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Radical Scavenging Potential And Cytotoxic Activity of Phenolic Compounds From *Tectona grandis* Linn.

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Abstract: Ten compounds including two phenolic acids and eight flavonoids were isolated from n-butanol and ethyl acetate fractions of *Tectona grandis* leaves part (Family Verbenaceae). All of the compounds were isolated and purified via different column chromatography. 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 to be identified as; quercetin (1), apigenin (2), gallic acid (3), quercetin-3-*O*-$^{1}C_{4}$-α-L- rhamnoside (quercetrin) (4), 5'-*O*-caffeoyl quinic acid (Chlorogenic acid) (5), diosmin (diosmetin-7-*O*-rutinoside) (6), kaempferol-3-*O*-α-$^{1}C_{4}$-L-rhamnopyranosyl-(1'''→6'')-*O*-β-D-$^{4}C_{1}$-glucopyranoside (7), 3'-methoxy-taxifolin-7-*O*-α-L-$^{1}C_{4}$-rhamnopyranosyl-(1'''→6'')-*O*-β-D-$^{4}C_{1}$-galactopyranoside (8), taxifolin (dihydroquercetine) (9) and hesperidin (3', 5, 7-trihydroxy-4'-methoxyflavanone-7-*O*-rhamnoglycoside) (10). 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 (HepG2) via Sulphorhodamine-B assay. The DPPH free radical SC_{50} values ranged from (3.85 µg/ml) to (14.75 µg/ml), while the total antioxidant capacity ranged from (710.23 mg AAE/g compound) to (386.45 mg AAE/gm compound). The results of the cytotoxic activity showed that all the tested compounds have cytotoxic activity with IC_{50} ranged from 3.53 µg/ml to 15.40 µg/ml. It was concluded that the isolated phenolic compounds from *Tectona grandis* possess a potent antioxidant and cytotoxic activities.

Key words: *Tectona grandis* · Phenolic Compounds · Antioxidant Activity · Cytotoxic Activity

INTRODUCTION

Free radicals are highly reactive unstable molecules containing an unpaired single electron; the presence of unpaired electrons allows a considerable extent of reactivity upon a free radical [1]. Organisms are exposed during their life to the effects of exogenous oxidizing agents from environmental pollutants, life style and to endogenous ones produced via metabolism. Reactive oxygen species (ROS) can be produced from both endogenous and exogenous agents. Potential endogenous sources include mitochondria, cytochrome P450 metabolism, peroxisomes and inflammatory cell activation. Additional endogenous sources of cellular reactive oxygen species are neutrophils, eosinophils and macrophages [2]. Chemical substrates that act as oxidizing agents contain reactive oxygen species (ROS), namely (oxygen-centered radicals) such as superoxide anion ($O2^{\bullet}$-), hydroxyl ($HO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals or reactive nitrogen species (RNS) which include agents like peroxynitrite anion ($ONOO^{-}$) and nitric oxide ($NO^{\bullet}$) radical. In addition, there are non-free radical species (oxygen-centered nonradical derivatives) such as hydrogen peroxide (H_2O_2), singlet oxygen $^{1}O_2$(O-O:), nitric oxide (NO) and hypochlorous acid (HClO) which also behave like oxidizing agents leading to initiation of cancer [3]. Cancer can be defined as a rapid abnormal uncoordinated proliferation of aberrant cells in any tissue

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or organ of the body which may mass together to form a growth or tumor or proliferate throughout the body indicating abnormal growth at other sites. If the process is not arrested it may progress until it causes the death of the organism [4, 5]. In fact, cancer is considered as one of the most fearsome causes of morbidity and mortality in all over the world. Although the disease has often been regarded principally as a problem of the developed world, more than half of all cancers occur in the developing countries [6]. From this point, natural products and their derivatives contribute more than half of all clinically administered drugs. They possess a significant position in drug discovery for treatment of cancer and other infectious diseases [7]. Antioxidants are defined as substances, when present at low concentrations compared to that of oxidizable substrates significantly delay or inhibit oxidization of those substrates [8]. Antioxidants can scavenge reactive oxygen species to stop radical chain reactions or inhibit the radical production [9]. Furthermore, antioxidant compounds in food play an important role as health-protecting materials, which reduce the risk for chronic diseases including cancer. Edible medicinal plants can act as an excellent source of useful food ingredients. Vitamin C, vitamin E, carotenoids and phenolic acids, have been reported to possess strong potential to reduce disease risk [10]. Verbenaceae, commonly known as the verbena family, is a family of mainly tropical flowering plants. It contains trees, shrubs and herbs notable for heads, spikes or clusters of small flowers, many of which have an aromatic smell. Verbenaceae family includes some 35 genera and 1200 species [11]. Essential oils [12], phenolic compounds [13], iridoids [14] and triterpenes [15] were isolated from different members of the family. Teak is the common name for the tropical hardwood tree species *Tectona grandis* (Linn.). *T. grandis* is native to South and Southeast Asia, mainly India, Indonesia, Malaysia and Myanmar, but is naturalized and cultivated in many countries, including Africa and the Caribbean. *T. grandis* is a large deciduous tree with small, fragrant white flowers and papery leaves. The plant used in treatment of urinary discharge, bronchitis, laxative, sedative, diuretic and in scabies [16]. The methanol extract of the plant leaves showed a significant wound healing, analgesic and anti-inflammatory activity [16, 17]. Therefore, the current study was undertaken to isolate and elucidate certain phenolic compounds from this plant as well as evaluation their antioxidant and cytotoxic activities.

