Tropical Journal of Pharmaceutical Research August 2020; 19 (8): 1661-1668
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v19i8.14

Original Research Article

Suppressive efficiency of Kojic acid from *Aspergillus tamarii* MM11 against HepG-2 cell line derived from human liver cancer

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Sent for review: 24 January 2020 *Revised accepted: 20 July 2020*

Abstract

Purpose: *To evaluate the antioxidant and cytotoxic properties of Kojic acid (KOJIC ACID) from Aspergillus tamarii MM11 against HepG-2 cell line derived from human liver cancer.*

Methods: *The crude extract of A. tamarii MM11 was dissolved in a mixture of CH_2Cl_2/MeOH (85:15) and separation was done using silica gel chromatography using gradient size exclusion chromatograph. The non-polar oily fractions were subjected to gas chromatography-mass spectrometric (GC-MS) analysis. Kojic acid structure was identified by x-beam crystallography and spectroscopic methods. Total antioxidant properties of KOJIC ACID were evaluated by using 1,1-diphenyl-2- picrylhydrazyl (DPPH) against ascorbic acid as a reference. The cytotoxic activity of KOJIC ACID from A. tamarii MM11 was investigated on the human cell line of liver cancer (HepG-2) using a sulforhodamine B (SRB) assay based on a cell density determination by the measurement of cellular protein content.*

Result: *Highly bioactive Kojic acid was isolated as the main product. A. tamarii MM11 Kojic acid showed good antioxidant activity with half-maximal inhibitory concentration of IC_{50} at concentrations of 10.34 compared to 6.79 µg/mL for ascorbic acid. Kojic acid also showed good cytotoxic activity against HepG-2 cell line of human liver cancer with IC_{50} at 6.20 compared to 3.25 µg/mL of reference drug doxorubicin.*

Conclusion: *Kojic acid produced naturally from A. tamarii MM11 shows good antioxidant and cytotoxic activity against HepG-2 cell line derived from human liver cancer. These findings suggest that Kojic acid can be therapeutically used as an antitumor drug after further in vivo studies.*

Keywords: *Aspergillus tamarii, Secondary metabolites, Kojic acid, Anticancer, Liver cancer*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Fungi this huge world produce multiple types of secondary metabolites, which including aromatic compounds, amino acids, anthracenones, butanolides, butenolides, cytochalasans, macrolides, naphthalenones, pyrones, terpenes,

etc. [1,2]. These compounds have numerous industrial, ecological and pharmaceutical uses.

Cancer is a life-threatening disease. Most of the successive anticancer medications currently used cause many undesirable side effects. For example, Doxorubicin can prompt cardiotoxicity and tumor drug resistance [3]. Methotrexate also can cause liver damage and portal hypertension and cirrhosis. While, Cisplatin administration can lead to nephrotoxicity and in some cases renal failure [4].

Therefore, new anticancer drugs with more efficiency and ability to mitigate side effects are needed. Fungal anticancer secondary metabolites are one of the very important targets for mycologist. In this connection, 5-hydroxy-2-hydroxymethyl-γ-pyrone (HMP) or Kojic acid (KOJIC ACID) is a major secondary metabolite produced by a limited range of microorganisms, including *Aspergillus oryzae*, *A. flavus*, and *A. tamarii*, as well as Penicillium species and certain bacteria [5].

Kojic acid possess strong antioxidant, antibacterial and antifungal activities. So, it is widely used in medical purposes and many other fields. It also used as a food flavor enhancer [6]. In agriculture, it used as anti-melanosis and insecticide activator [7]. In cosmetic, Kojic acid is well known as whitening agent, ultraviolet filter, tyrosinase inhibitor, and radio-protective agent [8]. Few studies were performed on anticancer activity of Kojic acid.

In the present investigation, most of the secondary metabolome of terrestrial *A. tamarii* MM11 was detected and the produced Kojic acid was isolated, purified and elucidated and its antioxidant properties were studied. Furthermore, the cytotoxic effect of Kojic acid on liver carcinoma cell lines were determined.

EXPERIMENTAL

Fungal strain

The terrestrial fungal isolate used in this study was isolated from tubers of rotten Jerusalem artichoke (*H. tuberosus*) identified by molecular sequencing of fungal Inter Transcribed Spacer (ITS). The fungus strain was identified as *A. tamarii* MM11 with the accession no. GU295949.

Fermentation

A. tamarii MM11 was inoculated from well grown agar plates colonies in 0.1 L sterilized glass bottles each containing sterilized rice. The medium composition was: 8 g commercial rice; 10 mL distilled water. The bottles were incubated for 15 days at 28 ± 2°C. After harvesting, 50 mL of 1:1 DCM/MeOH was added to each bottle, followed by vigorous shaking for two hours. The afforded organic extract was decanted, filtered and then concentrated *in vacuo* till dryness.

