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Antioxidant and Cytotoxic Activities of Flavonoidal Compounds from *Gmelina arborea* Roxb.

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Abstract: A bioassay-guided fractionation and chemical investigation of the *Gmelina arborea* leaves (Verbenaceae) resulted in the isolation and characterization of six known flavonoids (1, 3-7) along with new flavone glycoside (2), identified as; luteolin (1), luteolin-4'-*O*-β-D-$^4C_1$-galactoside (2), kaempferol (3), quercetin - 3 - *O* - β - D - $^4C_1$ -glucopyranoside (isoquercitrin) (4), quercetin - 3 - *O* - α - $^1C_4$ - L - rhamnopyranosyl - (1'''→6'') - *O* - β-D-$^4C_1$-glucopyranoside (rutin) (5), luteolin-7-*O*- β-D-$^4C_1$-galactoside (6) and quercetin-3-*O*-α-$^1C_4$-L-rhamnopyranosyl-(1'''→6'')-β-$^4C_1$-D-galactopyranoside (quercetin-3-*O*-robinobioside) (7). Their structures were elucidated via UV, IR and NMR spectral techniques as well as (Co-PC, Co-TLC and Co-m.p.) and acid hydrolysis. Their antioxidant activity (AOA) was evaluated via 1,1'-diphenyl-2-picraylhydrazyl free radical (DPPH) and phosphomolybdenum assays, while their cytotoxic activity was evaluated toward liver-carcinoma cell line (HepG-2) via Sulphorhodamine-B assay. The DPPH free radical SC_{50} values ranged from 5.70 to 14.40 µg/ml, while the total antioxidant capacity ranged from (630.75 to 403.66 mg AAE/gm compound). Compounds 1, 2 and 7 showed cytotoxic activity toward liver-carcinoma cell line (HepG2) with IC_{50}= 3.38, 8.98 and 15.70 μg/ml respectively. From the above results, the isolated flavonoidal compounds from the leaves of *Gmelina arborea* could be used as promising naturally occurring antioxidant and anticancer agents.

Key words: *Gmelina arborea* · Chromatographic Isolation · Phenolic Compounds · Structural Identification · Antioxidant Activity · Cytotoxic Activity · HEPG-2

INTRODUCTION

Free radicals especially reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been found to play a vital role in the initiation of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease via the oxidative stress mechanism [1]. The exposure to ROS leads to harmful changes of tissues function via a number of alterations like lipid peroxidation, enzyme inactivation, oxidative DNA damage and cancer [2]. Previous studies revealed that, in Egypt, liver cancer is the second cause of deaths from cancer after breast cancer and it is third frequent occurring cancer after bladder and breast cancer. Furthermore, hepatocellular carcinoma (HCC) is a major health problem. The load of hepatocellular carcinoma (HCC) has been increasing in Egypt with a doubling in the happening rate in the past 10 years [3]. Antioxidants can be defined as molecules that can delay or inhibit the oxidation of lipids or other molecules via inhibiting the initiation or propagation of oxidation chain reactions. Generally, the antioxidants are classified according to their origin into two basic types natural and synthetic. Recent studies revealed, that the interest has increased extremely in findings naturally occurring antioxidants for use in food or medicinal beneficial materials to displace synthetic antioxidants, which are being restricted due to their carcinogenic effect [4]. Natural products from medicinal plants are a rich resource used for centuries to fix various diseases. The use of bioactive plant-derived molecules increased, because the basic absorption with the use of synthetic drugs is the harmful side effects which can be

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even more dangerous than the diseases. On the other side, plant derived medicines are based upon the preamble that they contain natural compounds that can elevate health, alleviate illness and proved to be safe, better patient bearing, relatively less expensive and globally competitive [5]. Plant polyphenolic compounds are secondary metabolites with promising properties for human health. The beneficial characters of those compounds are related to their antioxidant activity [6]. Furthermore, previous studies have revealed that the antioxidant activity may be due to the presence of biologically active secondary metabolites such as flavonoids, tannins and phenolic acids via two modes of action involving both free radical scavenging and metal chelation mechanisms which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [4, 7]. *Gmelina arborea* (Roxb.) family verbenaceae is a fast growing deciduous tree occurring naturally throughout greater part of India. It also occurs naturally in Myanmar, Thailand, Cambodia, Vietnam and in southern provinces of China, Sierra Leone, Nigeria, Malaysia and on experimental basis in other countries as well [8]. *Gmelina arborea* was used in constructions, furniture, carriages, sports, musical instruments and artificial limbs. Pervious phytochemical studies on gmelina genus showed the presence of several compounds such as phenolic compounds [9, 10], iridoids [11, 12] and lignans [10]. Also, previous studies revealed that different parts of *Gmelina arborea* (leaf, root and bark) possess; antioxidant activity [13], antimicrobial activity [8, 13,14], anthelmintic activity [15], cytotoxic activity [16], antiulcer activity [17], diuretic activity [18], antidiabetic activity [19-21] and vasorelaxt activity [22]. Therefore, the purpose of this study is to analyse the antioxidant and cytotoxic activities along with the elucidation of chemical structures of the flavonoidal compounds from *Gmelina arborea*.

