Metabolites **8**, **7**, **1**, **3** and **2**, respectively presented more cytotoxic activity than that of curcumin, but relatively, less potent than doxorubicin, which was used as a positive control in this study. On the other hand, metabolites **4**, **5** and **6** presented lower activity with higher IC_{50} values than curcumin (table 2).

3.2.3. Antimicrobial Activity

The antibacterial and antifungal activities of curcumin and its isolated metabolites were assessed using agar well diffusion method. All tested compounds showed antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, and *Aspergillus niger* with variable degrees. Metabolites **8**, **7**, **1**, **2** and **3** respectively exhibited higher antibacterial activity than that of curcumin against *Escherichia coli*, while metabolite **5** showed activity similar to curcumin with the same inhibition zone (16.0 mm) at a dose of 1 mg/mL. Metabolites **7**, **8**, **1**, **2** and **3** respectively presented higher antimicrobial activity against *Staphylococcus aureus* than curcumin. Metabolite **4** showed activity similar to that of curcumin with the same inhibition zone (14.0 mm) at a dose of 1 mg/mL, while metabolites **5** and **6** showed less activity than curcumin against *Staphylococcus aureus* (table 3). Metabolites **7**, **8**, **1**, **2** and **3**, respectively exhibited higher antifungal activity against the two species of the tested fungi at a dose of 1mg/mL. Metabolite **6** presented similar activity to curcumin against *Aspergillus niger* (inhibition zone=12 at 0.5 mg/mL, and 13 mm at 1mg/mL). The other tested metabolites showed lower antifungal activity against the two tested species (table 4).

Table (1): Antioxidant activity of curcumin and its isolated metabolites

Compound	Conc. (µg/mL)					
	100	**50**	**25**	**12.5**	**6.25**	**IC_{50} (µg/ml)**
Curcumin	***69.82±0.02***	***67.16±0.02***	***37.24±0.02***	***33.64±0.83***	***31.95±0.81***	***33.91±1.85***
Metabolite **1**	62.94±0.53	61.12±0.63	54.78±0.13	35.31±0.09	30.10±0.05	18.56±0.77
Metabolite **2**	68.47±0.15	67.90±0.17	59.33±0.05	46.79±0.07	44.62±0.06	22.26±0.32
Metabolite **3**	66.61±0.07	64.45±0.11	54.73±0.06	43.64±0.09	41.28±0.06	24.00±0.27
Metabolite **4**	71.62±0.05	61.42±0.16	41.23±0.29	35.95±1.03	33.21±0.81	38.91±2.45
Metabolite **5**	77.91±0.02	62.73±0.05	40.13±0.21	36.80±1.13	34.37±0.95	44.14±2.32
Metabolite **6**	37.25±0.40	30.96±0.30	26.32±0.01	24.14±0.32	22.54±0.21	93.18±4.85
Metabolite **7**	67.15±0.54	65.42±0.71	63.27±0.19	46.39±0.02	10.36±0.01	8.62±1.44
Metabolite **8**	62.11±0.11	61.44±0.13	56.43±0.03	39.21±0.01	32.60±0.01	16.92±0.15
Quercetin	***76.96±0.51***	***76.72±0.62***	***63.12±0.15***	***27.18±0.08***	***14.03±0.04***	***17.64±0.37***

* Data obtained from triplicate determinations (n=3) and shown as mean±SE. IC_{50} value was calculated by four parameter logistic (4PL) nonlinear regression analysis and expressed as mean±SE (n=3).

Table (2): Cytotoxic activity of curcumin and its isolated metabolites against Caco-2 cell line

Compound	Conc.(µg/ml)				
	50	**25**	**12.5**	**5**	**IC_{50} (µg/ml)**
Curcumin	***0.43±0.05***	***0.54±0.02***	***0.59±0.02***	***0.84±0.07***	***33.80±0.94***
Metabolite **1**	0.36±0.07	0.45±0.01	0.53±0.04	0.76±0.03	16.80±0.81
Metabolite **2**	0.39±0.08	0.52±0.05	0.62±0.06	0.80±0.09	28.00±0.69
Metabolite **3**	0.39±0.04	0.46±0.02	0.55±0.01	0.61±0.03	19.46±0.78
Metabolite **4**	0.50±0.08	0.60±0.04	0.70±0.09	0.84±0.06	50.00±1.25
Metabolite **5**	0.49±0.07	0.53±0.03	0.64±0.08	0.67±0.04	47.80±1.02
Metabolite **6**	0.60±0.09	0.62±0.07	0.63±0.06	0.67±0.08	> 50.00
Metabolite **7**	0.24±0.05	0.24±0.05	0.46±0.07	0.66±0.02	11.12±0.38
Metabolite **8**	0.37±0.03	0.43±0.02	0.48±0.03	0.53±0.06	9.47±0.52
Doxorubicin	***0.35±0.04***	***0.39±0.04***	***0.40±0.05***	***0.41±0.01***	***4.19±0.33***

