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New Bioactive Metabolites from *Aspergillus flavus* 9AFL

Essam M. Eliwa[1,2], Mohammad M. El-Metwally[3], Ahmed H. Halawa [2], Ahmed M. El-Agrody[2], Ahmed H. Bedair [2], Mohamed Shaaban[1,4*]

[1]Organic and Bioorganic Chemistry, Department of Chemistry, Bielefeld University , D-33501 Bielefeld, Germany.
[2]Chemistry Department, Faculty of Science, Al-Azhar University, Nasr City-Cairo 11884, Egypt.
[3]Botany and Microbiology Department, Faculty of Science, Damanhour University, Damanhour, Egypt.
[4]Chemistry of Natural Compounds Department, Division of Pharmaceutical Industries, National Research Centre, El-Behoos St. 33, Dokki-Cairo 12622, Egypt.

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ABSTRACT:

*Fungi, the huge kingdom is a wide field for exploring of new and promising secondary metabolites. In this connection, the naturally new 5-chloro-2-methoxy-N-phenylbenzamide (**1**) and 5-acetoxy-3-hydroxy-3-methylpentanoic acid (**2**) were isolated from the terrestrial Aspergillus flavus 9AFL. This was together with linoleic acid, glycerol linoleate, cyclo(leucyl-prolyl), uracil and kojic acid methyl ether (**3**). Structures of the new compounds were confirmed by intensive studies of their 1D and 2D NMR spectral data, mass spectrometry, and comparison with the related literature. The cytotoxicity of the strain extract and compounds **1-2** were carried out against the human cervix carcinoma cell line (KB-3-1) in comparison with (+) – griseofulvin, showing negative potentiality.*

KEY WORDS: N-phenylbenzamide derivative, *Aspergillus flavus* 9AFL, Cytotoxicity

*** Corresponding author**

Mohamed Shaaban,
Email: mshaaba@gmail.com

INTRODUCTION:

Natural product research is a treasure trove for providing of novel and interesting chemical compounds. Microorganisms such as fungi and bacteria are rich sources for discovering of new bioactive natural compounds possessing unique skeletons with many functional groups [1]. The screening of fungal microorganisms is talented sources of interesting bioactive metabolites and became highly attractive after the discovery of penicillin G in 1928 by Alexander Fleming [2]. Hence, numerous pharmaceutical

companies and research groups were motivated to start sampling and screening large collections of fungal strains for antibiotics [3], antimycotics [4], antivirals [5], anticancers [6] and pharmacologically active agents [7].

In our continuous research program for new bioactive secondary metabolites from fungi [8-11], the terrestrial *Aspergillus flavus* 9AFL isolated from a soil sample in Egypt, was investigated. In the present article, the taxonomical characterization of the fungus, upscale fermentation, isolation, and structural identification of the produced new compounds (**1-2**) were discussed, and their cytotoxic activities were investigated as well.

MATERIALS AND METHODS:

General instrumental procedures

NMR spectra (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC) were measured on Bruker Avance DRX 500 MHz (125 MHz for ^{13}C NMR) spectrometer (Bruker, USA) using standard pulse sequences and referenced to residual solvent signals. Mass spectrometry experiments were performed using a Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer APEX III (Bruker Daltonik GmbH, Bremen, Germany) equipped with a 7.0 T, 160 mm bore superconducting magnet (Bruker Analytic GmbH -Magnetics, Karlsruhe, Germany), infinity cell, and interfaced to an external (Nano) ESI ion source. EI MS was recorded on a Finnigan MAT 95 spectrometer (70 eV) with per fluorokerosene as the reference substance for HR-EI-MS. The ultraviolet and visible (UV–Vis) spectra were measured on Spectro UV–Vis Double Beam PC8 scanning auto Cell UVD-3200, LABOMED, INC. Column chromatography was carried out on silica gel 60 (0.040–0.063 mm, Merck) and Sephadex LH-20 as the stationary phases. Preparative TLC (0.5 mm thick) and analytical TLC were performed with pre-coated Merck silica gel 60 $PF_{254+366}$. R_f values and visualisation of chromatograms was carried out under UV light (λ = 254 and 366 nm) and further by spraying with anisaldehyde/sulfuric acid followed by heating.