MATERIALS AND METHODS

Plant Materials: The leaves of the plant under investigation were collected from Zoo Garden, Giza, Egypt in June 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 TG) 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 *T. grandis* (2 kg) were extracted with 90% methanol at room temperature. The 90% methanolic extract was concentrated under reduced vacuum to yield 300 gm. 280 gm was defatted with petroleum ether (60-80°C) to give petroleum ether fraction (39.59 gm). The defatted material was fractionated via organic solvents; $CHCl_3$, EtOAc and n-BuOH. The obtained fractions were concentrated to afford 14.38, 5.48 and 96.31 gm respectively.

Antioxidant Activity

Determination of DPPH Radical Scavenging Activity: 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 [18].

Determination of Total Antioxidant Capacity: 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) [19, 20].

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 pure compounds was tested using method of Skehan *et al*., 1996, using cell line HEPG2. Cells were plated in 96-multiwell plate (104cells/well) for 24 hrs before treatment with the compound to allow attachment of cell to the wall of the plate. Different concentrations of the compound 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 [21].

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; 8.5:1.5 (**S4**), $CHCl_3$: MeOH; 8:2 (**S5**), $CHCl_3$: MeOH: H_2O; 7:3:0.5 (**S6**), $CHCl_3$: MeOH: H_2O; 16:9:2 (**S7**), $CHCl_3$: MeOH: H_2O; 65:45:12 (**S8**), n-BuOH: MeOH: H_2O; 4:1:0.5 (**S9**), EtOAc: HCOOH: H_2O; 18:1:1 (**S10**), EtOAc: $C_6H_5CH_3$: AcOH; 5:4:2 (**S11**), C_6H_6: MeOH: AcOH; 79:14:7 (**S12**) and $CHCl_3$: Me_2CO:MeOH: H_2O; 3:3:2:1 (**S13**). 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. Five major fractions I, II, III, IV and V were obtained via chromatographic isolation, fractions I and II were eluted with CH_2Cl_2: MeOH; 90:10; fraction III was eluted with CH_2Cl_2: MeOH; 85:15 and fractions IV, V

were eluted with CH_2Cl_2: MeOH; 70:30 to afford compounds (**1-8**). 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; 90:10; whereas fraction II was eluted with CH_2Cl_2: MeOH; 80:20 to afford compounds (**9-10**).

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 (**S13**) [22, 23].

RESULTS AND DISCUSSION

In the current research, ten phenolic compounds were isolated from the leaves parts of *T. grandis* 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 Phenolic Compounds

Compound 1: Dark yellow powder, m.p. 305-306°C, R_f: PC 0.69 (S1) and 0.06 (S2); TLC 0.88 (S4). UV spectral data, λ_{max} (nm) (Table 2). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3406.64 (-OH), 3320.82 (-CH-Ar), 1610.27 (-C=O), 1521.33 (Ar-C=C-) and 1145 (-C-O-). Compound 1 was identified as 3, 5, 7, 3', 4'- pentahydroxy-flavone (quercetin) via comparison it's chromatographic properties with authentic quercetin sample (Co-PC, Co-TLC and Co-m.p.) [22, 24, 25].

Compound 2: Yellow powder, m.p. 340-343°C, R_f: PC 0.83 (S1) and 0.11 (S2); TLC 0.80 (S7) and 0.92 (S10). UV spectral data, λ_{max} (nm) (Table 2). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3406.64 (-OH), 3320.82 (-CH-Ar), 1595 (-C=O), 1443.15 (Ar-C=C-) and 1039 (-C-O-). Via comparison its chromatographic properties with authentic apigenin sample (Co-PC, Co-TLC and Co-m.p.), compound 2 was identified as 5, 7, 4'- trihydroxy-flavone (apigenin) [22, 26, 27].

Compound 3: White powder, m.p. 251-252°C, R_f: PC 0.66 (S1) and 0.52 (S2); TLC 0.25 (S4). UV spectral data, λ_{max} (nm) (Table 2). IR ν_{max} (Kbr) cm^{-1} spectrum showed the absorption bands at: 3500 (Ar-OH), 3285 (carboxylic-OH), 3064 (Ar-CH-), 1725 (-C=O) and 1620 (Ar-C=C-). Compound 3 was identified as 3,4,5-trihydroxybenzoic acid (gallic acid) via comparison it's chromatographic properties with authentic gallic acid sample (Co-PC, Co-TLC and Co-m.p.) [24, 26, 28].

Compound 4: Dark yellow powder, m.p. 179-182°C, R_f: PC 0.47 (S1) and 0.29 (S2); TLC 0.72 (S5). UV spectral data, λ_{max} (nm) (Table 2). 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, L-rhamnose. Therefore, compound 4 was identified as quercetin – 3 – *O* – α – L - $^1C_4$ - rhamnoside (quercetrin) [22, 29].