Separation and purification of fungal secondary metabolome

The crude extract (7.79 g) was dissolved in a mixture of CH_2Cl_2/MeOH (85:15). Six gram of silica gel were added and this mixture was brought to dryness under reduced pressure. Separation was performed using a silica gel column (3 × 100 cm, 200 g) chromatography eluted with CH_2Cl_2-MeOH gradient (0.5 L 100:0, 0.5 L 98:2, 0.6 L 95:5, 0.5 L 93:7, 0.6 L 90:10, 0.3 L 80:20 and 0.3 L 50:50 v.v). After TLC monitoring, four fractions were afforded, FI (3.2 g), FII (1.2 g), FIII (3.2 g). Fraction I (3.2 g) was then applied to purification on Sephadex LH-20 (CH_2Cl_2-MeOH, 60:40) affording a large oily zone of fraction1 (2.1 g), which on application to GC-MS analysis afforded seventy-three compounds, including naphthalene (**1**), 1-methyl-naphthalene (**3**), 2,7-dimethyl-naphthalene (**9**), 1,3-dimethyl-naphthalene (**10**), 2,6-dimethyl-naphthalene (**11**) and penta-chloro-pyridine (**14**).

Fraction II (1.2 g) was purified on Sephadex LH-20 (CH_2Cl_2-MeOH, 60:40), yielding a large zone of oily mixture (0.9 g), which onapplication to GC-MS analysis delivered twenty-six compounds, including ethyl-2-methyl-3-Oxo-hexanoate (**71**), 1,2-dibromo-2-methyl-propane (**72**), azulene (**75**), bicyclo [4.3.0] nonane, 3-butyl-4-hexyl- (**83**), 6-nitroundec-5-ENE (**84**), 4,9-decadienoic acid, 2-nitro-, ethyl ester (**88**) and 1-chloro-octadecane (**89**). The last fraction FIII (3.2 g) applied to washing with dichloromethane followed by filtration to afford colourless needles of Kojic acid (**1**, 2.3 g).

The nuclear magnetic resonance (NMR) spectra were determined on Varian Unity 300 (300.145 MHz) and Varian Inova 600 (150.820 MHz) spectrometers. Electro spray ionization mass spectra (ESI MS) was recorded on a Finnigan LCQ with quaternary pump Rheos 4000 (Flux Instrument). Flash chromatography was carried out on silica gel (230-400 mesh).

The rate of flow (R_f) values were measured on Polygram SIL G/UV_{254} TLC cards (Macherey-Nagel & Co.). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie,

Steinheim, Germany). Mass spectra were recorded on a Finnigan MAT 95 spectrometer (70 eV). All solvents and chemicals were purchased from Sigma, Merck and Aldrich.

Determination of total antioxidant activity

The antioxidant activities of fungal KOJIC ACID were detected using 1,1-diphenyl-2-picrylhydrazyl (DPPH) in comparison with ascorbic acid as standard radical scavenging agent. The experiment was carried out by preparing solutions of 50 mg/mL, and then serial dilutions (5-50 mg/mL) of KOJIC ACID and the reference ascorbic acid were prepared. Then, 250 µL of each dilution was added to 1 mL DPPH solution (6 mg/50 mL). Control tube was also prepared using 1 mL of ethanol. The mixture was shaken and incubated for 30 min in the dark at room temperature. Absorbance was measured using a Genway spectrophotometer at 517 nm [9]. These steps were repeated 3 times and the radical scavenging (R) evaluated according to Eq 1.

$$R\ \% = 1-(A_s/A_c) \times 100 \ldots\ldots\ldots\ldots (1)$$

where, R is radical scavenging, A_s is the absorbance of the sample and A_c is the absorbance of the control.

Evaluation of cytotoxic activity

The cytotoxic activity of KOJIC ACID of *A. tamarii* MM11 was investigated on the human cell line of liver cancer (HepG-2) using sulforhodamine B (SRB) assay based on a cell density determination by the measurement of cellular protein content. HepG-2 monolayer was fixed on the 96-well plate with trichloroacetic acid (TCA). Then, SRB was added to each well and incubated at room temperature for 1h. SRB binds to basic amino acids in cellular proteins under mild acidic conditions. The excess dye was removed by washing repeatedly with acetic acid. The protein bound dye was dissolved by adding Tris-base solution (basic medium) to each well and shake the plate to solubilize the protein bound dye.