***** Data obtained from triplicate determinations (n=3) and shown as mean±SE. IC_{50} value is defined as the concentration of 50% survival of Caco-2 cells.

Table (3): Antibacterial activity of curcumin and its isolated metabolites

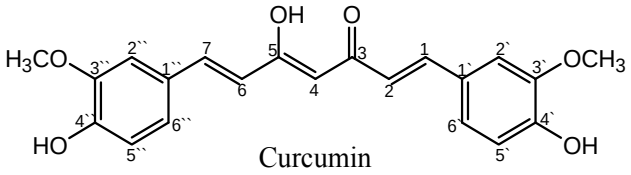
Compound	***Escherichia coli****		***Staphylococcus aureus****	
	0.5 mg/mL	**1 mg/mL**	**0.5 mg/mL**	**1 mg/mL**
DMSO	-	-	-	-
Curcumin	***14±0.0***	***16±0.0***	***13±0.0***	***14.5±0.5***
Metabolite **1**	22±0.0	24±0.0	23±0.0	24.5±0.5
Metabolite **2**	19.5±0.5	22±0.0	18±1.0	20±0.0
Metabolite **3**	15±0.0	17±0.0	17±0.0	18.5±0.5
Metabolite **4**	12±0.0	13±0.0	12.5±0.5	14±0.0
Metabolite **5**	13±0.0	16.5±0.5	11±0.0	12±0.0
Metabolite **6**	10.5±0.5	11±0.0	10±0.0	10.5±0.5
Metabolite **7**	23±0.0	24.5±0.5	22±0.0	26±1.0
Metabolite **8**	24±0.0	25±0.0	21.5±0.5	24±0.0
Gentamycin (10 µg/ml)	***31±0.0***		***32.5±0.5***	

Data obtained from triplicate determinations and shown as mean±SE.
*Zone of inhibition includes diameter of the well (10 mm).

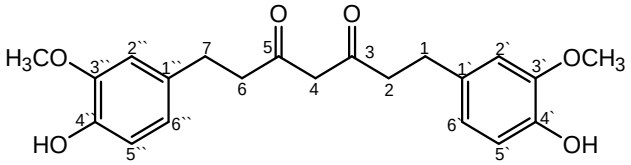
Table (4): Antifungal activity of curcumin and its isolated metabolites

Compound	***Candida albicans****		***Aspergillus niger****	
	0.5 mg/mL	**1 mg/mL**	**0.5 mg/mL**	**1 mg/mL**
DMSO	-	-	-	-
Curcumin	***14±0.0***	***15±1.0***	***12.5±0.5***	***13±0.0***
Metabolite **1**	20±0.0	22±0.0	19.5±0.5	21±1.0
Metabolite **2**	18±0.0	20±0.0	17±0.0	18.5±0.5
Metabolite **3**	15±0.0	16±1.0	14±1.0	17.5±0.5
Metabolite **4**	12±0.0	13±1.0	10±0.0	12.5±0.5
Metabolite **5**	13±0.0	14.5±0.5	10±0.0	11±1.0
Metabolite **6**	13±0.0	14.5±0.5	12.5±0.5	13±0.0
Metabolite **7**	21.5±0.5	23±0.0	20±0.0	23.5±1.5
Metabolite **8**	20.5±0.5	23±0.0	22.5±0.5	24±1.0
Fluconazole(30 µg/ml)	***30±1.0***		***29.5±0.5***	

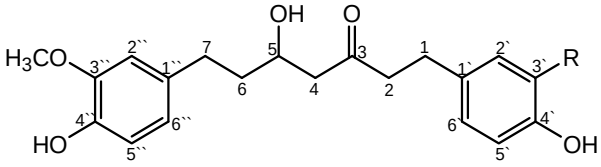
Data obtained from triplicate determinations and shown as mean±SE.
***** Zone of inhibition includes diameter of the well (10 mm).