Compound 5: White powder, m.p. 199-200°C, R_f: PC 0.68 (S1) and 0.86 (S2); TLC, 0.35 (S8), 0.2 (S11) and 0.25 (S12). It gave blue fluorescence under UV-light changing to yellowish green with UV/HCl and no change with $AlCl_3$ spraying reagents; also it gave rosy red colour with phenol and effervescence with $NaHCO_3$ reagents indicating the presence of hydroxyl and carboxylic groups as well as deep blue colour with $FeCl_3$ confirming presence of phenolic OH group. UV spectral data, λ_{max} (nm): 328, 297^{sh}, 245^{sh}, 218 (Table 2). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3349.75; 2952.48; 1250; 1687.41 and 1517.7. IR spectrum showed absorption bands indicated to the presence of; (–OH aromatic alcohol stretching, -CH=CH- stretching, -C–O- stretching, >C=O stretching and –C=C– aromatic ring) [30]. Based on chromatographic properties, color reaction with different spraying reagents, UV spectra and IR spectra, compound 5 was expected to be phenolic acid. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), indicate the characteristic resonance in the aromatic region for a caffeoyl moiety, represented by presence of an AX spin coupling system as olefinic doublets at 7.37 and 8.10 ppm with large *J*-values (each, d, *J*= 15.3) for H-7 and H-8, respectively to confirm a geometrical isomerism as *E*-form for the caffeic acid moiety. Moreover an ABM system at 6.99 (brs), 6.97 (brd, *J*= 8.6Hz) and 6.10 (d, *J*= 8.6 Hz) ppm, for H-1, H-4 and H-5 respectively. The quinic acid moiety was confirmed via the presence of four resonances in aliphatic region, among which the most downfield signal at about 5.01 for H-5' due to the location of the caffeoyl moiety at C-5' [24, 31]. Similarly, ^{13}C-NMR spectra (Table 1), showed two carbonyl carbons at 176.99 and 168.39 ppm. A part from these there were eight signals at 146.82, 145.91, 143.96, 126.66, 122.43, 115.93, 114.88 and

Table 1: ^{1}H-^{13}C-NMR (DMSO-d_6) spectral data for the isolated phenolic compounds

Carbon	δ_C (ppm)	5 δ_H (ppm)	δ_C	6 δ_H (ppm) (ppm)	δ_H	8 δ_C (ppm) (ppm)	δ_H	10 δ_C (ppm) (ppm)
1	126.08							
2	115.26	6.99 (1H,brs, H-1)	164.71		85.67		76.79	5.45 (1H,brd, H-2)
3	145.48		100.40	6.79 (1H, s, H-3)	131.41		40.34	3.70-2.46 (2H, m, H-3eq, ax)
4	146.07		182.48		197.57		197.56	
5	116.23	6.10 (1H, d, *J*= 8.6 Hz, H-5)	161.73	12.90 (1H, s, 5-OH)	163.54	11.99 (1H, s, 5-OH)	163.56	11.95 (1H, s, 5-OH)
6	121.90	6.97 (1H, dd, *J*= 8.6 Hz, H-6)	100.10	6.42 (1H, d, *J*= 2.0 Hz, H-6)	96.05	6.08 (1H, H-6)	96.90	6.10 (2H, H-6,8)
7	148.86	7.37 (1H, d, *J*= 15.3 Hz, H-7)	163.46		163.54		165.65	
8	114.76	8.10 (1H, d, *J*= 15.3 Hz, H-8)	95.31	6.72 (1H, d, *J*= 2.0 Hz, H-8)	96.05	6.08 (1H, H-8)	96.06	
9	166.24		157.47		163.01		163.01	
10	------		101.05		103.83		103.83	
1′	73.94		119.48	7.40 (1H, d, *J*= 2.0 Hz, H-2')	114.66		131.40	
2′	39.82		112.75		109.81	6.89 (3H, m, H-2', 5', 6')	118.30	6.89 (3H, m, H-2',5',6')
3′	70.80		147.29		146.96	3.38 (3H, s, 3'-OCH_3)	148.48	
4′	68.48		151.83	7.10 (1H, d, *J*= 8.4 Hz, H-5')	148.47		146.96	
5′	71.41	5.59 (1H,m, H-5')	113.65	7.52 (1H, dd, *J*= 8.4, 2.0 Hz, H-6')	112.53		112.53	
6′	39.99	1.88-1.99 (2H, d, *J*= 15, 10 Hz, H-6')	123.39	7.40 (1H, d, *J*= 2.0 Hz, H-2')	118.47		118.48	
7′/3'-OCH_3	175.48				56.20			
7-*O*-			105.97	5.04 (1H, d, *J*= 6.85 Hz, H-1")				
1″			73.61		101.13	5.12 (1H, brd, H-1")	99.95	4.95 (1H, brd, H-1" Glc)
2″			76.10		76.03		72.58	
3″			68.86		76.78		76.03	
4″			76.77		70.78		70.79	
5″			66.56		78.91		73.50	
6″			105.97		68.84		68.84	
Rha (1→6)								
1‴			104.35	4.50 (1H, d, *J*= 1.25 Hz, H-1‴)	100.79	4.47 (1H,s, H-1‴)	101.12	4.67 (1H, s, H-1‴ Rha)
2‴			70.07		71.21		70.79	
3‴			71.25		71.46		70.10	
4‴			72.55		72.57		72.58	
5‴			68.86		70.09		68.84	
6‴			18.35	1.04 (3H, d, *J*= 6.7 Hz, 6‴)	18.37	1.03 (3H, brs, 6‴)	18.37	1.04 (3H, brd, Rha-6‴)
4'-OCH_3			56.31	3.83 (3H, s, 4'-OCH_3)		4.47 (1H,s, H-1‴)	56.20	3.76 (3H, s, 4'-OCH_3)