The amount of bound dye can be determined by measuring the absorbance at 510 nm in a microplate reader. It can then be extrapolated to measure cell proliferation [10]. HepG-2 used in this study was obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). The tumor cell line was maintained at the National Cancer Institute, Cairo, Egypt, through serial sub-culturing. Doxorubicin was used as the reference drug.

Statistical analysis

Statistical analysis of the results was carried out using GraphPad instant, Version 3.06 (GraphPad Software Inc, San Diego, California, USA). The data are expressed as mean ± standard deviation (SD). Curves plotting were performed with Origin 6.0

RESULTS

Phytochemical profile

Non-polar fractions

GC-MS analysis of the un-polar oily fraction of fraction I (Figure 2) afforded the shown below listed of compounds (Table 1). In accordance, seventy three compounds were identified namely, naphthalene (2), n-dodecane (3), 1-methyl-napthalene (4), n-tridecane (5), 3-methyl-hexadecane (6), 1,1'-biphenyl (7), 7-tetradecene (8), tetradecane (9), 2,7-dimethyl-napthalene (10), 1,3-dimethyl-napthalene (11), 2,6-dimethyl-napthalene (12), n-dotriacontane (13), pentadecane (14), penta-chloro-pyridine (15), 7-methyl-pentadecane (16), 5-methyl-decane (17), 3-methyl-eicosane (18), 9h-fluorene (19), 1-hexadecene (20), hexadecane (21), 7-hexadecene (22), 1-hexyl-3-methyl-cyclopentane (23), butyl-tridecyl-sulfurous acid ester (24), 4,6-dimethyl-dodecane (25), 1,3-dibutenyl-4-phenyl-benzene (26), 5-methyl-tridecane (27), phensuximide (28), phenanthrene (29), e-15-heptadecenal (30), octadecane (31), pentacosane (32), (2-propyl) octadecyl sulfurous acid (33), nonadecane (34), allyl-tridecyl-oxalate (35), methyl-hexadecanoate (36), 10-methyl-nonadecane (37), 2-ethylhexylheptadecyl sulfurous acid (38), dodecyl-hexyl-oxalate (39), hexyl-tetradecyl-oxalate (40), ethylhexadeca-noate (41), eicosane, 5-methyl-1-heptene (42), 1,2-dibromo-dodecane (43), 2-chloroethyl-linoleate (44), methyl-10-methyl-heptadecanoate (45), 3-decen-1-ol (46), 9,12-octadecadienoic acid (z,z) (47), 12-methyl-e,e-2,13-octadien-1-ol (48), pentadecyl-3-bromo-benzoate (49), ethyllinoleate, ethyloleate, methyl-17-methyl-octadecanoate (50), 1,13-tetradecadien-3-one (51), n-[4-bromo-n-butyl]-2-piperidinone (52), 3-ethyl-1-octene (53), 3,4-dimethyl-heptane (54), 2-fluoro-1-triacetylribofuranosyl-imidazole (55), 1-fluoro-tetradecane (56), 1,2-epoxy-hexadecane (57), 7-hexadecyne (58), 2-tridecyloxirane (59), trans-cinnamonitrile (60), (9e)-9-hexacosene (61), dotriacontane (62), 3-(2,5-dimethyl-1h-pyrrole-3-yl)-1,3-dihydro-indol-2-one (63), 1-(dodecyloxy)-2,3-epoxy- propane (64), 1-bromopentadecane (65), squalene (66), tetradecanal (67), Sulfurous acid, butyl

heptadecyl ester (68), 2-(4-hydroxybutyl)-2-nitrocyclodecanone (69), 1,2-Epoxy-1-vinylcyclododecane (70), 17-Pentatriacontene (71).

Alternatively, an analysis of the un-polar oily sub-fraction by GC-MS (Fig. 3) established the existence of twenty six metabolic compounds (Table 2), namely: Ethyl-2-methyl-3-oxo-hexanoate (72), 1,2-dibromo-2-methyl-propane (73), benzeneacetic acid, methyl ester (74), Azulene (75), n-tetradecane (76), pentadecanoic acid, ethyl ester (77), Hexadecanoic acid, methyl ester, hexadecanoic acid, ethyl ester, 10,13-octadecadienoic acid, methyl ester (78), octadecanoic acid, methyl ester (79), heptadecanoic acid, 16-methyl-, methyl ester (80), ethyl linoleate (81), 9-octadecenoic acid (z)-, ethyl ester (82), bicyclo[4.3.0]nonane, 3-butyl-4-hexyl- (83), 6-nitroundec-5-ene (84), 1,2-benzenedicarboxylic acid, bis (2-ethylhexyl) ester (85), di-n-octyl phthalate (86), 1,2-benzenedicarboxylic acid, diisooctyl ester (87), 4,9-decadienoic acid, 2-nitro-, ethyl ester (88), octadecane, 1-chloro- (89), 1,2-benzenedicarboxylic acid, diisononyl ester, phthalic acid, nonyl tridec-2-yn-1-yl ester (90), didodecyl phthalate.