Fungal isolation and purification:

The dilution plate method was used as presented by Johnson et al. (1960) [12]. Ten grams of soil sample (cultivated loamy soil in Dakahliya, Egypt) was added to cylinder and water was added to the volume to reach 100 ml. The suspension is stirred and pours into 500 ml flask and shacked well for 10 min. Ten ml of this spore suspension was immediately drawn into 10 ml pipette and translocate into 90 ml of sterile tap water, 10 ml sample was then transferred immediately through successive 90 ml and so on till 10^{-6} dilution. One ml of the final dilution was transferred into a series Petri dishes and 15-20 ml of Czapek–Dox Agar medium (CDA) (g/L): sucrose (30), $NaNO_3$ (3),K_2HPO_4 (1), KCl (0.5), $MgSO_4$ (0.5), $FeSO_4$ (0.01), agar (20) and distilled water (1 L) at pH 7.3, after incubation period at 28°C for 3-5 days plate were examined and fungal colonies develop were identified.

Morphologically different fungal colonies were selected for purification. Purification of fungal colonies was carried out by repeated streaking on CDA plates, and incubating at 28°C for 7 days. The pure colonies obtained were transferred to fresh slants and stored at 4°C.

Phenotypic characterization of fungi

The fungus was identified on the basis of macroscopic and microscopic characteristics after culture on CDA media at 28°C for 7 days [13-16]. On CDA, colonies attaining 5-6 cm in diameter after 7 d at 28 C; margin

white; thin, conidial area yellow-green; exudate lacking; reverse colourless. Conidiophores colourless, long, usually arising from substrate hyphae, up to 1 mm x 12 µm wide, walls coarsely roughened; conidial heads typically radiate splitting into poorly-defined columns, small heads also present in the same culture; vesicles globose, sub-globose or flask-shaped, up to 45 µm in diameter; metulae absent in small heads present or absent in large ones. Conidia globose to sub-globose, nearly colourless to yellow-green, conspicuously echinulate, 3.5-5.0 µm in diameter, sometimes broadly elliptical, 4-5 x 3-4 µm; some isolates produce brownish sclerotia. The fungus 9AFL identified as *Aspergillus flavus*. The strain was deposited at the collection of Dr M.M. El-Metwally, Botany and Microbiology Department, Faculty of Science, Damanhour University, Egypt.

Fermentation on Czapek–Dox medium

Well-grown small pieces (2 cm^2) of 7-day-old *Aspergillus flavus 9AFL* colonies (grown at CDA) were inoculated into sterilised 50 of 0.5L Erlenmeyer flasks, each containing 200 mL of production CD medium. The inoculated flasks were statically incubated for 10 days at 28°C. After harvesting, the obtained yellowish-green broth was filtered over celite under vacuum. The mycelial extract was macerated in methanol (3 L), then the methanolic extract was concentrated in vacuo and the remaining water residue was re-extracted by ethyl acetate and concentrated.

The crude extract (3 g) was separated by column chromatography on silica gel (100× 10 cm) with a cyclohexane-CH_2Cl_2-MeOH gradient (0.5 L cyclohexane, 0.5 L cyclohexane-CH_2Cl_2 [1:1], 0.5 L CH_2Cl_2, 0.5 L CH_2Cl_2-MeOH [97:2], 1 L CH_2Cl_2-MeOH [95:5], 0.5 L CH_2Cl_2-MeOH [93:7], 1 L CH_2Cl_2-MeOH [90:10], 0.5 L CH_2Cl_2-MeOH [80:20], 0.5 L CH_2Cl_2-MeOH [50:50]; 0.5 L MeOH). According to TLC monitoring, six fractions were obtained: FI (0.5 g), FII (0.5 g), FIII (0.3 g), FIV (0.47 g), FV (0.53 g), FVI (0.7 g). Further column chromatography of fraction III on Sephadex LH-20 (CH_2Cl_2/40% MeOH) followed by silica gel (column 100 × 2 cm) with a cyclohexane-CH_2Cl_2 gradient to afford 5-chloro-2-methoxy-N-phenyl-benzamide as a colourless solid (**1**, 9 mg). Fraction IV delivered on silica gel linoleic acid as a colourless solid (10 mg). Purification of fraction V using Sephadex LH-20 (CH_2Cl_2/40% MeOH) afforded glycerol linoleate as a colourless oil (40 mg), Cyclo (Leucyl-Prolyl) (15 mg), and 5-acetoxy-3-hydroxy-3-methylpentanoic acid as a colourless semi-solid (**2**, 1 mg). Finally, purification of fraction VI on Sephadex LH-20 (MeOH) led to a colourless solid of kojic acid methyl ether (**3**, 2 mg), and a colourless semi-solid of uracil (10 mg).

