ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
Essa et al., 2018, 2 (1), 44-55

Journal of Advanced Pharmacy Research

Flavonolignans and Biological Activity of *Nannorrhops ritchiana* Leaves

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Submitted on: 04-10-2017; Revised on: 26-10-2017; Accepted on: 31-10-2017

ABSTRACT

Objectives: This study aimed at isolating the polyphenolic compounds of 70% methanol extract of *Nannorrhops ritchiana* Griff. leaves and assaying the antioxidant and cytotoxic activities of the extract and main fractions. **Methods:** The methanol extract of the leaves of *N. ritchiana* was chromatographically fractionated using a bioactivity-guided approach. The isolated compounds were spectroscopically elucidated by UV, MS, $^{1}H/^{13}C$ NMR and 2D NMR spectroscopic techniques. The radical scavenging activity of the methanol extract and its fractions was evaluated using DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging assay and their cytotoxic activity was assayed using SRB (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) test against human hepatocellular liver carcinoma (HepG2), human alveolar adenocarcinoma (A549) and human prostate carcinoma (PC3) cell lines. **Results:** Eight compounds were isolated and identified for the first time from the leaves of *N. ritchiana*, comprising five flavonolignans; Indocalatin A **(4)** which is a rare compound that has been reported only once in nature, 9″-*O*-glucopyranosyl Salcolin A **(5),** 9″-*O*-glucopyranosyl Salcolin B **(6)** together with their aglycones, Salcolin A **(7)** and Salcolin B **(8)** along with three flavone glycosides; Tricin-7-*O*-rutinoside **(1),** Orientin **(2)** and Isoorientin **(3).** The methanol extract of *N. ritchiana* showed antioxidant activity with SC_{50} 39.4 ± 1.06 µg/ml, while fraction **II** showed significant effect with SC_{50} of 6.1 ± 0.24 µg/ml in comparison with ascorbic acid (SC_{50} 1.8 ± 0.35 µg/ml). The methanol extract and the fraction **III** showed a promising cytotoxic activity against selected cell lines especially the A549 with IC_{50} 9.5 ± 1.98 µg/ml and HepG2 with IC_{50} 8.15 ± 1.3 µg/ml, respectively. **Conclusion:** *N. ritchiana* leaf methanol extract is a new source of biologically active compounds, including flavonolignans.

Keywords: *Antioxidant; Cytotoxicity; DPPH*; *Flavonolignan; HPLC; Nannorrhops ritchian Griff.; SRB*

INTRODUCTION

The Arecaceae (Palmae) is a botanical family of perennial climbers, shrubs, a caules and trees commonly known as palm trees. The family with great importance in landscaping and gardening contains 181 genera with around 2600 species which are found throughout equatorial, tropical, and subtropical areas of the world. The abundant presence of C-glycosylflavones, leucoanthocyanins, sulphated flavonoids and tricin derivatives provides interesting chemosystematic marker of the family[1]. The genus *Nannorrhops* is one of palm trees belonging to this family where *Nannorrhops ritchiana* Griff. is the sole species of this genus. The species commonly known as Mazari palm is a shrub-like clumping palm, with blue-green to grey-green fan-like leaves and several stems slowly growing and connected to form a single base. It is native to Southwestern Asia, from Southeast of the Arabian Peninsula to east through Iran and Afghanistan to Pakistan[2]. The young leaves of the plant with sweet astringent taste have been used as purgative in livestock[3]. The fruit is edible and used by local communities for the treatment of alimentary tract complaints[4] and other infectious disorders, in Baluchistan, Pakistan[5]. The petroleum ether, butanol, ethyl acetate and methanol extracts of both roots and leaves of *N. ritchiana* showed good antifungal and weak antibacterial activities against several strains[6-8].

Moreover, the phytochemical screening of leaves alcoholic extract of the plant revealing the presence of flavonoids, tannins, alkaloids, cardiac glycosides, saponnins and terpenoids[8], has encouraged the authors to phytochemically investigate the biologically active fractions.

MATERIALS AND METHODS

Apparatus

UV–visible spectrophotometer (Shimadzu UV 240, Koyoto, Japan) was used for recording UV spectra and measuring the absorbance in UV and visible range. The mass spectra were recorded on GC-MS (GCMS-QP 1000EX, Shimadzu, Koyoto, Japan), ESI-MS Negative ion acquisition mode (XEVO TQD triple quadruple instrument Waters Corporation, Milford, MA 01757, U.S.A) and Atmospheric Pressure Chemical Ionization (APCI-MS); JMS-700 mass spectrometer, JEOL, Japan).