As colourless needles, kojic acid (KOJIC ACID) (**1**) was afforded from the polar fraction FIII after washing with hot dichloromethane. The structure of KOJIC ACID (**1**) was definitely deduced on the basis of X-ray crystallography and spectroscopic means. Kojic acid (1): $C_6H_6O_4$ (142). UV absorbing, colorless solid, R_f = 0.15 ($CHCl_3$/10% MeOH), turned blue on spraying with anisaldehyde/sulfuric after heating. -^{1}HNMR (DMSO-*d6*, 300 MHz): δ = 8.99 (brs, 1H, OH), 7.97 (s 1H, CH-2), 6.34 (s, 1H, CH-5), 5.69 (brs, 1H, OH), 4.28 (s, 2H, CH_2-7). -^{13}C/APT NMR (DMSO-*d6*, 125 MHz) δ = 174.2(C_q-4), 168.2 (C_q-6), 153.0 (C_q-3), 138.9 (CH-2), 110.0 (CH-5), 59.7 (CH_2-7). -EI MS *m/z* (%): 142 ([M+.], 100), 113 ([M-CHO] +, 33), 97 (15), 85 (18), 69 (74), 57 (25), 39 (42), 29 (52).

The following data for the unit cell were obtained from X-ray oscillation photographs: *a* = 3·85, *b* = 18·4, *c* = 8·84 A.; *β* = 74°, correct to about ± 1 per cent as typical to those reported one. The measured density of 1.58 g. cm-3 gave 3.98~4 molecules/cell. These results are in accord with previous measurements [11,12].

According to EI MS, the molecular weight of Kojic acid was established as 142 Dalton with a corresponding molecular formula of $C_6H_6O_4$. Based on the proton nuclear magnetic resonance spectroscopy (^{1}H NMR, DMSO-d_6), two singlets were visible at 7.97 and 6.34, being for aromatic/olefinic attached protons, together with two broad singlets at δ 8.99 and 5.69 ppm being for phenolic and aliphatic hydroxyl protons, respectively, in addition to an sp^2-attached oxy-methylene protons were shown at δ 4.28 ppm. On the bases of ^{13}C NMR/APT spectra of Kojic acid, six carbon signals, as matched with the afforded molecular formula, were deduced, being for one γ-lactone carbonyl (δ 174.2), three sp^2-quaternary Oxy-carbons (δ 168.2 and 153.0), two sp^2-CH carbon signals (δ 138.9 and 110.0) and one sp^2-attached Oxy-methylene (59.7). According to these data, searching in AntiBase and comparison with literature, Kojic acid structure was confirmed.

Kojic acid from *A. tamarii* MM11 recorded strong antioxidant activities with IC_{50} value reached to 10.34 µg/mL in comparison with the reference compound (ascorbic acid) which recorded IC_{50} of 6.79 µg/mL. These values indicated potent radical scavenging activities of KOJIC ACID (Table 3). Kojic acid also showed excellent cytotoxic activities against cancerous human liver cell line (HepG-2) with IC_{50} equals to 6.20 µg/mL in comparison with 3.25 µg/mL for the reference drug Doxorubicin (DOX) (Figure 6)