MATERIALS AND METHODS

Plant Material: The leaves of the plant under investigation were collected from Zoo Garden, Giza, Egypt in July 2011. The identity of the plant was established by Prof. Dr. Wafaa Amer, Professor of Plant Taxonomy, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimen (given number GA) was kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI). The plant material was air-dried in shade place at room temperature, then powdered by electric mill and kept in tightly closed container in dark places until subjected to the extraction process.

General Experimental Procedures: ^{1}H-NMR (500 MHZ, DMSO-d_6) and ^{13}C-NMR (125 MHZ, DMSO-d_6) spectra were recorded on JEOL-GX-spectrometer National Research Center (NRC), Giza-Egypt. The chemical shifts were expressed in δ (ppm) with reference TMS and coupling constant (*J*) in Hertz. UV spectra were determined in methanol as well as diagnostic shift reagents (Micro Analytical Center, Faculty of Science, Cairo-Egypt). Infrared spectra were determined in Fourier Transform Infrared Spectrometer (FT/IR)-6100 JASCO, National Research Center (NRC). Melting points were determined on an electrothermal apparatus. Silica gel 60 GF254 (Fluka) were used for analytical TLC. Sephadex LH-20 (25-100 μm, Sigma) and silica gel (70-230 mesh, Merck) were used for column chromatography. Paper chromatography was carried out on Whatman No. 1 and No. 3 paper sheets (Whatman, England). Spots were visualized by absorption of UV radiation and spraying with methanolic 1% $FeCl_3$ and/or 5% $AlCl_3$ and/or Naturstoff (NA) and 40% H_2SO_4 followed by thermal activation.

Material and Chemicals: All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picraylhydrazyl (DPPH$^{\bullet}$) free radical was purchased from (Sigma-Aldrich Co.). Aluminum chloride, ferric chloride, Naturstoff, sodium phosphate, ammonium molybdate, ascorbic acid and gallic acid were purchased from (Merck Chemical Co.), all solvents and acids [petroleum ether, chloroform, methylene chloride, ethyl acetate, n-butanol, acetone, methanol, acetic acid and sulphuric acid], were purchased from (Sigma-Aldrich Co.). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

Extraction and Fractionation: The air-dried powdered leaves of *Gmelina arborea* (2 kg) were extracted with 90% methanol at room temperature. The 90% methanolic extract was concentrated under reduced vacuum to yield 400 gm. The dried 90% methanol extract 300 gm was defatted with petroleum ether (60-80°C) to give petroleum ether fraction (30.69 gm). The defatted material was fractionated via organic solvents; $CHCl_3$, EtOAc and n-BuOH. The obtained fractions were concentrated to afford 10.73, 7.35 and 86.06 gm respectively.

Antioxidant Activity Measurements:

***Evaluation of Dpph Radical Scavenging Activity* (RSA):** The scavenging activity of the stable 1,1'-diphenyl-2-picraylhydrazyl (DPPH) free radical was determined via the method described by Marwah *et al*. 2007. Briefly, the reaction medium contained 2 ml of 100 µM DPPH purple solution in methanol and 2 ml of the compound, ascorbic acid was used as standard, the control consist of 2ml of DPPH and 2ml methanol. The reaction mixture was incubated in the dark for 20 min and the absorbance was recorded at 517 nm. The assay was carried out in triplicate. The decrease in absorbance on addition of test samples was used to calculate the antiradical activity, as expressed by the inhibition percentage (%IP) of DPPH radical, following the equation: %IP = [Ac - As]/ Ac X 100; where Ac and As are the absorbencies of the control and of the test sample after 20 min, respectively [23].

Evaluation of Total Antioxidant Capacity (TAC): The total antioxidant capacity of each compound was determined according to phosphomolybdenum method, using ascorbic acid as standard. In this method, 0.5 ml of each compound (200 µg /ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials containing the reaction mixture were capped and incubated in a thermal block at 95°C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the compounds was expressed as the number of ascorbic acid equivalents (AAE) [24,25].

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Statistical Analysis: All data were presented as mean ± SD using SPSS 13.0 program.

Cytotoxic Activity

Liver Carcinoma Cell Line (HepG2): Potential cytotoxicity of the isolated compounds was tested using method of Skehan *et al*. [26], using cell line HEPG-2. Cells were plated in 96-multiwell plate (104cells/well) for 24 hrs before treatment with the compounds or extract to allow attachment of cell to the wall of the plate. Different concentrations of the compounds under test (0, 1, 2.5, 5 and 10 µg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 hrs at 37°C and atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is platted to get the survival curve of each tumor cell line after the specified compound.