Table 2: UV spectral data λ_{max} (nm) for the isolated phenolic compounds

Compound	MeOH	MeOH + NaOMe	MeOH + $AlCl_3$	MeOH + $AlCl_3$ + HCl	MeOH + NaOAc	MeOH + NaOAc+H_3BO_3
1	255, 269^{sh}, 301^{sh}, 370	270^{sh}, 329, 415	272, 304^{sh}, 333, 454	265, 304^{sh}, 354, 428	257^{sh}, 275, 320, 380	261, 304^{sh}, 388
2	265, 295^{sh}, 339	278^{sh}, 330^{sh}, 395	275, 298^{sh}, 392	275, 393^{sh}, 388	277^{sh}, 306^{sh}, 383	268, 299^{sh}, 344
3	216, 274					
4	256, 264^{sh}, 358	271, 351^{sh},405	274, 334^{sh}, 429	271, 357^{sh}, 401	273, 324^{sh}, 390	260, 299, 385
5	328, 297^{sh}, 245^{sh}, 218					
6	254, 268^{sh}, 346	262, 345^{sh}, 408	273, 315^{sh}, 401	270, 355^{sh}, 388	266, 362^{sh}, 403	266, 307^{sh}, 374
7	267, 326^{sh}, 356	267, 274, 347^{sh}, 412	267, 298^{sh}, 304^{sh}, 401	267, 298^{sh}, 301^{sh}, 401	267, 273, 320^{sh}, 392	267, 308^{sh}, 372
8	254, 268^{sh}, 298^{sh},354	269, 330^{sh},412	271, 306^{sh}, 431	268, 359, 405	270, 310^{sh}, 398	260, 290, 372
9	255, 297^{sh}, 370	247^{sh}, 329	272, 304^{sh}, 454	265, 354^{sh}, 428	275, 320^{sh}, 390	261, 304^{sh}, 388

114.16 ppm corresponding to six aromatic and two olefinic carbons. The six carbons from the cyclohexane ring appeared at 74.69, 71.15, 70.40, 68.92, 36.31 and 36.19 ppm [24, 31]. Therefore, based on Co-PC with the authentic sample, compound 5 was identified as 5'-*O*-E-caffeoyl-quinic acid (chlorogenic acid).

Compound 6: Pale yellow powder, m.p. 292-294°C, R_f: PC 0.35 (S1) and 0.45 (S2). It gave dark purple fluorescence under UV-light, changed to greenish yellow with NA and faint green with $AlCl_3$ spraying reagents; also it gave positive Molisch's test indicating its glycosidic nature. UV spectral data, λ_{max} (nm) (Table 2). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3414.35; 2937.06; 1291; 1661.37; 1611.23, 1565.92, 1449.24 and 2937.06. R_f value of the aglycone (Co-PC) after complete acid hydrolysis of 6 was identical with diosmetin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standards, D-glucose and L- rhamnose. The UV spectral data of 6 with diagnostic shift reagents indicated a flavone substituted at position C-7 and presence of and of free 4'-OH at B-ring [22]. IR spectrum showed absorption bands indicated to the presence of; (-OH aromatic alcohol stretching, -CH=CH- stretching, -C–O- stretching, >C=O stretching and Ar–C=C–, -OCH_3). Based on chromatographic properties, color reaction with different spraying reagents, UV spectra and acid hydrolysis products, compound 6 was expected to be luteolin-7-*O*-glycosides skeleton [22]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), exhibited an AMX spin coupling system of three aromatic protons at 7.52 (dd), 7.40 (d) and 7.10 (d) ppm assigned for H-6', H-2' and H-5',