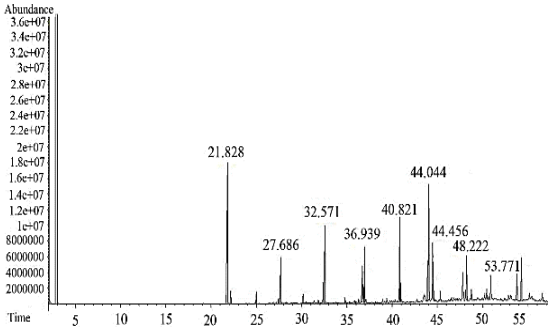

Figure 1: GC-MS chromatogram of oily fraction of fraction I

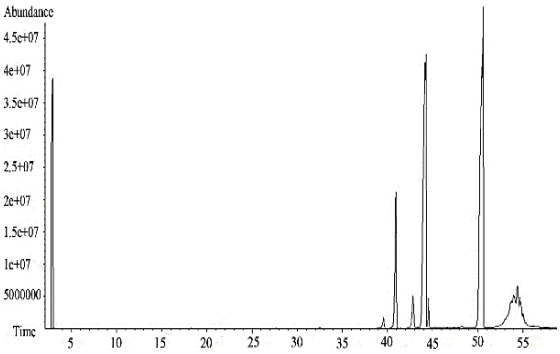

Figure 2: GC-MS chromatogram of fraction II

Table 1: Compounds detected in oily fraction I by GC-MS

No.	Name	MF*	MWt	R_T	A%
1	Naphthalene (**2**)	$C_{10}H_8$	128	21.83	11.03
2	n-Dodecane (**3**)	$C_{12}H_{26}$	170	22.18	0.38
3	1-Methyl-napthalene (**4**)	$C_{11}H_{10}$	142	24.89	0.06
4	n-Tridecane (**5**)	$C_{13}H_{28}$	184	25.01	0.40
5	3-Methyl-hexadecane (**6**)	$C_{17}H_{36}$	240	26.88	0.05
6	1,1'-Biphenyl (**7**)	$C_{12}H_{10}$	154	27.18	0.04
7	7-Tetradecene (**8**)	$C_{14}H_{28}$	196	27.45	0.20
8	Tetradecane (**9**)	$C_{14}H_{30}$	198	27.69	1.66
9	2,7-Dimethyl-napthalene (**10**)	$C_{12}H_{12}$	156	27.85	0.1
10	1,3-Dimethyl-napthalene (**11**)	$C_{12}H_{12}$	156	28.24	0.05
11	2,6-Dimethyl-napthalene (**12**)	$C_{12}H_{12}$	156	28.32	0.06
12	n-Dotriacontane (**13**)	$C_{32}H_{66}$	450	29.23	0.05
13	Pentadecane (**14**)	$C_{15}H_{32}$	212	30.15	0.33
14	Penta-chloro-pyridine (**15**)	C_5Cl_5N	249	30.46	0.08
15	7-Methyl-pentadecane (**16**)	$C_{16}H_{34}$	226	31.26	0.08
16	5-Methyl-decane (**17**)	$C_{11}H_{24}$	156	31.39	0.13
17	3-mMethyl-eicosane (**18**)	$C_{21}H_{44}$	266	31.83	0.16
18	9H-Fluorene (**19**)	$C_{13}H_{10}$	166	32.22	0.04
19	1-Hexadecene (**20**)	$C_{16}H_{32}$	224	32.37	0.88
20	Hexadecane (**21**)	$C_{16}H_{34}$	226	32.57	2.90
21	7-Hexadecene (**22**)	$C_{16}H_{32}$	224	32.64	0.01
22	1-Hexyl-3-methyl-cyclopentane (**23**)	$C_{12}H_{24}$	168	33.79	0.05
23	Butyl-tridecyl-sulfurous acid ester (**24**)	$C_{17}H_{36}O_3S$	320	33.94	0.03
24	4,6-Dimethyl-dodecane (**25**)	$C_{14}H_{30}$	198	34.88	0.13
25	1,3-Dibutenyl-4-phenyl-benzene (**26**)	$C_{20}H_{22}$	262	35.30	0.08
26	5-methyl-tridecane (**27**)	$C_{14}H_{30}$	198	35.88	0.11
27	Phensuximide (**28**)	$C_{11}H_{11}NO_2$	189	36.01	0.11
28	Phenanthrene (**29**)	$C_{14}H_{10}$	178	36.68	2.09
29	E-15-Heptadecenal (**30**)	$C_{17}H_{34}$	238	36.78	1.31
30	Octadecane (**31**)	$C_{18}H_{38}$	254	36.94	1.96
31	Pentacosane (**32**)	$C_{25}H_{52}$	352	37.09	0.22