Chromatographic Isolation

Chromatographic Isolation of N-butanol and Ethyl Acetate Fractions: The n-butanol fraction (35 gm) was subjected to column chromatography packed with silica gel 60 adsorbent (70-230 mesh, Merck; 750 gm). Elution was started with petroleum ether followed by gradient mixtures of petroleum ether: CH_2Cl_2 then CH_2Cl_2 followed by gradient mixtures of CH_2Cl_2: MeOH up to methanol. Fractions (500 ml) were collected and monitored via paper chromatography (PC) in eluent systems BAW (n-BuOH: AcOH: H_2O; 4:1:5 top layer; S1) and 15%AcOH (S2) as well as via thin layer chormatography (TLC), in eluent systems; $CHCl_3$: MeOH, 9:1 (S3), $CHCl_3$: MeOH; 9.5: 0.5 (S4), $CHCl_3$: MeOH; 8.5:1.5 (S5), $CHCl_3$: MeOH: H_2O; 7:3:0.5 (S6), $CHCl_3$: MeOH: H_2O; 16:9:2 (S7), n-BuOH: MeOH: H_2O; 4:1:0.5 (S8), EtOAc: HCOOH: H_2O; 18:1:1 (S9), $CHCl_3$: MeOH: HCOOH; 8:5:0.1 (S10) and $CHCl_3$: Me_2CO: MeOH: H_2O; 3:3:2:1 (S11). The PC chromatograms were examined under UV light and sprayed with $AlCl_3$ and/or Naturstoff and/or $FeCl_3$ and/or ammonia spraying reagents; while TLC chromatograms were examined under UV light and sprayed with MeOH/H_2SO_4 (60/40 v/v) reagent, similar fractions were collected together. Four major fractions I, II, III and IV were obtained via chromatographic isolation, fraction I was eluted via 95:5; fraction II was eluted via 910:10; fraction III was eluted via 85:15 and fraction IV was eluted via CH_2Cl_2: MeOH; 75:25 to afford the compounds (1-5). The ethyl acetate fraction (5 gm) was similarly chromatographed to afford two major fractions I and II, fraction I was eluted with CH_2Cl_2: MeOH; 85:15; whereas fraction II was eluted with CH_2Cl_2: MeOH; 75:25 to afford the compounds (6-7).

Complete Acid Hydrolysis: The compound (3-5 mg) was hydrolyzed with 10% HCl (3.5 ml) in aqueous methanol at 100°C for 2 hrs, after the removal of the solvent, hydrolysate was exhaustively extracted with ethyl acetate in separating funnel. Aglycones identified via Co-PC with authentic aglycone sample. The aqueous phase was neutralized with 5% sodium bicarbonate and used for investigation of the sugar moieties via Co-TLC with authentic sugar markers in eluent system (S11) [27,28].

RESULTS AND DISCUSSION

In the current research, seven flavonoidal compounds were isolated from the leaves parts of *G.arborea* plant growing in Egypt. The structural identification of those compounds was carried out on the basis of R_f values, products of acid hydrolysis, Co-PC, Co-TLC, m.p. and spectroscopic tools (UV, IR, ^{1}H NMR and ^{13}C NMR).

Identification of the Isolated Flavonoidal Compounds

Compound 1: Pale yellow powder, m.p. 325-328°C, R_f: PC 0.76 (S1) and 0.076 (S2); TLC 0.93 (S5), 0.78 (S7) and 0.85 (S9). It gave a dark purple florescence under UV-light, yellow colour under UV/NH_3 and no change with $AlCl_3$ [27]. UV spectral data, λ_{max} (nm) (Table 3) [27, 29]. Compound 1 was expected to be luteolin aglycone on the basis of its chromatographic properties (R_f values, colour under UV/NH_3 and $AlCl_3$) and UV spectra [27]. IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3505.95-3421.1 (-OH aromatic alcohol stretching), 3071.08 (Ar-CH-), 1267 (-C-O- stretching), 1656.55 (>C=O stretching), 1610.27, 1502.28 and 1442.43 (Ar-C=C-) [30]. ^{1}H, ^{13}C-NMR (500, 125 MHz, DMSO-*d*6) δ in ppm (Table 1), were agreement with data given in the literature [31,32]. On the basis of the above data compound 1 was identified as 5, 7, 3', 4'- tetrahydroxy-flavone (luteolin).