Curcumin

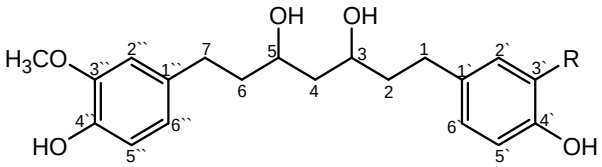


Tetrahydrocurcumin (metabolite **1**)



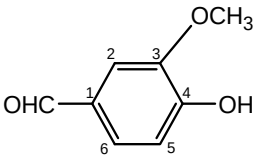
R= OCH3 Hexahydrocurcumin (metabolite **2**)

R= H Demethoxyhexahydrocurcumin (metabolite **4**)

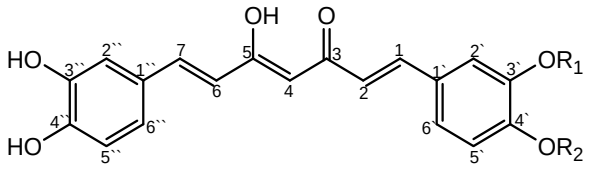


R= OCH3 Octahydrocurcumin (metabolite **3**)

R= H Demethoxyoctahydrocurcumin (metabolite **5**)



Vanillin (metabolite **6**)



$R_{1=}$ R_2= H didesmethylcurcumin (metabolite **7**)

R_1= CH_3 , R_2= Glu Curcumin-4`-*O*-*β*–*D*-glucoside (metabolite **8**)

Figure (1): Chemical structures of curcumin and its isolated metabolites.

4. DISCUSSION

Microbial transformation of curcumin was carried out after screening of more than forty strains of fungi of different classes. The study resulted in isolation of eight metabolites. The structures of these metabolites were established using physical and spectroscopic techniques including: melting points, UV, IR, ^{1}H-NMR, ^{13}C-NMR and ESI-MS**.** Identification of the isolated metabolites (figure 1) was based on comparison of the spectral data with those previously published.

(+) ESI-Mass spectrum of metabolite **1** showed the presence of a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 373 presumably corresponding to a molecular formula of $C_{21}H_{24}O_6$. The UV spectrum (MeOH) of metabolite **1** showed λ_{max}at 217, 246 and 280 nm. ^{1}H-NMR spectrum showed the absence of two doublet signals due to the four olefinic protons of curcumin (H-1, 2, 6, and 7) which suggests reduction of the two terminal double bonds at C-2 and C-6 of the aliphatic moiety. The two triplet signals interpreted for eight protons at 2.57 and 2.87 ppm could be assigned to protons at position H-1,7 and H-2,6, respectively. The presence of a singlet signal of two protons intensity at 3.50 ppm corresponding to two H-4 protons instead of methine proton singlet signal of curcumin at 5.85 ppm and downfield to typical methylene protons (< 3 ppm) suggested that the structure was in keto rather than enol form. The presence of signals of ten aliphatic protons at 2.57-3.50 ppm compared to five protons in curcumin confirmed reduction to the tetrahydro derivative. ^{13}C-NMR spectrum showed the high field resonances at 45.7 ppm (C-4), 45.4 ppm (C-2,6) and 29.1 ppm (C-1,7) substantiated the reduction of curcumin. A carbonyl signal at 203.5 ppm assignable to C-3,5 as aliphatic ketone in distinction of conjugated ketone at 183.3 ppm which confirmed the keto form of the structure. The ^{1}H and ^{13}C-NMR data of metabolite **1** were coincident with published data for tetrahydrocurcumin [15-17] and the chemical structure of the metabolite was elucidated as 1,7-bis(4-hydroxy-3 methoxyphenyl)heptan-3,5-dione or tetrahydrocurcumin in keto form.