32	(2-propyl) octadecyl sulfurous acid (**33**)	$C_{21}H_{44}O_3S$	376	38.17	0.08
33	Nonadecane (**34**)	$C_{19}H_{40}$	268	38.92	0.16
34	Allyl-tridecyl-oxalate (**35**)	$C_{18}H_{32}O_4$	312	39.15	0.12
35	Methyl-hexadecanoate (**36**)	$C_{17}H_{34}O_2$	270	39.44	0.09
36	10-methyl-nonadecane (**37**)	$C_{20}H_{42}$	280	39.72	0.08
37	2-ethylhexylheptadecyl sulfurous acid (**38**)	$C_{25}H_{52}O_3S$	432	39.77	0.11
38	Dodecyl-hexyl-oxalate (**39**)	$C_{20}H_{38}O_4$	342	39.92	0.09
39	Hexyl-tetradecyl-oxalate (**40**)	$C_{22}H_{42}O_4$	370	40.18	0.17
40	Ethylhexadecanoate (**41**)	$C_{18}H_{36}O_2$	284	40.82	4.63
41	Eicosane	$C_{20}H_{42}$	282	40.91	1.17
42	5-Methyl-1-heptene (**42**)	C_8H_{16}	112	41.04	0.10
43	1,2-Dibromo-dodecane (**43**)	$C_{12}H_{24}Br_2$	328	41.59	0.05
44	2-Chloroethyl-linoleate (**44**)	$C_{20}H_{35}ClO_2$	341	42.64	0.09
45	Methyl-10-methyl-heptadecanoate (**45**)	$C_{19}H_{38}O_2$	298	43.21	0.11
46	3-Decen-1-ol (**46**)	$C_{10}H_{20}O$	156	43.42	0.33
47	9,12-octadecadienoic acid (Z,Z) (**47**)	$C_{18}H_{32}O_2$	280	43.51	0.52
48	12-Methyl-E,E-2,13-octadien-1-ol (**48**)	$C_{19}H_{36}O$	280	43.55	0.09
49	Pentadecyl-3-bromo-benzoate (**49**)	$C_{22}H_{35}BrO_2$	280	43.60	0.55
50	Ethyllinoleate	$C_{20}H_{36}O_2$	308	43.90	2.07
51	Ethyloleate	$C_{20}H_{38}O_2$	310	44.04	6.31
52	Methyl-17-methyl-octadecanoate (**50**)	$C_{20}H_{40}O_2$	312	44.46	3.12
53	1,13-Tetradecadien-3-one (**51**)	$C_{14}H_{24}O$	208	44.68	0.16
54	N-[4-bromo-n-butyl]-2-piperidinone (**52**)	$C_9H_{16}BrNO$	234	45.14	0.10
55	3-Ethyl-1-octene (**53**)	$C_{10}H_{20}$	140	45.31	0.45
56	3,4-Dimethyl-heptane (**54**)	C_9H_{20}	128	45.37	0.10
57	2-Fluoro-1-triacetylribofuranosyl-imidazole (**55**)	$C_{11}H_{17}FN_2O_7$	344	46.19	0.10
58	1-Fluoro-tetradecane (**56**)	$C_{14}H_{29}F$	344	46.33	0.04
59	1,2-epoxy-hexadecane (**57**)	$C_{16}H_{32}O$	240	46.45	0.12
60	7-Hexadecyne (**58**)	$C_{16}H_{30}$	222	48.14	0.77
61	2-Tridecyloxirane (**59**)	$C_{13}H_{26}O$	198	49.50	0.77
62	Trans-Cinnamonitrile (**60**)	C_9H_7N	129	50.43	0.66
63	(9E)-9-Hexacosene (**61**)	$C_{26}H_{52}$	364	50.88	1.33
64	Dotriacontane (**62**)	$C_{32}H_{66}$	450	50.93	0.43