Compound 2: Dark yellow powder, m.p. 225-227°C, R_f: PC 0.84 (S1) and 0.50 (S2); TLC 0.16 (S5). It gave a dark purple spot under UV-light turned to yellow after spraying with $AlCl_3$, yellow fluorescence on exposure to ammonia vapors and orange with Naturstoff (NA) spraying reagents. It gave positive Molisch's test indicating its glycosidic nature [27]. UV spectral data, λ_{max} (nm) (Table 3), indicated a flavone substituted at position C-4'. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 2 was identical with luteolin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standard, D-galactose. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), revealed ABX spin coupling system at δ ppm 7.44 (1H, dd, *J*=8.4 and 2.0 Hz, H-6'), 7.41 (1H, d, *J*= 2.0 Hz, H-2') and 7.20 (1H, d, *J* = 8.6 Hz, H-5') for B-ring. Moreover AM spin-coupling system of two meta-coupled protons (H-6 and H-8) at 6.13 and 6.42 ppm for 5, 7-dihydroxy A-ring. There is also a singlet signal attributed to H-3 at 6.74 ppm for C-ring. The presence of galactose moiety on 4'-OH was concluded through intrinsic upfield location of H-1" at 4.83 ppm (brd) [27, 33]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 1), showed 15 carbon signals characteristic for luteolin moiety. The location of D-galactose moiety on 4' position was confirmed from the alternative α-upfield/β-downfield effect on the resonances of B-ring. This was observed from upfield of C-4' (149.07), slight downfield of C-3' (147.53) and C-5' at (116.47), upfield of C-2' (114.09) and C-6' (118.89) and downfield of C-1' at (125.25 → Δ 4 ppm) [27, 33]. Configuration and confirmation of galactose moiety was identified as β-D-$^4C_1$-galactopyranoside on the basis of δ- and *J*-values of its proton resonances [31,32, 34]. From the above data compound 2 was identified as luteolin-4'-*O*-β-D-$^4C_1$-galactopyranoside.

Compound 3: Yellow powder, m.p. 277-279°C, R_f: PC 0.79 (S1) and 0.05 (S2); TLC 0.71 (S4). It appeared as yellow spot on PC under UV-light, gave bright yellow with NH_3 vapor, bright yellow with $AlCl_3$ and pale yellow with Naturstoff (NA) spraying reagents. UV spectral data, λ_{max} (nm) (Table 3). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3425 (-OH aromatic alcohol stretching); 1295, (-C-O-stretching); 1650 (>C=O stretching) and 1475 (Ar-C=C-) [35]. Compound 3 was expected to be a flavonol like structure based on its chromatographic properties and UV spectra [27]. In addition to comparative study with published data as well as via comparison its chromatographic properties with authentic kaempferol sample through (Co-PC, Co-TLC and Co-m.p.) [31,32], therefore compound 3 was identified as 3, 5, 7, 4'-tetrahydroxy-flavone (kaempferol).

Compound 4: Dark yellow powder, m.p. 192-194°C, R_f: PC 0.65 (S1) and 0.45 (S2). It gave faint yellow spot on PC on visible-light, under UV-light showed dark purple fluorescence, when fumed with ammonia vapor turned to yellow, changing to yellow with $AlCl_3$ and orange with Naturstoff (NA) spraying reagents. Also, it gave positive Molisch's test indicating its glycosidic nature; also it gave positive Shinoda test indicating its flavonol skeleton [27, 31]. UV spectral data, λ_{max} (nm) (Table 3) [27, 36]. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 4 was identical with quercetin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standard, D-glucose. Based on chromatographic properties, UV-spectral data and acid hydrolysis products, compound 4 was expected to be quercetin-3-*O*-glycoside [27]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), showed a characteristic five proton resonances of spin coupling systems for quercetin 3-*O*-glycoside structure were assigned to H-2', H-6', H-5', H-8 and H-6. Concerning the sugar moiety, signal anomeric

Table 1: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds 1, 2 and 4 in (DMSO-d_6) (δ in ppm).

	1		2		4	
Carbon	δ_c(ppm)	δ_H(ppm)	δ_c(ppm)	δ_H(ppm)	δ_H(ppm)	δ_c(ppm)
1						
2	164.40		163.51		156.85	
3	103.30	6.64 (1H, s, H-3)	104.36	6.74 (1H, s, H-3)	133.80	
4	182.18		182.12		177.91	
5	161.94	12.92 (1H, s, 5-OH)	161.91	12.84 (1H, s, 5-OH)	161.75	12.61 (1H, s, 5-OH)
6	99.38	6.36 (1H, d, J= 2.1 Hz, H-6)	99.73	6.13 (1H, d, J= 2.0 Hz, H-6)	99.22	6.15 (1H, d, J= 1.9 Hz, H-6)
7	164.74		163.51		161.75	
8	94.42	6.41 (1H, d, J= 2.1 Hz, H-8)	94.72	6.42 (1H, d, J= 2.0 Hz, H-8)	94.04	6.35 (1H, d, J= 1.9 Hz, H-8)
9	157.81		157.94		156.63	
10	104.13		104.36		104.40	
1'	121.93		125.25		121.65	
2'	113.76	7.35 (2H, d, J= 8.4, 2.1 Hz, H-2', 6')	114.09	7.41 (1H, d, J= 2.0 Hz, H-2')	115.70	7.53 (1H, dd, J=7.65, 2.0 Hz, H-2', 6')
3'	146.25		147.53		145.34	
4'	150.26		149.07		149.01	
5'	116.52	6.85 (1H, d, J= 8.4 Hz, H-5')	116.47	7.20 (1H, d, J= 8.4 Hz, H-5')	116.68	6.80 (1H, d, J= 8.6 Hz, H-5')
6'	119.52		118.89	7.44 (1H, dd, J= 8.4 and 2.0 Hz, H-6')	122.11	
1"			101.63	4.84 (1H, d, J= 6.85 Hz , H-1" Galac)	101.35	5.44 (1H, d, J= 7.6 Hz , H-1" Glc)
2"			73.73		74.60	
3"			76.36		77.01	
4"			70.25		70.44	
5"			77.76		78.10	
6"			61.17		61.47	
1"'						
2"'						
3"'						
4"'						
5"'						
6"'						