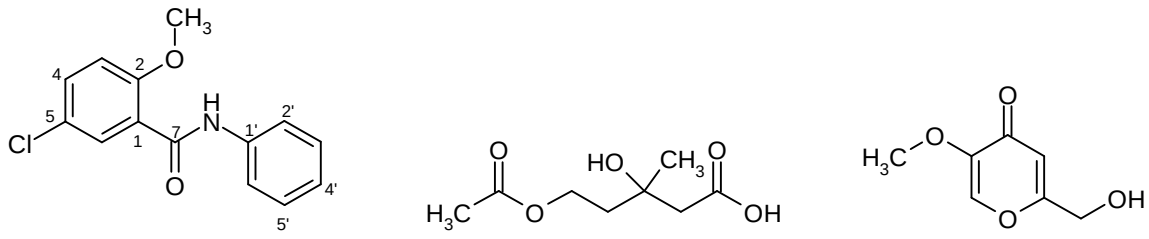
Cytotoxicity resazurin assay

KB 3-1 cells were cultivated as a monolayer in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/L), L-glutamine, sodium pyruvate and phenol red, supplemented with 10 % of KB 3-1 and foetal bovine serum (FBS). Cells were maintained at 37 °C and 5.3 % CO_2- humidified air. One day prior to the test, cells of 70 % confluence were detached with 0.05 % / 0.02 % of trypsin-ethylendiaminetetraacetic acid solution in Dulbecco´s Phosphate Buffered Saline (DPBS) and placed in sterile 96-well plates in a density of 10000 cells in 100 µL medium per well. The dilution series of the compounds were prepared from stock solutions in DMSO of concentrations of 100 µM, 50 µM or 25 µM. The stock solutions were diluted with culture medium (10 % FBS [KB-3-1]) down to pM range and dilutions added to the wells. Each concentration was

tested in six replicates. Dilution series were prepared by pipetting liquid from well to well, with the control containing the same concentration of DMSO as the first dilution. After incubation for 72 h at 37 °C and 5.3 % CO_2-humidified air, 30 µL of aqueous resazurin solution (175 µM) was added to each well. The cells were incubated under the conditions mentioned above for further 5 h and fluorescence was measured (λ_{ex} = 530 nm, λ_{em} = 588 nm). Results are given as IC_{50}, calculated as average of two determinations by sigmoidal dose-response curve fitting model using Graph Pad Prism software version 4.03 [17, 18].

RESULTS AND DISCUSSION:

The terrestrial *Aspergillus flavus* 9AFL was applied to upscale cultivation on rice solid medium, followed by working up and successive purification using different chromatographic techniques affording the two naturally new compounds, 5-chloro-2-methoxy-N-phenylbenzamide (**1**) and 5-acetoxy-3-hydroxy-3-methylpentanoic acid (**2**). Further five known compounds were isolated and characterized as linoleic acid [19], glycerol linoleate [9], cyclo-(Leu-Prol) [9], uracil [19], and kojic acid methyl ether (**3**) [20]. Structures of the new metabolites (**1**, **2**) along with **3** (Figure 1), were fully assigned using intensive examination of 1D, 2D-NMR, and MS spectrometric data. The physico-chemical properties of 5-chloro-2-methoxy-N-phenylbenzamide (**1**) and 5-acetoxy-3-hydroxy-3-methylpentanoic acid (**2**) are listed in Table 1.



1 **2** **3**

Fig 1: Structure of compounds 1-3 isolated from *Aspergillus flavus* 9AFL

Table 1 Physico-chemical properties of compounds 1-2

	1	2
Appearance	colourless solid	colourless semi-solid
R_f[a]	0.66[b]	0.28[c]
Staining with anisaldehyde/ sulfuric acid	-	violet
Molecular formula	$C_{14}H_{12}ClNO_2$ (261)	$C_8H_{14}O_5$ (190)
(+)-ESI-MS: *m/z* (%)	284/286 ($[M+Na]^+$, 100/30), 545/547 ($[2M+Na]^+$, 31/10)	-
(-)-ESI-MS: *m/z* (%)	260/262 $[M-H]^-$	189 $[M-H]^-$
EI-MS: m/z (%)	261/263 ($[M]^{+\cdot}$, 25/8), 169/171 ($[M-NHPh]^+$, 100/33), 126 (14), 111 (12)	-
HREI-MS: (*m/z*)	261.05403 (calcd. 261.05511 for $C_{14}H_{12}ClNO_2$)	-
UV/VIS λ_{max} (log ε) nm	($CHCl_3$, c = 5): 240.5 (3.25)	-