Metabolite **2** displayed a pseudo-molecular ion $[M+H]^+$ at *m/z* 375 presumably corresponding to a molecular formula of $C_{21}H_{26}O_6$. UV spectrum (MeOH) of metabolite **2** showed λ_{max}at 216, 237, 279 and 368 nm. ^{1}H-NMR spectrum showed the absence of the two doublets integrated for four protons characteristic of the four olefinic protons of curcumin (H-1, 2, 6, 7) which suggests reduction of the two terminal double bonds at C-2 and C-6 of the aliphatic chain. The absence of methine proton singlet at 3.50- 4.50 ppm suggests reduction of central double bond at C-4 also, instead a multiplet signal at 4.03 ppm integrating for one proton characteristic of -CHOH or H-5. The presence of eleven protons resonating between 1.69 and 4.03 ppm compared to five protons in curcumin, substantiates the reduction to the hexahydro derivative. ^{13}C-NMR spectrum showed the presence of high field signals of the six aliphatic carbons (C-1, 2, 4, 5, 6, 7) at 29.5, 45.6, 49.9, 67.2, 38.6, and 31.6 ppm, respectively, indicating the reduction of the double bonds at these aliphatic carbons. The ^{1}H and ^{13}C-NMR data of metabolite **2** were identical to those reported for hexahydrocurcumin [17-18]. The chemical structure of the metabolite was elucidated as 5-hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-one or hexahydrocurcumin.

Metabolite **3** showed a pseudo-molecular ion peak $[M+H]^+$ at *m/z* 377 in (+)ESIMS presumably corresponding to a molecular formula of $C_{21}H_{28}O_6$, which was more than curcumin by 8 d, which can be attributed to reduction to the octahydro derivative. UV spectrum (MeOH) of metabolite **3** showed λ_{max} at 217, 234 and 284 nm. IR spectrum revealed the absence of carbonyl (C=O) peak at 1706 cm^{-1}, which suggests also the reduction of carbonyl group. ^{1}H-NMR spectrum revealed the appearance of a triplet at 2.65 ppm and a multiplet at 1.75 ppm each integrating for four protons (lacking in curcumin) which can be ascribed to four equivalent protons at C-1 and C-7 and four protons at C-2 and C-6 respectively. The absence of one proton intensity methine singlet of curcumin and appearance of a multiplet signal at 3.88 ppm integrating for two protons characteristic of -CHOH or H-3, 5 suggest reduction of both central double bond at C-4 and carbonyl group at C-3. The presence of thirteen signals between 1.60 and 3.88 ppm, compared to five protons in curcumin support the reduction to the octahydro derivative. ^{13}C-NMR showed the lack of low field carbonyl signal at183.3 ppm, which confirms reduction of carbonyl group. Appearance of four high field signals at 31.6, 40.2, 72.7 and 43.3 ppm, not encountered in curcumin confirms reduction of double bonds and can be assigned to seven aliphatic carbon atoms. ^{1}H and ^{13}C-NMR data of metabolite **3** were consistent with those published for octahydrocurcumin [17-18]. The chemical structure of the metabolite was thus established as 1,7-bis(4-methoxy-3-hydroxphenyl)heptan-3,5-diol or octahydrocurcumin.

Metabolite **4** exhibited a pseudo-molecular ion $[M+H]^+$ in (+) ESIMS at *m/z* 345, presumably corresponding to a molecular formula of $C_{20}H_{24}O_5$ (less than metabolite **2** by 30 d). The UV spectrum (MeOH) of metabolite **4** showed λ_{max}at 218, 238 and 281 nm.^{1}H-NMR spectrum showed a singlet signal at 3.85 ppm integrating for three protons suggesting the presence of only one methoxy group at C-3" of aromatic ring. This was confirmed by presence of five aromatic signals with a coupling constant of 8.0 Hz (four trans olefinic protons of curcumin were absent since they have a coupling constant of 16.0 Hz).^{13}C-NMR spectrum revealed the upfield shift of C-3` signal from 147.9 to 115.5 ppm suggests the absence of methoxy group at C-3`. The^{1}H and ^{13}C-NMR data of metabolite **4** were identical to those previously reported for demethoxyhexahydrocurcumin [17-18] and the chemical structure of the metabolite was elucidated as 5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1-(4-hydroxyphenyl)heptan-3-one or demethoxyhexahydrocurcumin.