Table 2: ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data for compounds 5-7 in (DMSO-d_6) (δ in ppm).

	5		6		7	
Carbon	δ_c(ppm)	δ_H(ppm)	δ_c(ppm)	δ_H(ppm)	δ_H(ppm)	δ_c(ppm)
1						
2	157.13		163.50		157.11	
3	133.80		101.73	6.75 (1H, s, H-3)	133.78	
4	177.89		182.12		177.88	
5	161.69	12.55 (1H, s, 5-OH)	161.93	12.85 (1H, s, 5-OH)	161.69	12.53 (1H, s, 5-OH)
6	99.22	6.17 (1H, d, *J*= 1.9 Hz, H-6)	99.72	6.13 (1H, d, *J*= 1.9 Hz, H-6)	99.20	6.16 (1H, d, *J*= 1.9 Hz, H-6)
7	164.66		163.50		164.65	
8	94.13	6.38 (1H, d, *J*= 1.9 Hz, H-8)	94.70	6.42 (1H, d, *J*= 1.9 Hz, H-8)	94.11	6.34 (1H, d, *J*= 1.9 Hz, H-8)
9	156.91		157.94		156.90	
10	101.24		103.91		101.25	
1'	121.66		125.27		121.65	
2'	115.79	7.50 (2H, d, *J*= 7.65 Hz, H-2', H-6')	114.10	7.45 (2H, d, *J*= 1.9 Hz, H-2', 6')	115.77	7.51 (2H, d, *J*= 1.9 Hz, H-2', 6')
3'	145.27		147.55		145.27	
4'	148.94		149.07		148.94	
5'	116.77	6.83 (1H, d, *J*= 8.6 Hz, H-5')	116.48	7.20 (1H, dd, *J*= 8.6 and 1.9 Hz, H-5')	116.75	6.82 (1H, d, *J*= 8.75 Hz, H-5')
6'	122.10		118.88		122.10	
1"	104.44	5.29 (1H, d, *J*= 6.7 Hz, H-1" Glc)	104.39	4.83 (1H, d, *J*= 6.7 Hz, H-1" Galac)	101.66	5.29 (1H, d, *J*= 7.8 Hz, H-1" Galac)
2"	74.35		73.76		72.33	
3"	76.39		76.36		74.57	
4"	70.87		70.26		70.58	
5"	76.46		77.81		76.19	
6"	68.74		61.20		68.75	
1"'	101.69	4.41 (1H, d, *J*= 1.2 Hz, H-1"' Rha)			104.43	4.38 (1H, d, *J*= 1.5 Hz, H-1"' Rha)
2"'	71.07				71.05	
3"'	71.07				70.86	
4"'	72.36				72.01	
5"'	70.49				70.48	
6"'	18.24	0.95 (3H, d, *J*= 5.7 Hz, Rha-6"')			18.26	0.93 (3H, d, *J*= 6.8 Hz, Rha-6"')

Table 3: UV spectral data λ_{max} (nm) for the isolated flavonoidal compounds 1-7.

Compound	MeOH	MeOH + NaOMe	MeOH + $AlCl_3$	MeOH + $AlCl_3$ + HCl	MeOH + NaOAc	MeOH + NaOAc+H_3BO_3
1	267, 275^{sh}, 339	277, 328^{sh}, 392	275, 305^{sh}, 492	276, 302^{sh}, 345, 388	275, 308^{sh}, 385	268, 306^{sh}, 344
2	259, 274^{sh}, 352	263, 318^{sh}, 398	279, 339^{sh}, 399	278, 308^{sh}, 355	269, 310^{sh}, 386	269, 309^{sh}, 365
3	267, 293^{sh}, 367	272, 300^{sh}, 418	274, 345^{sh}, 368	271, 350^{sh}, 420	275^{sh}, 318, 385	267, 315^{sh}, 370
4	256, 265^{sh}, 297^{sh}, 357	272, 325 ^{sh},408	274, 307 ^{sh}, 430	269, 300^{sh}, 363, 400	273, 322, 408	261, 323^{sh}, 397
5	254, 268 ^{sh}, 298 ^{sh},354	269, 330 ^{sh},412	271, 306 ^{sh}, 408	268, 359, 405	270, 310^{sh}, 398	260, 290, 372
6	255, 269^{sh}, 348	263, 348^{sh}, 403	270, 313^{sh}, 400	268, 353^{sh}, 386	264, 360^{sh}, 401	264, 305^{sh}, 372
7	254, 275^{sh}, 350	268, 347 ^{sh}, 409	272, 314 ^{sh}, 410	261, 324 ^{sh}, 361, 402	268, 321^{sh}, 395	268, 306 ^{sh}, 370