[a]Silica gel G/UV254, [b] (CH_2Cl_2 100%), [c](CH_2Cl_2/ 10% MeOH)

5-Chloro-2-methoxy-N-phenylbenzamide

Compound **1** was isolated as a colourless solid, showing UV absorbance on TLC, nevertheless it showed no colour staining by spraying with anisaldehyde/sulfuric acid. The UV spectrum of **1** displayed a peak at λ_{max} = 240.5 nm, referring to its aromatic nature. According to (+) ESI-MS, two doublet *pseudo*-ion peaks were shown at *m/z* = 284/286 $[M+Na]^+$ and 545/547 $[2M+Na]^+$, while an ion peak was observed at *m/z* = 260/262 $[M-H]^-$ in negative mode, indicating the molecular weight of **1** as 261/263 (3:1) Dalton. The odd mass number confirmed the existence of odd number of nitrogen atoms, and the intensity (3:1) of shown doublet ion confirmed the presence of one chlorine atom. This conclusion was further confirmed by the HR-EI-MS, establishing the molecular formula as $C_{14}H_{12}ClNO_2$ with 9 double bond equivalents (DBE) (Table 1).

During the study of the ^{1}H NMR spectrum, eight signals integrated for 12 protons, were visible as matching with the revealed molecular formula. In the downfield region, a broad singlet ^{1}H resonance was shown at $\delta = 9.73$ ppm, being for an acidic proton (NH or OH). In the aromatic region, six signals were observed between $\delta = 8.28$ and 6.99 ppm, two among them integrated with 2H ($\delta = 7.67$, 7.39 ppm), constructing 8 aromatic methines. In the aliphatic region, 3H singlet was observed at $\delta = 4.07$ ppm is attributed to an aromatic-bounded methyl ether (OCH_3). Based the ^{1}H NMR spectrum and according to the number 9 DBE aforementioned, two aromatic rings (A and B) were proposed.

The ^{13}C NMR/HMQC spectra showed 12 carbon signals representing 14 carbons (as matched with the molecular formula) in the region between $\delta = 161.8$ and 56.6 ppm, classified into one carbonyl group (161.8 ppm) of ester or amide (Table 2), eight aromatic methines, four sp^2 quaternary carbons, and an aromatic bound methoxy (56.6 ppm).

According to ^{1}H,^{1}H-COSYspectrum, the first aromatic reside (A) was deduced as 1,2,5-trisubstituted system, at where the *o*-coupled protons at $\delta = 7.45$ (H-4) and 6.99 (H-3) exhibited a strong COSY connectivity. The earlier proton (H-4) showed a further COSY coupling with the *m*-coupled proton at δ = 8.28 (H-6). The second aromatic system (B) was alternatively concluded as mono-substituted phenyl ring, such that multiplet protons at δ = 7.39 (3′/5′-H) showed COSY connectivity with those at δ = 7.16 (4′-H) and 7.67 (2′/6′-H).

Based on the HMBC spectrum (Figure 2), the three aromatic protons (H-3, H-4 and H-6) of ring A showed vital correlations *versus* the quaternary carbons at δ = 123.2 (C-1), 127.1 (C-5), and 155.6 (C-2). The last carbon (155.6, C-2) is attached to the methoxy group (4.07) during a shown ^{3}J correlation from the OCH_3 (4.07). Additionally, the downfield *m*-coupled proton (δ 8.28, H-6) exhibited a vital ^{3}J correlation at the carbonyl carbon (161.8, C-7). In the second aromatic spin system (ring B), the H-2′/-6′ and H-3′/5′ aromatic ^{1}H signals exhibited ^{2}J and ^{3}J correlations, respectively at quaternary carbon at δ = 138.0 (C-1′), which is typically showing proximity to N atom, constructing an anilide moiety. Consequently, the two aromatic residues (A and B) are mostly attached *via* an anilide connectivity of ring B with the carbonyl group of ring A. Based on the aforementioned spectroscopic study in agreement with chromatographic property, compound **1** is deduced as 5-Chloro-2-methoxy-N-phenylbenzamide. A search in different data bases (DNP, AntiBase and Scifinder) confirmed the natural novelty of **1**. Alternatively, compound **1** was reported as synthetic product [21], however, no spectroscopic assignments were reported.