NMR spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on NMR spectrometer (JNM-LA400, JEOL, Japan) and at 500 MHz for ^{1}H and 125 MHz for ^{13}C (Bruker 500 MHz Avance DRX, USA). The δ values were reported as ppm relative to TMS in DMSO-*d6* and CD_3OD and *J* values in Hz. Determination and quantification of phenolic compounds was performed using HPLC apparatus (Agilent 1200 Series, Agilent Technologies, CA, USA) equipped with autosampling injector, solvent degasser, quaternary HP pump (series 1200) and ultraviolet (UV) detector set at 280 nm for phenolic acids and 330 nm for flavonoids. ELISA micro plate reader spectrophotometer (Tecan Group Limited.-Sunrise, Crailsheim**,** Germany) was used for measuring color intensity (optical density) of cells in cytotoxicity assay.

Plant material

Leaves of *Nannorrhops ritchiana* Griff. were collected from the garden of Manial Palace- El-Manial district- Cairo, Egypt in July 2014 and were identified by both Consultants of Plant Taxonomy; Dr. Mohamed El-Gebaly at the Ministry of Agriculture and Mrs. Therese Labib, at Orman Botanical Garden, Giza, Egypt. Voucher specimens (# M 126) were kept at the herbarium of the National Research Centre, Giza, Egypt.

Chemicals

All solvents of HPLC and analytical grade were purchased from Fisher Scientific, Dorset, UK. The deuterated solvents used for NMR were purchased from Sigma-Aldrich Co., Saint Louis, Missouri, USA. Chemicals used for evaluating the antioxidant activity, DPPH (2,2-Diphenyl-1-picrylhydrazyl) and ascorbic acid were purchased from Sigma-Aldrich Co., Saint Louis, Missouri, USA. Chemicals used in evaluating the cytotoxicity of the extract and derived fractions, SBR stain (Sulforhodamine-B; (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma-Aldrich Co., Saint Louis, Missouri, USA. Adriamycin® (Doxorubicin 10 mg vials) were obtained from Pharmacia, Uppsala, Sweden. Authentic reference flavonoid and phenolic compounds were supplied by Agriculture Research Centre, Food Technology Research Institute, Giza, Egypt.

Cell line and culture medium

Human hepatocellular liver carcinoma (HepG2), human alveolar adenocarcinoma (A549) and human prostate carcinoma (PC3) cell lines were obtained in frozen state under liquid nitrogen (-180ºC) from the American Type Culture Collection (ATCC, VA, USA). The cells were suspended in Roswell Park Memorial Institute medium (RPMI 1640) (Sigma-Aldrich Co., St Louis, Missouri, USA) supplemented with 10% fetal calf serum (SIGMA, USA) in presence of 1% antibiotic antimycotic mixture (10.000 U/ml K-penicillin, 10.000 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B) and 1% L-glutamine (all purchased from Lonza, Bornem, Belgium) in a humidified, 5% (v/v) CO_2 atmosphere at 37°C.

HPLC analysis of flavonoid and phenolic compounds

Flavonoid and phenolic compounds of the samples were detected and determined according to the method described by Goupy *et al*[9] and Mattila *et al*[10] using HPLC instrument composed of column C_{18} hypersil BDS with particle size 5 µm., the solvent system used was a gradient of A (CH_3COOH 2.5%), B (CH_3COOH 8%) and C (acetonitrile). The best separation was obtained with the following gradient: at 0 min, 5% B; at 20 min, 10% B; at 50 min, 30% B; at 55 min, 50% B; at 60 min, 100% B; at 100 min, 50% B and 50%C; at 110 min, 100% C until 120 min., flow with 1 mL/min where the retention times were compared with those of standards injected in the same run. Quantification was carried out using external standard calibration and expressed in mg/g dry matter of equivalent flavonoid and phenolic compounds.

Extraction and isolation of phenolics from *N. ritchiana* leaves

The air dried ground leaves (1kg) were exhaustively extracted with 70% methanol by soaking at room temperature for 24 h, thereafter the solvent was evaporated under reduced pressure affording 250 g of methanol concentrate. Sugar content was removed from the methanol extract by dissolving the residual concentrate in least amount of distilled water followed by addition of excess absolute ethanol. Fifty grams of dry concentrate were loaded on 750 g polyamide 6 column (5 cm W. x 120 cm L.) which was then eluted using water then H_2O/MeOH mixtures with 20% stepwise decreasing