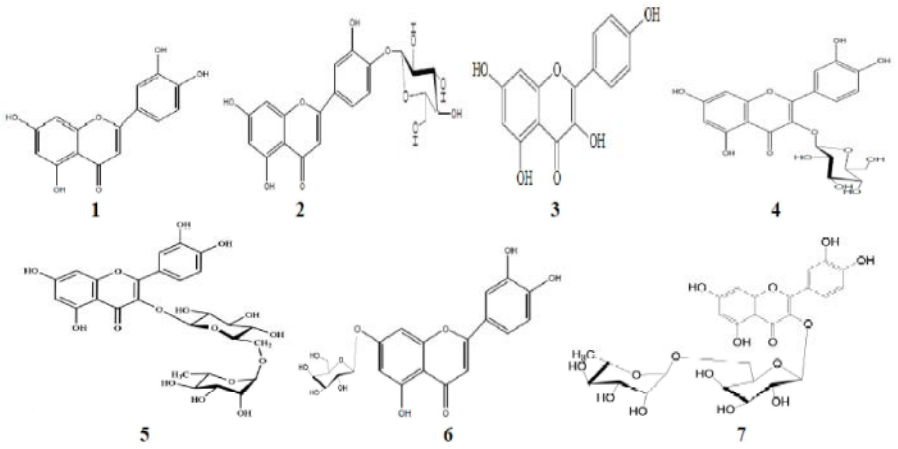

Fig. 1: Chemical skeletons of flavonoidal compounds isolated from *G. arborea* leaves.

proton at 5.43 ppm (assignable to β-proton, d, *J*= 7.6 Hz) [31,32, 37]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 1), showed typical 15 carbon signals for quercetin aglycone moiety, among them C-4' and C-3' at 149. 01 and 145.34 ppm which can be consider the key signals of 3', 4' at dihydroxy B-ring in all quercetin glycosides. As well as, it showed six ^{13}C-resonances of an *O*-glucopyranoside moiety. Glycosidation at 3-OH was proved by relative upfield shift of C-3 to 133.80 ppm and downfield shift of C-2 (+10 ppm) relative to those of free aglycone. Assignment of all other ^{13}C-resonances was proved by their comparison with reported data in the literature [31,32, 37]. Hence, compound 4 was identified as quercetin - 3 - *O* - β - D - $^4C_1$ - glucopyranoside (isoquercetrin).

Compound 5: Yellow fine crystal, m.p. 188-189°C, R_f: PC 0.49 (S1) and 0.55 (S2); TLC 0.50 (S10). It gave dark purple spot under UV-light, turned yellow fluorescence with ammonia vapors and $AlCl_3$ as well as orange colour with Naturstoff (NA) spraying reagents. Also, it gave positive Molisch's test indicating its glycosidic nature; also it gave positive Shinoda test indicating its flavonol skeleton [27]. UV spectral data, λ_{max} (nm) (Table 3) [27, 38]. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 5 was identical with quercetin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standards, D-glucose and L-rhamnose. On the bases of its chromatographic properties, UV-spectral data and acid hydrolysis products, compound 5 was expected to be quercetin-3-*O*-glycoside. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 2), showed the splitting pattern characteristic for quercetin type as two spin coupling systems. The first one, an ABX of three types of protons at 7.66, 7.51 and 6.83 ppm which were assignable to H-2'/H6' and 5' of 3', 4'-dihydroxy B-ring respectively, the second system was described as AM of two meta coupled doublets at 6.17 and 6.38 ppm (each doublet *J*= 1.9 Hz), respectively for H-8 and H-6 of 5, 7-dihydroxy A-ring. Glycosidation at C-3 was deduced from the upfield shield of C-3 at 133.80 ppm. Rutinoside moiety was deduced from the two anomeric proton signals at 5.31 (d, *J*=6.7 Hz) and 4.34 (d, *J*= 1.5 Hz) together with a doublet of three protons at 9.45 ppm (d, *J*= 5.7 Hz) assigned for a β-glucopyranoside and α-pyranoside moieties, respectively, 1'''→ 6″ interglycosidic linkage was followed from the relative downfield location of (CH_2-6") as two broad doublets at 3.67 and 3.35 ppm [29, 39-41]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 2), twelve carbon resonances were assigned for a rutinoside moiety, among which the most downfield signals at 104.44 and 101.69 ppm assigned to the two anomeric carbons C-1" and C-1''', respectively together with the down field shift of C-6″ (68.74) to

confirm the 1'''→6″ interglycosidic linkages. The sugar moieties were deduced to have α-$^1C_4$ and β-$^4C_1$-pyranose stereo structure in case of rhamnosyl and glucosyl moieties, respectively [29, 39-41]. All spectral data of the compound with in full agreement with reported data [31-32, 42]. Therefore, via comparison of it's chromatographic properties with authentic rutin sample through (Co-PC, Co-TLC and Co-m.p.), compound 5 was identified as quercetin - 3 -*O* - α - L - $^1C_4$ - rhamnopyranosyl - (1''' → 6'') - *O* - β - D - $^4C_1$ glucopyranoside (rutin).