65	3-(2,5-Dimethyl-1H-pyrrole-3-yl)-1,3-dihydro-indol-2-one (**63**)	$C_{14}H_{14}N_2O$	226	51.75	0.15
66	1-(dodecyloxy)-2,3-epoxy- Propane (**64**)	$C_{16}H_{32}O_2$	256	52.46	0.12
67	1-Bromopentadecane (**65**)	$C_{16}H_{31}Br$	291	52.85	0.45
68	Squalene (**66**)	$C_{30}H_{50}$	410	54.26	1.54
69	Tetradecanal (**67**)	$C_{14}H_{28}O$	212	54.49	0.11
70	Sulfurous acid, butyl heptadecyl ester (**68**)	$C_{21}H_{44}O$	312	55.14	0.49
71	2-(4-hydroxybutyl)-2-nitrocyclodecanone (**69**)	$C_{14}H_{25}NO_4$	271	55.30	0.11
72	1,2-Epoxy-1-vinylcyclododecane (**70**)	$C_{14}H_{24}O$	208	56.45	0.12
73	17-Pentatriacontene (**71**)	$C_{35}H_{70}$	490	56.57	0.63

*MF = molecular formula; Mwt = molecular weight; Rt = retention time; A%= abundance

Table 2: Compounds detected in fraction II by GC-MS

No.	Name	MF	MWt	R_T	A%
1	Ethyl-2-methyl-3-oxo-hexanoate (**72**)	$C_9H_{16}O_3$	172	2.92	9.75
2	1,2-Dibromo-2-methyl-propane (**73**)	$C_4H_8Br_2$	216	18.27	0.01
3	Benzeneacetic acid, methyl ester (**74**)	$C_9H_{10}O_2$	150	21.58	0.02
4	Azulene (**75**)	$C_{10}H_8$	128	21.72	0.09
5	n-Tetradecane (**76**)	$C_{14}H_{30}$	198	22.17	0.02
6	Pentadecanoic acid, ethyl ester (**77**)	$C_{17}H_{34}O_2$	270	36.86	0.07
7	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	39.56	0.59
8	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	40.86	3.12
9	10,13-Octadecadienoic acid, methyl ester (**78**)	$C_{19}H_{34}O_2$	294	42.83	2.19
10	Octadecanoic acid, methyl ester (**79**)	$C_{19}H_{38}O_2$	298	43.34	0.05
11	Heptadecanoic acid, 16-methyl-, methyl ester (**80**)	$C_{19}H_{38}O_2$	298	43.37	0.04
12	Ethyl linoleate (**81**)	$C_{20}H_{36}O_2$	308	44.20	4.77
13	9-Octadecenoic acid (Z)-, ethyl ester (**82**)	$C_{20}H_{38}O_2$	310	44.27	4.76
14	Bicyclo[4.3.0]nonane, 3-butyl-4-hexyl- (**83**)	$C_{19}H_{36}$	264	48.20	0.09
15	6-Nitroundec-5-ene (**84**)	$C_{10}H_{19}NO_2$	185	48.24	0.06
16	1,2-Benzenedicarboxylic acid, bis (2-ethylhexyl) ester (**85**)	$C_{24}H_{30}O_4$	390	50.33	14.26
17	Di-n-octyl phthalate (**86**)	$C_{24}H_{30}O_4$	390	50.41	5.25
18	1,2-Benzenedicarboxylic acid, diisooctyl ester (**87**)	$C_{24}H_{30}O_4$	390	50.46	2.03
19	4,9-Decadienoic acid, 2-nitro-, ethyl ester (**88**)	$C_{12}H_{19}NO_4$	241	52.06	0.02
22	Octadecane, 1-chloro- (**89**)	$C_{18}H_{37}Cl$	288	52.45	0.01
24	1,2-Benzenedicarboxylic acid, diisononyl ester	$C_{26}H_{34}O_4$	418	53.27	0.34
25	Phthalic acid, nonyl tridec-2-yn-1-yl ester (**90**)	$C_{30}H_{46}O_4$	470	54.35	0.98
26	Didodecyl phthalate	$C_{28}H_{38}O_4$	446	54.60	0.10

Table 3: IC_{50} of *A. tamarii* MM11 kojic acid

Extract	IC_{50} (µg/mL)
Kojic acid of *A. Tamarii* MM11	10.34
Reference (ascorbic acid)	6.79

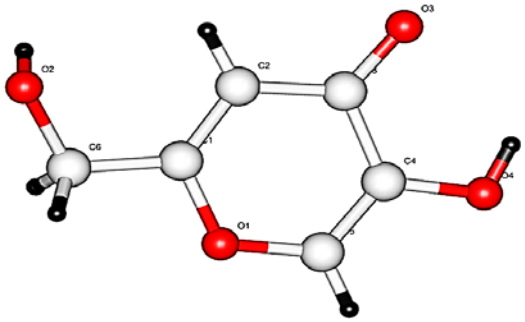

Figure 3: Crystallographic structure of Kojic acid

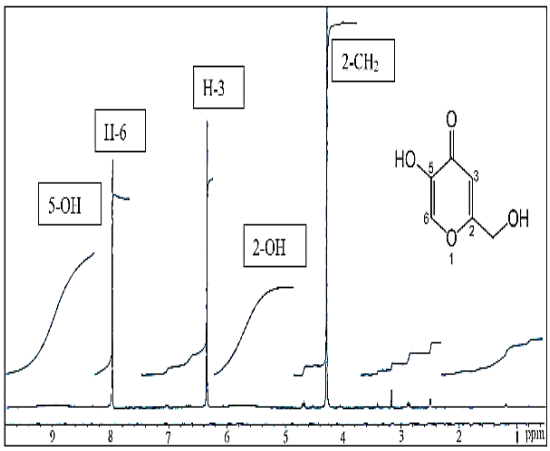

Figure 4: ^{1}H NMR spectrum for kojic acid (1) (DMSO-d6, 300MHz).