Metabolite **5** displayed a (+) ESI-Mass pseudo-molecular ion at *m/z* 347 presumably corresponding to a molecular formula of $C_{20}H_{26}O_5$. UV spectrum (MeOH) of metabolite **5** showed λ_{max} at 216, 233, 258 and 317 nm. The IR spectrum revealed the absence of carbonyl (C=O) peak at 1706 cm^{-1}, which suggests the reduction of carbonyl group at C-3.^{1}H-NMR data of metabolite **5** showed a singlet signal at 3.86 ppm integrating for three protons suggesting presence of only one methoxy group at C-3" of aromatic ring (similar to metabolite **4**). Absence of methine proton singlet of curcumin in the range of 3.50- 4.50 and appearance of a multiplet signal at 4.05 ppm integrating for two protons characteristic of -CHOH or H-3 and 5 suggest reduction of both central double bond at C-4 and carbonyl group at C-3. The presence of twelve aliphatic protons at1.58- 4.03 ppm, compared to five protons of curcumin confirms reduction to the octahydro derivative. ^{13}C-NMR spectrum showed the absence of a low field carbonyl signal at 183.3 confirming reduction of carbonyl group. The presence of two hydroxylated carbons at 65.2 and 67.3 ppm of C-3 and C-5 also confirms the reduction of carbonyl group. The UV, IR, (+) ESI-Mass and ^{1}H and ^{13}C-NMR data of metabolite **5** were consistent with those of demethoxyoctahydrocurcumin [17-18] and the chemical structure of the metabolite was elucidated as 7-(4-hydroxy-3 methoxyphenyl)-1-(4-hydroxyphenyl)heptan-3,5-diol or demethoxyoctahydrocurcumin.

Metabolite **6** displayed a (+) ESI-Mass pseudo-molecular ion at *m/z*153 presumably corresponding to a molecular formula of $C_8H_8O_3$. The UV spectrum (MeOH) of the compound showed λ_{max} at 314 nm. ^{1}H-NMR spectrum showed seven protons signals suggesting that the compound was a degradation product of curcumin. The characteristic signals at 3.96 ppm of three proton intensity assigned to methoxy group at C-3 and a one singlet signal at 9.81 ppm indicative of aldehyde proton. The remaining three signals at 7.04, 7.40 and 7.43 ppm were for aromatic ring protons. UV, IR, (+) ESI-Mass and ^{1}H-NMR profile of metabolite **6** is consistent with those previously reported for vanillin [19].^{13}C-NMR spectrum of the metabolite showed a total of eight signals corresponding to eight different carbon atoms. A low field aldehyde carbon signal at 190.8 ppm and a characteristic high field methoxy carbon peak at 56.2 ppm were present with the remaining six signals

belonging to six aromatic carbons. Based on the ^{1}H and ^{13}C-NMR data and the aforementioned results, the chemical structure of metabolite **6** was established as 4-hydroxy-3-methoxybenzaldehyde or vanillin [19-20].

Metabolite **7** showed a pseudo-molecular peak $[M+H]^{+}$ at *m/z* 341 corresponding presumably to a molecular formula of $C_{19}H_{16}O_6$. UV spectrum (MeOH) of metabolite **7** showed λ_{max} at 250 and 419 nm. ^{1}H-NMR data of the compound showed almost complete resemblance to that of curcumin, the difference was the absence of the six protons singlet due to two methoxy group protons at C-3` and C-3``, suggesting demethylation of the two methoxy groups. This was confirmed by almost complete match of ^{1}H-NMR data of metabolite **7** with that of synthetic didesmethylcurcumin [21]. The ^{13}C-NMR data of the compound was similar to that of curcumin [17] with the sole distinction being the lack of methoxy carbon signal of curcumin at 55.4 ppm. Metabolite **7** was thus identified as 5-hydroxy-1,7-bis(3,4-dihydroxyphenyl)hept-1,4,6-triene-3-one or didesmethylcurcumin [21]. This metabolite was obtained for the first time via microbial transformation and it is the first time to report as a natural product.

Metabolite **8** gave positive Molisch's test. ESI-Mass spectrum of metabolite **8** displayed a pseudo-molecular ion peak $[M+H]^{+}$ at *m/z* 531 presumably corresponding to a molecular formula of $C_{27}H_{30}O_{11}$. UV spectrum (MeOH) of metabolite **8** showed λ_{max} at 266 and 428 nm. ^{1}H-NMR spectrum showed six proton multiplet at 3.99-4.26 ppm due to the sugar protons and the doublet of the anomeric proton (Glu-H-1`) resonating at 5.05 ppm. The anomeric proton chemical shift and the large coupling constant (*J*=7.2 Hz) indicate the *β*-linkage of glucose to curcumin. ^{13}C-NMR showed twenty seven carbon signals, which correspond to twenty one carbon atoms of curcumin and six carbons of glucose. There is slight downfield shift of C-4` signal providing evidence for glucosylation of the 4` hydroxyl group. Both ^{1}H-NMR and ^{13}C-NMR data of metabolite **8** were identical to those reported for curcumin-4`-*O*-*β*-D-glucoside [22].