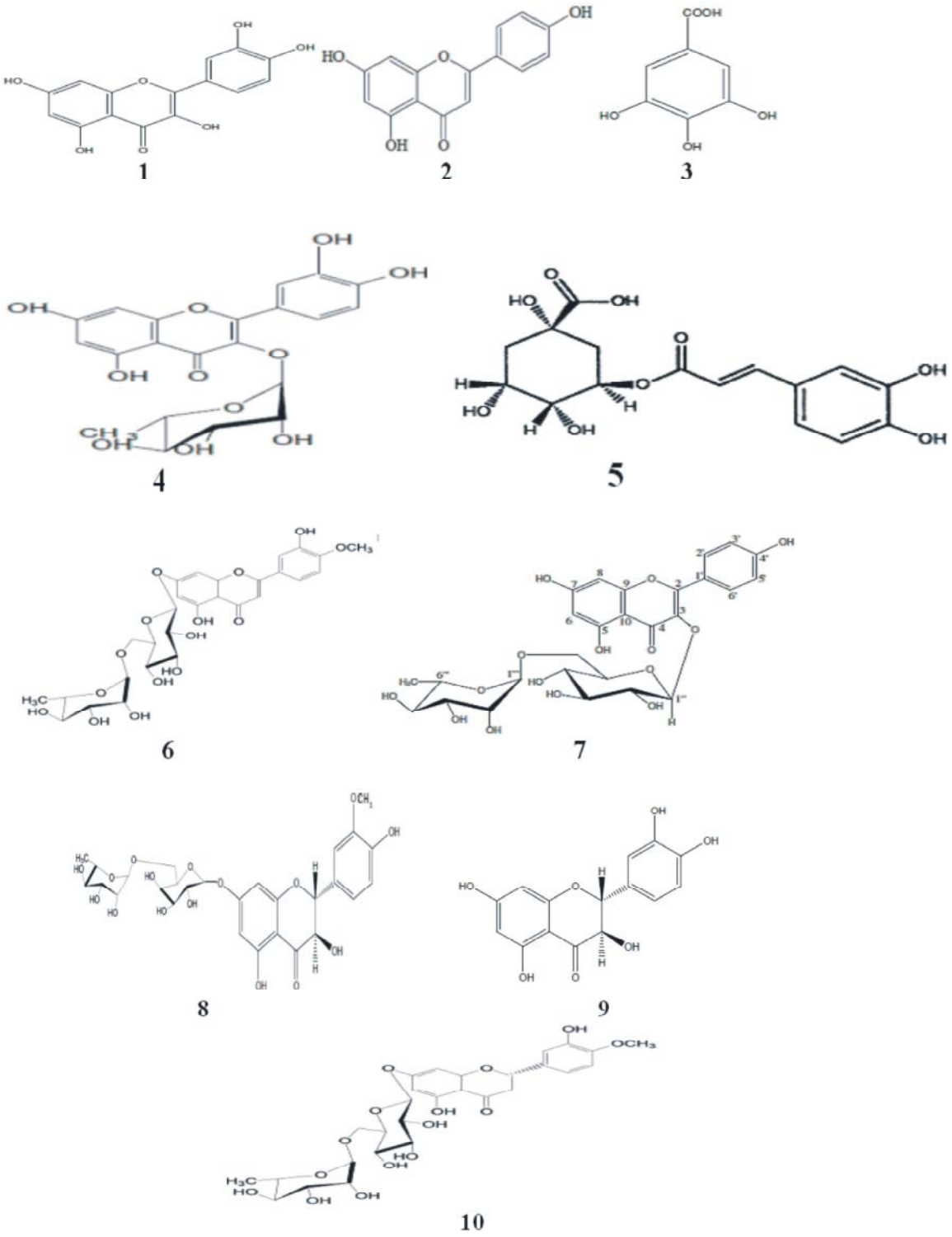

Fig. 1: Chemical skeletons of phenolic compounds isolated from *T. grandis* leaves

respectively besides an AM spin coupling system of two meta doublet protons assigned for H-8 and H-6 at 6.72 and 6.42 ppm in 5,7 di-hydroxy A-ring, respectively. The downfield location of H-5' (+0.2) indicated substitution at 4'-OH. As well as the downfield shift of both H-8 and H-6 along with doublet of H-1" at 5.04

confirmed the glycosidation at C-7. Presence of a terminal rhamnosyl moiety on C-6" was downfield from the characteristic position of H-1'" at 4.50 and downfield shift of CH_2-6" protons at 3.41 and 3.31 ppm. A single at 3.83 ppm was characteristic for -OCH_3 group on 4'-OH [32,33]. ^{13}C-NMR (125 MHz, DMSO-*d*6) (Table 1), showed characteristic signal at 56.31 ppm for -OCH_3 group on 4'-OH, which reflected on a downfield shift of C-4' and C-1'. Characteristic position of C-7 resonance 163.46 ppm confirmed the location of sugar moiety at this carbon. The downfield shift of C-6" (68.86 ppm) explained the attachment of rhamnosyl moiety at C-6" of glucose. δ and *J*-values of ^{1}H-NMR and ^{13}C-NMR spectra for the two sugar moieties referred to the β-$^4C_1$ and α-$^1C_4$ pyranose of the glucosyl and rhamnosyl moieties, respectively [32, 33]. Therefore, compound 6 was identified as luteolin-4'-methyl ether 7-*O*-α-L-$^1C_4$-rhamnopyranosyl (1‴→6″)-β-D-$^4C_1$-glucopyranoside = diosmetin-7-*O*-β-D-$^4C_1$-rutinoside = diosmin.

Compound 7: Dark yellow powder, m.p. 180-182°C, R_f: PC 0.49 (S1) and 0.57 (S2). UV spectral data, λ_{max} (nm) (Table 2). R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 7 was identical with kaempferol, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standards, D-glucose and L- rhamnose. Therefore, compound 7 was identified as kaempferol – 3 – *O* – α – L - $^1C_4$ -rhamnopyranosyl-(1‴→6″)- *O* – β – D - $^4C_1$ -glucopyranoside [22, 24, 26, 34].