Biologically, N-phenylbenzamide derivatives possess important role as therapeutic agents in medicinal chemistry involving antimicrobial, antimalarial, antiviral, anticonvulsant, antidiabetic, analgesic, antiulcer and anti-allergy agents [22-27]. Further investigation showed that, when N-phenylbenzamide scaffold coupled with sulphonamide moieties, they provide new and potent anti-cancer agents [21]. Recently, N-phenylbenzamides are used as potent inhibitors of the mitochondrial permeability transition pore as well [28].

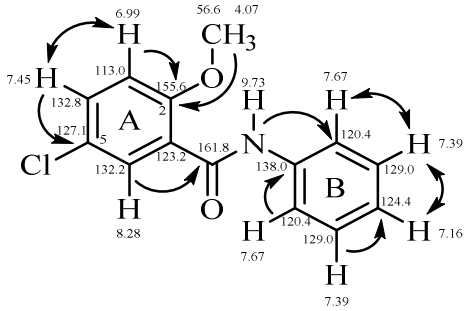


Fig 2: Key ^{1}H,^{1}H-COSY (↔) and HMBC (→) correlations of 5-Chloro-2-methoxy-N-phenylbenzamide (1)

Table 2:^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of 5-Chloro-2-methoxy-N-phenylbenzamide (1) in $CDCl_3$

No.	δ_C	δ_H (mult., *J* in Hz)
1	123.2	-
2	155.6	-
2-OCH_3	56.6	4.07 (s)
3	113.0	6.99 (d, 8.8)
4	132.8	7.45(dd, 8.8, 2.8)
5	127.1	-
6	132.2	8.28 (d, 2.8)
7	161.8	-
1′-NH	-	9.73 (s)
1′	138.0	-
2′, 6′	120.4	7.67 (m)
3′, 5′	129.0	7.39 (m)
4′	124.4	7.16 (tt, 7.3, 1.1)

5-Acetoxy-3-hydroxy-3-methylpentanoic acid

As a colourless semi-solid, compound **2** was obtained, showing no UV absorbing, however, it has been detected on faint violet on spraying with anisaldehyde/sulfuric acid and heating. According to (-) ESI-MS, an ion peak was shown at *m/z* = 189 $[M-H]^-$ indicating the molecular weight of **2** as 190 Dalton. Inspection of the ^1H NMR and ^{13}C/ HMQC spectra (Table 3) of **2**, no resonating signals were visible in the aromatic region. Evidently, two methylene groups being for an ethanediyl group (according to ^1H, ^1H COSY experiment, Fig. 3) were exhibited, the first of them was shifted at δ_H 4.56 and 4.38 (δ_C 66.1 ppm), while the other one at δ 1.99 and 1.85 ppm (δ_C 35.0), confirming their flanking by oxygen and sp^3 carbon atoms, respectively. An additional sp^2-bounded methylene group was observed as multiplet at δ_H = 2.58 ppm (δ_C 43.6), which could be attached directly to carbonyl group (Table 3). Finally, two methyl groups were observed at δ_H 2.60 (δ_C 28.3 ppm), being mostly for an acetyl group, and the other one was shown at δ_H = 1.34 ppm (δ_C 27.9 ppm). Further three quaternary carbons were shown at δ 172.4, 172.2 and 67.7, representing two carbonyls of acid and/or ester and an sp^3 oxygenated carbon (67.7), respectively. In accordance, compound **2** is bearing 3 CH_2 (one of them is oxygenated), 2 CH_3, two carbonyls and one sp^3 C_q-O, constructing the empirical formula $C_8H_{12}O_5$, revealing two 2H less than the reported molecular weight (190). Hence the molecular formula of **2** is concluded as $C_8H_{14}O_5$, and hence two DBE.