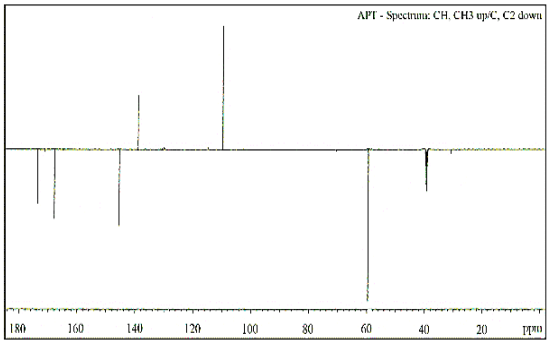

Figure 5: 13 C/APT NMR spectrum for kojic acid (DMSO-d_6, 500MHz)

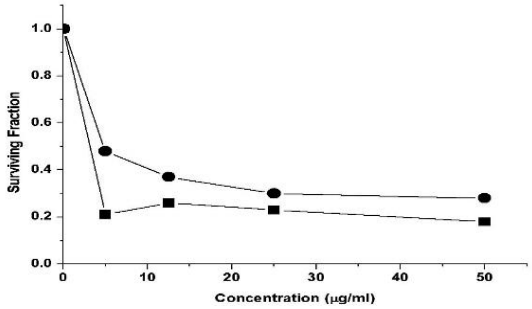

Figure 6: IC_{50} of Kojic acid from *A. tamarii* MM11 (●) and DOX (■) against HepG-2 cell line.

DISCUSSION

The great progress in instrumental analysis devices contributed greatly in understand the secondary metabolome map of active fungi. These maps give a platform of secondary metabolites informatics which can be used in many applications. In this article, 100 organic secondary metabolites were identified using GC mass from *A. tamarii* MM11 extract. Generally, *A. tamarii* produces vast types of secondary metabolites including fumigaclavine A, aflatoxin, cyclopiazonic acid, speradine A and kojic acid. *A. tamarii* produces a considerable amount of Kojic acid, [13]. Many studies evaluated antimicrobial and antioxidant properties of KOJIC ACID, but only few studies investigated its anticancer activities. In the 1950s, Gerschman *et al* [14] demonstrated that oxygen-containing free radicals. have hazardous effects on all living cells. Reactive oxygen species (ROS) are endogenous, very active oxygen bearing atoms, which can be divided into enzymatic and non-enzymatic classes [15]. ROS have been believed to be the main cause of various diseases as cancer, sclerosis, Parkinson's, Alzheimer's, immune system ailment, stroke, and others [16]. Antioxidants are the compounds that can neutralize ROS and provide protection against cancer by lowering the peril of tumor development [17].

From the results, purified Kojic acid from *A. tamarii* MM11 gave relatively high DDPH radical scavenging activity. It showed potent antioxidant properties with a very close IC_{50} to that of the reference ascorbic acid. Kojic acid can be effectively served as nontoxic naturally occurring antioxidant, blocking the action and side effects of many routinely ROS produced during the photodynamic therapy of neoplastic diseases and others such as arteriosclerosis and diabetes [18]. As a promising result, Kojic acid showed highly cytotoxic effects on HepG-2 cells that suggesting strong antitumor effects of Kojic acid against hepatocellular carcinoma. These results were previously observed by another study indicated that Mannich indicated by a study documented that the combination therapy of Mannich base containing ciprofloxacin and Kojic acid structural units showed antitumor activity in HepG-2 [19]. Kojic acid is a potent inhibitor for cellular NF- kappaB activity in different cell types. It is documented that KOJIC ACID has this inhibitory effect in transfectant HaCaT cells, SCC-13 cells and in human keratinocytes. It was found to be more effective than other antioxidants as ascorbic acid and N-acetyl-L-cysteine which suggested that Kojic acid induced anti-melanogenic effect [20]. Previous studies showed that KOJIC ACID also inhibit cell growth of A375 melanoma cells. So, it is used now as a treatment for many types of melanoma [21,22].

There is argument about the effect of Kojic acid administration and DNA mutations. There was a study suggested the ability of Kojic acid to cause mutations in salmonella bacteria [3]. However, other *in vivo* mammalian studies proved KOJIC ACID as a safe drug at relatively high concentrations that is not significant acute oral toxicant in mice and rats with LD_{50} value greater than 1 g/kg [6,23].

CONCLUSION

The purified form of Kojic acid isolated from *Aspergillus tamarii* MM11 shows radical scavenging activity close to that of ascorbic acid. It also exhibits good cytotoxic activity in HepG-2 cell lines. Thus, kojic acid is a potentially safe, natural antioxidant and antitumor agent.

DECLARATIONS

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Khalid

University, Abha, KSA for funding this work through Research Groups Program (grant no. R.G.P.1/117/40).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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