Compound 8: Pale yellow powder, m.p. 220-222°C, R_f: PC 0.56 (S1) and 0.50 (S2). It gave yellow fluorescence under UV-light, turned to bright yellow with ammonia vapors, yellow fluorescence with Naturstoff (NA) and no change with $AlCl_3$ spraying reagents, also it gave positive Molisch's test indicating its glycosidic nature. UV spectral data, λ_{max} (nm) (Table 2). R_f value of the aglycone (Co-PC) after complete acid hydrolysis of compound 8 was identical with taxifolin, while R_f values of sugar moiety (Co-TLC) after hydrolysis were identical with the standards, D-galactose and L- rhamnose. Based on its chromatographic properties (R_f-values, fluorescence under UV-light, color reaction with different spray reagents and acid hydrolysis products) compound 8 was expected to have taxifolin-7-*O*-glycosides skeleton [22]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), showed typical dihydroflavonol skeleton. The ^{1}H-NMR also showed a pair protons of A-ring at δ 6.08 ppm (2H, H-6 and H-8), three protons of 1,3,4-trisubstituted B-ring at δ 6.89 ppm (3H, m, H-2',5',6'), one methoxy signal at δ 3.38 ppm (3H, s, 3'-OCH_3), one methyl signal at δ 1.03 ppm (3H, brs, H-6') and two anomeric protons of galactose and rhamnose at δ 5.12 (1H, brd, H-1") and 4.47 (1H, s, H-1'"), respectively [24, 26]. ^{13}C-NMR (125 MHz, DMSO-*d6*) (Table 1), showed the typical 15 carbon signals for taxifolin aglycone moiety. The down field shift of both H-8 and H-6 along with doublet of H-1" at 5.12 ppm confirmed the glycosidation at C-7. The connection of the methoxy group of C-3' at 56.20 ppm deduced from the downfield shift of C-2' and C-3' at 109.61 and 146.96 ppm, respectively. The connection (1'" → 6") of sugar moiety was confirmed via the chemical shift of the CH_2-6" (68.84 ppm). δ and *J*-values for ^{1}H and ^{13}C-NMR of two sugar moieties referred to the β-$^4C_1$ and α-$^1C_4$ pyranoside of the glucosyl and rhamnosyl moieties, respectively [24]. All other carbon resonances have been assigned through a comparative study with the previously published data [26]. Therefore, compound 8 was finally identified as 3'-methoxy-taxifolin-7-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'" → 6")-*O*-β-D-$^4C_1$ galactopyranoside.

Compound 9: Yellow powder, m.p. 235-238°C, R_f: PC 0.75 (S1) and 0.05 (S2). UV spectral data, λ_{max} (nm) (Table 2). IR ν_{max} (KBr) cm^{-1} spectrum showed the absorption bands at: 3500 (Ar-OH), 3285 (carboxylic-OH), 3064 (Ar-CH-), 1725 (-C=O) and 1620 (Ar-C=C-). Via comparison it's chromatographic properties with authentic taxifolin sample (Co-PC, Co-TLC and Co-m.p.), compound 9 was identified as 3,5,7,3',4'- pentahydroxy-flavanone (dihydroquercetine or taxifolin) [22, 25].

Compound 10: Pale yellow powder, m.p. 252-254°C, R_f: PC 0.54 (S1) and 0.80 (S2); TLC, 0.62 (S1). It gave yellowish green fluorescence under UV-light, yellow with ammonia vapors and turned to pale yellow with $AlCl_3$ spraying reagents. IR ν_{max} (KBr) cm^{-1}: spectrum showed the absorption bands at: 3425; 1295; 1657.32; 1614.13, 1500, 1450 and 2925.48. IR spectrum showed absorption bands indicated to the presence of; (–OH aromatic alcohol stretching, -C–O- stretching, >C=O stretching and Ar–C=C–,-O-CH_3) [35]. ^{1}H-NMR (500 MHz, DMSO-*d*6) δ in ppm (Table 1), showed three typical flavanone skeleton signals at δ 5.45 (1H, brd, H-2), a pair protons of A-ring at δ 6.10 (2H, H-6, 8). Three protons of 1,3,4-trisubstituted B-ring at δ 6.89 (3H, m, H-2',5',6'), one methoxy signal at δ 3.76 (3H, s, 4'-OCH_3), one methyl signal at δ 1.04 (3H, brd, H-6'"), two anomeric protons of glucose and rhamnose at δ 4.95 (1H, brd, H-1") and 4.67 (1H, s, H-1'"), respectively [35, 36]. This was confirmed via interpretation of ^{13}C-NMR spectrum of compound 10 (Table 1), which

Table 3: Free radical scavenging potential (DPPH) and total antioxidant capacity of the isolated pure compounds from *T. grandis*

Compound	[a]DPPH SC_{50} [µg/ml]	[b]Total antioxidant capacity (mg AAE /g ext.)
1	3.85 ± 1.20	710.23 ± 2.1
2	12.0± 1.55	485.35 ± 1.65
3	4.30 ± 1.75	680.10 ± 1.78
4	6.50 ± 2.35	540.53 ± 1.29
5	5.20 ± 3.25	550.25 ± 1.82
6	13.65± 1.35	500.35 ± 1.90
7	12.45 ± 1.85	582.56 ± 2.30
8	14.0 ± 1.50	386.45 ± 1.45
9	10.30 ± 2.85	418.23 ± 3.15
10	14.75 ± 3.0	463.46 ± 1.49
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)

showed the typical 15 carbon signals for aglycone moiety. For hesperidin moiety among them C-4' and C-3' at 148.48 and 146.96 ppm which can be considered the key signal of 3',4'-disubestitued B-ring. The down field shift of both H-8 and H-6 along with doublet of H-1" at 4.95 ppm confirmed the glycosidation at C-7. The connection of the methoxy group at 56.20 ppm deduced from the downfield shift of C-4' and C-1'. The connection (1‴ and 6″β) of sugar moiety was confirmed via the chemical shift of the CH_2-6" (68.48 ppm). δ and *J*-values for ^{1}H and ^{13}C-NMR of two sugar moieties referred to the β-$^{4}C_1$ and α-$^{1}C_4$ pyranoside of the glucosyl and rhamnosyl moieties, respectively. All other carbon resonances have been assigned through a comparative study with the previously published data [35,36]. Thus, this compound was identified as hesperidin (3', 5, 7 - trihydroxy-4'-methoxyflavanone-7-*O*-rhamnoglycoside).