Biological study

Antioxidant activity

The antioxidant mechanism of curcumin was attributed to its unique conjugated structure, which includes two methoxylate phenols and an enol form of β-diketone; the structure shows typical radical-trapping ability as a chain-breaking antioxidant [23-24]. In the present study, the radical scavenging activity of the substrate and its isolated metabolites was evaluated using DPPH radical scavenging method. The results revealed the dose dependent activity of all the tested compounds (table 1). Metabolite **7** presented the most potent activity (two fold to that of quercetin), followed by metabolite **8**, which was more potent than quercetin, then metabolites **1**, **2**, **3**, **4**, **5**, and **6** respectively. Attachment of glucose part to curcumin to give curcumin-4`-*O*-*β*–D-glucoside (metabolite **8**) lowered IC_{50} from 33.9 to 16.9 µg/mL together with significant increase in water solubility which can be utilized as a good antioxidant agent. Vanillin (metabolite **6**) has an IC_{50} almost three fold to that of curcumin which can be attributed to degradation of the substrate and loss of keto-enol aryl conjugated system [25-26].

Cytotoxic activity

Curcumin was proven to possess antiproliferative activity against different types of cancer. Colorectal cancer was chosen for investigating cytotoxic activity of curcumin and its metabolites since it is one of the leading cause of death worldwide responsible for about 10% of cancer-related mortality [19]. Caco-2, a human colorectal cancer cell line, was chosen to conduct this study [20]. Curcumin and its metabolites formed in intestine and liver and mainly excreted in feces, so colon is the most likely target for curcumin's cytotoxic activity. The results showed that all tested compounds had cytotoxic activity and they were dose dependent. Metabolites **8**, **7**, **1**, **3** and **2** respectively presented more cytotoxic activity than that of curcumin, but relatively, less potent than doxorubicin, which was used as a positive control in this study. These results suggested that curcumin and its metabolites may possess a chemotherapeutic role against colorectal cancer.

Antimicrobial Activity

It has been reported that curcumin possesses antibacterial and antifungal activities [27]. The antibacterial and antifungal activity of curcumin and its isolated metabolites were assessed using agar well diffusion method. All tested compounds showed antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger* with variable degrees. Metabolites **7**, **8**, **1**, **2** and **3** respectively presented higher antimicrobial activity and inhibition zone than those of curcumin. However, the activity of these metabolites were less potent than the synthetic Gentamycin at a dose of 10 µg/mL which was used as a positive control in this study. It is interesting to annotate that Gram+ve bacterial isolates were sensitive to curcumin. Present study is in agreement with Chattopadhyay et.al who reported that curcumin extract exhibited antibacterial activity against a broad range of microbes and especially Gram positive strains and multiple antibiotic resistant bacteria [28]. These results, also correlates with Shahi et.al who reported that curcumin exhibited potent growth inhibitory effect against Gram positive bacteria (*Staphylococcus aureus* and *Strepococcus mutans*), Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and pathogenic yeast *Candida albicans* [29].

The available experimental evidence suggests that although the promising pharmacological effects of curcumin, microbial transformation can produce more biologically active metabolites, which could exceed that of curcumin.

5. CONCLUSION

Eight metabolites namely: tetrahydrocurcumin (**1**), hexahydrocurcumin (**2**), octahydrocurcumin (**3**), demethoxyhexahydrocurcumin (**4**), demethoxyoctahydrocurcumin (**5**), vanillin (**6**), didesmethylcurcumin (**7**), and curcumin-4`-*O*-*β*-D-

glucoside (**8**) were isolated for the first time from curcumin biotransformation using the tested species (or strains). Metabolite **7** was obtained for the first time from natural sources. All the tested metabolites and the substrate showed dose dependent activity. Metabolite **7** exhibited more potent antioxidant activity (IC_{50} 8.62 µg/mL) than curcumin (IC_{50} 33.91 µg/mL) and quercetin (IC_{50} 17.64 µg/L. Metabolites **8**, **7**, **1**, **3** then **2** exhibited more potent cytotoxic effect (IC_{50} 9.47, 11.12, 16.80, 19.46 and 28.0 µg/mL respectively) than curcumin (IC_{50} 33.80µg/mL). Metabolites **7**, **8**, **1**, **2**, and **3**, respectively showed the highest antimicrobial activity, which was higher than that of curcumin.

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