Compound 6: Pale yellow powder, m.p. 270-272°C, R_f: PC 0.48 (S1) and 0.35 (S2). It gave dark purple florescence in UV-light, converted to yellow colour upon exposure to ammonia vapor and changed to yellow with $AlCl_3$ spraying reagents [27]. UV spectral data, λ_{max} (nm) (Table 3) [27]. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 6 was identical with luteolin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standard, D-galactose. Compound 6 was expected to be luteolin-*O*-glycoside on the basis of its chromatographic properties (Rf-values, fluorescence under UV-light, color reaction with different spraying reagents, UV spectra and acid hydrolysis products) [27]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 2), showed a multiplet signal for two protons at ˜ 7.45 and a doublet at 7.20 ppm for H-2'/6' and H-5' characteristic for 3', 4'-dihydroxy B-ring of luteolin nucleus. The down shift of the two resonances, H-6 and H-8 at 6.13 and 6.42 ppm respectively, was indicative of glycosidation at 7-OH. Galactosyl moiety in the structure of compound 6 was deduced from the anomeric proton at 4.83 ppm (brd.) [31-32]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 2), showed in the aromatic region a typical pattern resolved fifteen carbon resonances of 7-*O*-substituted luteolin. Six carbon resonances were assigned for a galactosyl moiety. Assignment of all other carbon resonances was confirmed depending on a comparison study with those of the previous published data of structural related compounds [31-32, 42]. Therefore compound 6 was identified as luteolin-7-*O*-β-D-$^4C_1$-galactoside.

Compound 7: Yellow powder, m.p. 182-184°C, R_f: 0.52 (S1) and 0.61 (S2). It gave dark purple florescence in UV-light, converted to yellow colour upon exposure to ammonia vapor, orange colour with Naturstoff (NA) and yellow with $AlCl_3$ spraying reagents [27]. UV spectral data, λ_{max} (nm) (Table 3) [27]. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 7 was identical with quercetin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standards, D-galactose and L-rhamnose. IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3455 (-OH aromatic alcohol stretching); 1285, (-C-O-stretching); 1665 (>C=O stretching) and 1620, 1500, 1480 (Ar-C=C-). Therefore, the compound 7 was expected to be quercetin-3-*O*-rhamnosylgalactoside on the bases of its chromatographic properties (Rf-values, fluorescence under UV-light, color reaction with different spraying reagents, UV spectra and acid hydrolysis products) [27]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 2), exhibited am ABX coupling system of three types of protons at 7.51 (m), 7.49 (m) and 6.82 ppm (d, *J*= 8.6) assignable for H-2', H-6' and H-5', respectively beside an AM coupling system of two meta coupled protons at 6.89 (brd, d) and 6.10 ppm (brd) assignable to H-8 and H-6. ^{1}H-NMR also showed a β- anomeric proton signal of inner galactoside moiety at 5.17 ppm (brs) with a characteristic anomeric doublet signal and CH_3-6''' as a doublet at 1.04 ppm (brd) of a terminal rhamnosyl moiety. The δ-values of H-1''' at 4.45 ppm (brs) were confirmative evidence for (1'''→6″) rhmnosylgalactoside connection [42]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 2), exhibited typical 15 carbon signals characteristic for a 3-*O*-substitued quercetin; δ-values for 12 carbons of sugar moiety ^{13}C-resonance were in complete agreements with those of a robinobioside moiety, particularly the down field shift of C-6'' (+7ppm) and upfield shift of C-5'' (1ppm) due to 1'''→6''-glycosedation [31-32, 42]. The large difference between δ-values of C-3″ and 5″ (≈ 2 ppm) was diagnostic to galactose moiety, as compared with that of glucose (≈ 1 ppm) [31-32, 42]. Assignment of all ^{1}H and ^{13}C resonances was achieved through a comparison with the reported data in the literature [31-32, 42]. Hence, compound 7 was identified as quercetin 3-*O* -α - L-$^1C_4$-rhamnopyranosyl - (1'''→ 6″)-β - D -$^4C_1$-galactopyranoside = quercetin 3 - *O* - β - D - robinobioside.