Antioxidant Activity of the Isolated Compounds: 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 3), these results indicated that compounds 1, 3 and 5 exhibited marked scavenging activity compared to the standard ascorbic acid (SC_{50}= 8.0 µg/ml). The DPPH free radical antioxidant activity ranged from (3.85 µg/ml) to (14.75 µg/ml), while the total antioxidant capacity ranged from (710.23 mg AAE/g compound) to (386.45 mg AAE/g compound). The results indicated that the free-radical scavenging activity of these compounds is due to its hydrogen-donating ability, provided by the ease of stabilization of the phenoxyl radical after reduction of DPPH radical as well as their abilities to reduce Mo (VI) to Mo (V), also the activity is enhanced by the presence of catechol groups and the α, β-unsaturated carbonyl moiety, as evidenced by their low IC_{50} values [37]. It is also evident that *ortho*-dihydroxy moiety plays a vital role in enhancement of the antioxidant activity as observed in compounds 1, 4 and 9 than the α, β-unsaturated carbonyl as observed for compounds 2 and 7 due to the missing catechol group. It was reported that the antioxidant activity of flavonoids and phenolic acids depends upon the arrangement of functional groups about the nuclear structure [38, 39]. Previous studies reported that the primary mechanism of the radical scavenging activity of flavonoids is hydrogen atom donation, also may act by single-electron transfer. Furthermore, structural requirements for the hydrogen-donating antioxidant activity basically depend upon the presence of *ortho*-dihydroxy substitution in the B-ring, C_2-C_3 double bond and C_4 carbonyl group (keto group) in the C-ring. In the hydrogen atom transfer mechanism, hydroxy groups donate hydrogen to a radical, stabilizing it and giving rise to a relatively stable flavonoid phenoxyl radical (Ph-O$^{\bullet}$). The flavonoid phenoxyl radical may react with a second radical, acquiring a stable quinone structure [39,40]. Furthermore, in the current study the radical scavenging activity of the two isolated phenolic acids (3 and 5), was depends on the number and position of hydroxyl (-OH) groups in the molecules as well as the presence or absence of CH=CH-COOH group. The phenolic acids like gallic acid (e.g. 3) with most hydroxyl groups had the strongest radical scavenging activity because of pyrogallol structural advantage and potent H-donating ability. For hydroxycinnamic acids (e.g. chlorogenic acid 5), we observed that dihydroxyl cinnamates showed promising activity in scavenging radicals, due to the influence of hydroxyl position and -CH=CH-COOH group in hydroxycinnamic acids [39-42].

Table 4: Potential cytotoxicity (IC_{50}) of the some isolated compounds

	SF (HEPG2)					
Conc. µg/ml	1	3	5	6	9	10
0.000	1.000	1.000	1.000	1.000	1.000	1.000
5.000	0.511	0.309	0.307	0.677	0.504	0.720
12.500	0.227	0.195	0.148	0.350	0.310	0.575
25.000	0.161	0.181	0.168	0.243	0.238	0.248
50.000	0.193	0.191	0.373	0.318	0.241	0.159
IC_{50} µg/ml	5.48	3.53	3.53	8.93	5.18	15.40