Examination of HMBC spectrum (Fig. 3) of **2** revealed the presence of ^3J correlation from the oxy-methylene protons (4.56 and 4.38 ppm) towards the carbonyl carbon shown at δ = 172.2, to which the singlet methyl (δ = 2.60) is correlated affording an acetoxy group bounded to the last oxy-methylene carbon (66.1). Protons of the last oxy-methylene (4.56 and 4.38 ppm) showed a further vital ^3J correlation versus the quaternary sp^3 carbon (67.1), and the latter is flanked by the complementing ethanediyl methylene protons (1.99, 1.85), sp^2-bounded methylene group (δ = 2.58 ppm) and the singlet methyl visible at 1.34 ppm). The observed ^1H and ^{13}C chemical shifts of the last methyl group (δ_H: 1.34, δ_C: 27.9) are indicative for its attaching to an oxygenated sp^3 carbon as well. Finally, the methylene group at 2.58 is terminated by the free carboxylic group (δ_C: 172.4). Based on its revealed intensive spectroscopic and chromatographic studies, compound **2** was finally deduced as 5-acetoxy-3-hydroxy-3-methylpentanoic acid, a new natural product according to our searching in different data bases. Nevertheless, compound **2** was previously obtained as a synthetic product [29].

5-Acetoxy-3-hydroxy-3-methylpentanoic acid (**2**) is served in the synthesis of mevalonic acid (Mevalonolactone / Hiochic acid lactone) and its derivatives [29], important bio-regulators for the biosynthesis of e.g. cholesterol, dolichol, ubiquinone, and vitamin D [30, 31].

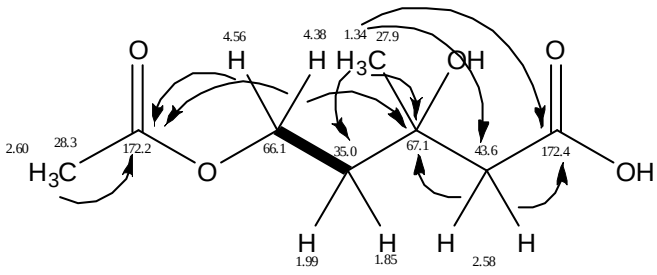


Fig 3: Key ^1H,^1H-COSY (—) and HMBC (→) correlations of 5-Acetoxy-3-hydroxy-3-methylpentanoic acid (2)

Table 3:^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of 5-Acetoxy-3-hydroxy-3-methylpentanoic acid (2) in MeOH-d_4

No.	δ_C	δ_H (mult., *J* in Hz)
1	172.4	-
2	43.6	2.58 (m)
3	67.1	-
4	35.0	1.99 (ddd, 14.3, 10.7, 5.5), 1.85 (dtd, 14.4, 3.9, 2.1)
5	66.1	4.56 (td, 10.9, 4.2), 4.38 (ddd, 11.2, 5.5, 3.6)
7	172.2	-
8	28.3	2.60 (s)
9	27.9	1.34 (s)

Kojic acid methyl ether (3)

The molecular structure of kojic acid methyl ether (**3**) was elucidated based on NMR spectra (Fig.4, Table 4), ESI-MS spectrum, and by comparison with reported literature data [20].

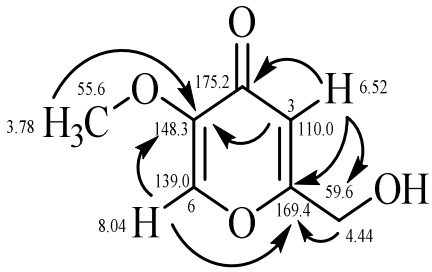


Fig 4: Key HMBC (→) correlations of kojic acid methyl ether (3)

Table 4:^{13}C (125 MHz) and ^{1}H (500 MHz) NMR data of kojic acid methyl ether (3) in MeOH-d_4

No.	Exp.		Lit.[20] *(in DMSO-d_6)*	
	δ_C	δ_H (mult, *J* in Hz)	δ_C (100 MHz)	δ_H(400 MHz) (mult., *J* in Hz)
2	169.4	-	168.0	-
2-CH_2	59.6	4.44 (d, 1)	59.3	4.29 (d, 5.5)
3	110.0	6.52 (d, 1)	110.7	6.29 (s)
4	175.2	-	172.8	-
5	148.3	-	147.9	-
5-OCH_3	55.6	3.78 (s)	56.1	3.64 (s)
6	139.0	8.04 (s)	139.0	8.08 (s)

Pharmacological applications:

Cytotoxicity

The *in vitro* cytotoxicity of the new compounds was studied against the human cervix carcinoma cell line (KB-3-1) using (+) – griseofulvin as a reference, but the compounds did not show noticeable activity (Table 5).

Table 5: *In vitro* cytotoxicity against KB-3-1 cell line

Compound	Molecular weight (g/mol)	IC_{50} KB-3-1 (µmol/L)
1	261	-
2	190	-
(+) – Griseofulvin^{R}	353	19.2

^{R}reference

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