Antioxidant Activity: The antioxidant activities of the isolated compounds were evaluated via 1,1'-diphenyl-2-picryl-hydrazyl free radical and phosphomolybdenum antioxidant assays. The antioxidant activity results of the isolated compounds were summarized in (Table 4), these results indicated that the seven compounds exhibited marked scavenging activity compared to the standard ascorbic acid (SC_{50}= 8.0 µg/ml). The DPPH free radical antioxidant activity (SC_{50}) ranged from 5.70 to 14.40 µg/ml, while the total antioxidant capacity ranged from

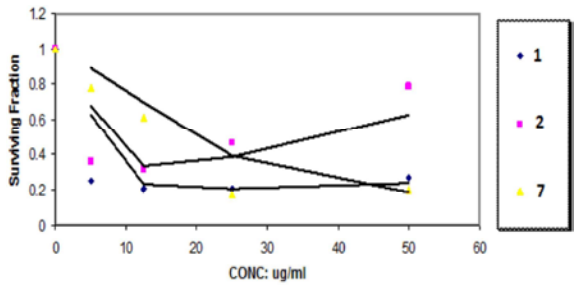

Fig. 2: Potential cytotoxicity of the isolated compounds 1, 2 and 7.

Table 4: Free radical scavenging potential (DPPH) and total antioxidant capacity of the isolated compounds from *G.arborea*.

Compound	[a]DPPH SC_{50} [µg/ml]	[b]Total antioxidant capacity (mg AAE /g ext.)
1	9.20 ± 2.0	600.30 ± 1.49
2	14.40 ± 1.50	403.66 ± 2.46
3	10.25 ± 3.45	520.45 ± 1.53
4	5.70 ± 1.20	541.87 ± 1.70
5	8.35 ± 2.15	630.75 ± 2.33
6	10.65 ± 1.55	491.84 ± 2.34
7	9.40 ± 2.65	590.20 ± 1.20
Ascorbic acid	8.0 ± 1.30	

Results are expressed as mean values ± standard deviation (n = 3).
[a] DPPH values are expressed as µg compound/ml (µg/ml).
[b] Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g compound).

Table 5: Potential cytotoxicity (IC_{50}) of the isolated compounds 1, 2 and 7.

	SF (HEPG2)		
Conc. µg/ml	1	2	7
0.000	1.000	1.000	1.000
5.000	0.252	0.360	0.780
12.500	0.208	0.313	0.614
25.000	0.209	0.465	0.175
50.000	0.274	0.789	0.198

SF = Surviving fraction; IC_{50}= Dose of the extract which reduces survival to 50%.

(630.75 mg AAE/g compound) to (403.66 mg AAE/g compound). The antioxidant assays could be based on the measurement of free radical scavenging potency (hydrogen-donating ability). The radical scavenging via the polyphenolic molecules leading to more stable phenoxy radicals. The use of DPPH radical provides an easy, rapid and convenient method to evaluate the antioxidants and radical scavengers [43,44]. Therefore, in this study, the isolated flavonoidal compounds were investigated for their possible antioxidant and radical scavenging activity by DPPH assay and their SC_{50} values were calculated for further comparisons. The antioxidants activity of the naturally occurring phenolic compounds is predominantly owing to their redox properties (the ability to act as reducing agent, hydrogen donors and singlet oxygen quenchers and to some extent could also be due to their metal chelation potential [43,44].

Cytotoxic Activity: The cytotoxic activity using HepG-2 assay showed that all the tested compounds have cytotoxic activity with IC_{50} ranged from 3.38 to 15.70 µg/ml (Table 5 and Figure 2). From the above results, the tested compounds exhibited cytotoxic activity against HepG-2 cells in the order 1, 2 and 7. The hydroxylation pattern of the B- and C-rings of the flavonoidal compounds, like luteolin and quercetin aglycones as well as their glycosides, play a vital role in their cytotoxic activities, especially the inhibition of protein kinase antiproliferation activity [45]. For example, luteolin (1) is an important member of the flavonoid family, it has been reported that it able to inhibit the proliferation of a variety of tumor cells, including solid tumors, ascites cancer and human myeloid leukemia as well as sensitize a number of apoptosis-inducing factors via unique mode of actions [45]. Furthermore, the previous reported studies revealed that numerous flavonoids have been isolated as cytotoxic antitumor agents [46]. In summary, we focused our investigation on cytotoxic principles from *G.arborea* and found a new flavone glycoside (2) with selective cytotoxic activity against the HepG-2 cell line and six known flavonoids (1, 3-7). Thus, it is possible to demonstrate that isolated compounds (1, 2 and 7) might possess beneficial therapeutic potential against tumors.

CONCLUSION

The present study revealed that the 90% methanolic extract of *Gmelina arborea leaves* exhibited high *in vitro* anticancer activity toward hepatocellular carcinoma cell line (HepG-2). Seven flavonoidal compounds were isolated from the n-BuOH and EtOAc derived fractions from 90% methanolic extract, all of them showed antioxidant activity in different percentages and three of these compounds showed high cytotoxic activity. From the above results the 90% methanol extract of *Gmelina arborea* leaves could be used as promising antioxidant and cytotoxic agent.

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