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

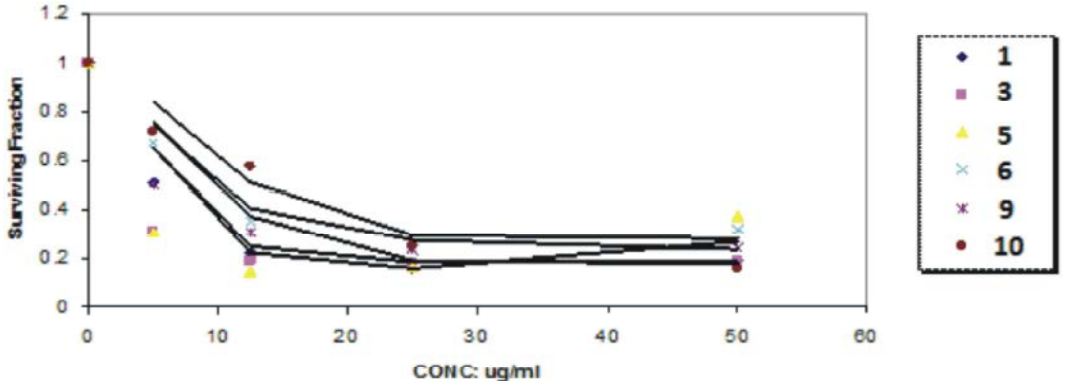

Fig. 2: Potential cytotoxicity of the some isolated compounds

Cytotoxic Activity of the Isolated Compounds: According to previous studies, the phenolic compounds have vital role on cancer chemoprevention and chemotherapy. Additionally, many mechanisms and modes of action have been identified, involving carcinogen inactivation, antiproliferation, cell cycle arrest, induction of apoptosis and differentiation, inhibition of angiogenesis and antioxidation or a combination of these mechanisms [43]. Also, it has been revealed that the antioxidant compounds (especially naturally occurring type), have a vital role in cancer prevention and treatment [44]. For the screening of novel naturally occurring anticancer agents with potent cytotoxic activity and high selectivity (unaffected the normal cells), the current study was directed to the exploration of pure isolated compounds from *T. grandis* plant. In our previous study, Sulphorhodamine-B assay was used to evaluate the cytotoxicity of the n-BuOH and EtOAc fractions against liver carcinoma cell line (HepG2), which revealed that both fractions have high cytotoxic activity than the remaining tested fractions with IC_{50} 11.6 and 22.1 µg/ml respectively, with respect to Doxorubicin IC_{50}4 µg/ml, this prompts us to follow up the fractionation procedure in parallel with the bio-assay activity of the isolated compounds; this resulted in the isolation of three tested compounds for first time were isolated from the plant identified as; 5, 6 and 10 with IC_{50} 3.53, 8.93 and 15.40 µg/ml respectively and untested one identified as 8 together with three tested known compounds identified as; 1, 3 and 9 with IC_{50} 5.48, 3.53 and 5.18 µg/ml respectively (Table 4 and Figure 2). From these results, we can conclude that the kind of flavonoid aglycon and the number of sugar units play important role in the cytotoxic activity. The structure-activity relationship (SAR) of phenolic compounds as anticancer agents was related to the variation in hydroxylation and oxidation pattern of the flavonoid and phenolic acids nucleus. It was reported that the existence of sugar moiety reduce the cytotoxic activity of the aglycone on tumor cell lines and this may be due to the fact that the hydrophilic nature of sugars or the increased bulky size of glycosides could interfere with the drug entering through the cellular membrane [45]. Also, it was reported that the presence of the hydroxyl group at C-3 as well as the C-4 oxo groups is required for maximal biological activity of flavonoids [45]. In some cases, the cytotoxic activity of the crud extract or fraction was more potent than some of its isolated compound, which may be return to the synergistic Co-activity (synergetic activity), in which the plant crude extracts are multi-composed complexes that can be subdivided into principal active constituents as well as concomitant compounds; these concomitant compounds are termed as co-effectors due to their abilities to change the physicochemical features of the basic active compounds, accordingly lead to dramatic changes in the biopharmaceutical parameters.

Furthermore, concomitant molecules able to change or modify the physical and chemical stability of the tested samples [46]. The weak cytotoxic activity of some isolated phenolic compounds may be attributed to the presence of sugar units as well as the polyhydroxylation pattern upon the flavonoid nucleus which reduce the hydrophobicity of such compounds, therefore making difficult flavonoids entry into the cell [46, 47]. The increasing hydrophobicity of the tested flavonoids molecules is vital to obtain strong interaction between the flavonoid and a determined target implicated in cancer. The presence of 2,3 double bond and the 4-oxo group is required for maximal biological activity of flavonoids. Both of the aromatic substituents and the keto-enol functionality can acts as targets for future structure activity relationship (SAR) studies of flavonoid compounds [46, 48].

Abbreviations:

µM	:	Micro Molar.	IC_{50}	:	Median Inhibitory concentration.
^{13}C-NMR	:	Carbon Nuclear Magnetic Resonance.	IP	:	Inhibition Percentage.
^{1}H-NMR	:	Proton Nuclear Magnetic Resonance.	IR	:	Infra Red.
AAE	:	Ascorbic Acid Equivalent.	*J*	:	Coupling Constant.
AOA	:	Antioxidant Activity.	L	:	Levavo.
BAW	:	n-Butanol: Acetic acid: Water.	ELISA	:	Enzyme-Linked Immuno-Sorbent Assay.
EDTA	:	Ethylene Diamine Tetra Acetic Acid.	PC	:	Paper Chromatography.
FTIR	:	Fourier Transform Infra Red.	ppm	:	Part Per Million.
Galac	:	Galactose.	R_f	:	Retention Factor.
Glc	:	Glucose.	Rha	:	Rhamnose.
HEPG-2	:	Liver Cancer Cell Line.	RNS	:	Reactive Nitrogen Species.
m	:	Meta.	ROS	:	Reactive Oxygen Species.
M	:	Molar.	s	:	Singlet.
mg	:	Milligram.	SAR	:	Structure Activity Relationships.
MHz	:	Mega Hertz.	SC_{50}	:	Median Scavenging Concentration.
mM	:	Milli Molar.	SF	:	Survival Fraction.
NA	:	Naturstoff.	SRB	:	Sulphorodamine B.
nm	:	Nanometer.	TAC	:	Total Antioxidant Capacity.
NRC	:	National Research Center.	δ	:	Chemical Shift.
o	:	Ortho	λ_{max}	:	Wave Length Maximum.
p	:	Para.	ν_{max}	:	Frequency Maximum.
sh	:	Shoulder.	Co-m.p.	:	Co-melting point.
TLC	:	Thin Layer Chromatography.	Co-PC	:	Co-Paper Chromatography.
TMS	:	Tetramethyl Silane.	Co-TLC	:	Co- Thin Layer Chromatography.
UV	:	Ultraviolet.	D	:	Dextro.
brd	:	Broad Doublet.	d	:	Doublet.
brs	:	Broad Singlet.	dd	:	Doublet of Doublet.
cm	:	Centimeter.	DMSO- d_6	:	Dimethyl Sulfoxide-deutrated$_6$.
cm^{-1}	:	Centimeter^{-1}.	DPPH	:	1,1'- Diphenyl-2-PicrylHydrazyl.
brd	:	Broad Doublet.			

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