polarity, that yielded 34 fractions of 500 ml each, and similar PC fractions were combined together affording into five major collective fractions; **I** (7 g) from 100% H_2O (1-5), **II** (3.6 g) from 20% MeOH/H_2O (6-12), **III** (1.2 g) from 40% MeOH/H_2O (13-20), **IV** (1.8 g) from 60-80% MeOH/H_2O (21-29) and **V** (4 g) from pure MeOH (30-34). The interesting biological (antioxidant and cytotoxic) activities of fractions **II**, **III** and **IV** were encouraging for further processing of these fractions mainly by successive column chromatography on Sephadex and preparative paper chromatography using different solvent systems S_1 (n-Butanol: Acetic acid: Water 4: 1: 5) and S_2 (acetic acid: water 15:85). Fraction **II** was further applied to subcolumn Sephadex (LH-20) using H_2O, then MeOH/H_2O mixtures with decreasing polarity to give two major subfractions **IIa** (eluted with 20% MeOH) and **IIb** (eluted with 40-60% MeOH). Subfraction **IIa** was chromatographed by preparative paper chromatography (PPC) using S_2 solvent system and finally was purified on column Sephadex using MeOH to give compound **1**. Fractionation of subfraction **IIb** on column Sephadex using 20% MeOH/H_2O as an eluting solvent with stepwise decrease in polarity followed by further purification on column Sephadex using MeOH afforded two pure compounds **2** and **3**. Fraction **III** was subjected to Sephadex column chromatography using 30% MeOH/H_2O with increasing amount of MeOH to give major subfraction which was applied to PPC using S_2 solvent system to give two bands where the first band was further purified on Sephadex column using methanol resulting pure compound **4.** The second band in the same manner afforded isomeric mixture of compounds **5** and **6**. Fraction **IV** was applied to column Sephadex using 40% MeOH/H_2O with increasing the ratio of MeOH to give major subfraction which was applied to PPC using S_2 and then purified on column Sephadex using MeOH to give epimers of compounds **7** and **8**.

Antioxidant assay

DPPH free radical scavenging activity was evaluated by measuring the scavenging activity of the extract on stable 2.2-diphenyl-1-picryl hydrazylradical (DPPH)[11]. A solution of DPPH (0.25 mM) in 70% methanol was prepared. Stock solutions of extract (1.0 mg/ml) and fractions **II, III** and **IV** (0.5 mg/ml) in 70% methanol were prepared. Different concentrations of extract (10–100 µg/ml) and fractions (5-25 µg/ml) solutions were added to 0.335 ml (0.25 mM DPPH) and final volume was made to 1 ml with 70% methanol. The mixture was shaken vigorously and kept standing at room temperature for 10 min. Thereafter the absorbance of the mixture was measured at 517 nm on UV-spectrophotometer. The decrease in the absorbance indicates an increase in DPPH-radical scavenging activity. The percentage inhibition was calculated by the following equation: DPPH radical scavenging (%) = [($A_{blank} - A_{sample}$)/ A_{blank}] ×100 where A_{blank} is the absorbance of control and A_{sample} is absorbance of sample. The experiment was performed in triplicate and Vitamin C (ascorbic acid; 0.5-2.5 µg/ml) was used as a standard drug. The mean values were calculated and SC_{50} value was calculated as the concentration of sample required to scavenge 50% of DPPH free radicals.

Cytotoxicity Assay

The cytotoxicity against Hep-G2, A549 and PC3 cells were tested according to the SRB (Sulforhodamine B) assay method[12] where doxorubicin was used as the reference drug. Briefly, cells grown in T-75 flasks of stock solution were used when 70% confluence was reached in T-75 flasks. The attached cell line was collected with 0.025% trypsin then plated in 96-multiwell plates at densities of 10^4cells/well in a fresh media and incubated under normal growth condition for approximately 24 h before treatment with the tested sample to allow adherence of cells to the wall of the plate. The *N. ritchiana* leaves methanol extract and fractions **II, III** and **IV** were diluted serially with DMSO-*d6* (100%). Then, 200 µl of each aliquot were added in several concentrations (0, 1, 2.5, 5 and 10 µg/ml) and the plates were incubated for 48 h at 37°C in a humidified incubator containing 5% CO_2 in air. Control cells were treated with vehicle alone. Each individual concentration was added to three wells. Following 48 h treatment, cells were fixed, washed and stained with Sulforhodamine B stain. Wells were repeatedly washed with 1% (v/v) acetic acid to remove excess dye and treated with Tris EDTA buffer to recover attached stain. The optical density (O.D.) of each well was measured in an ELISA reader spectrophotometer. The amount of dye extracted from the stained cells is directly proportional to the protein content of cells and the survival cell mass. Negative control was treated with the vehicle (0.1% DMSO-*d6*) used for diluting the tested samples. Doxorubicin (1.0 µg/ml) was used as the positive control.

Statistical analysis

All the aforementioned experiments were conducted in triplicates. Data were expressed as mean ± standard deviation (SD) and at $P<0.05$. Data were analyzed by using one-way ANOVA followed by Duncan's multiple range tests using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

HPLC analysis of flavonoid and phenolic compounds

The experiment revealed the identification of twenty two phenolic compounds in which case, the most abundant one was ellagic acid (7670.04 ppm), while twenty one flavonoid glycosides and aglycones were

Table 1. HPLC analysis of phenolic and flavonoid compounds of methanol *N. ritchiana* leaf extract

Identified phenolic compound	RRT[a] (min)	Amount (ppm)	Identified flavonoid compound	RRT[b] (min)	Amount (ppm)
Pyrogallol	0.52	1499.07	Luteolin 6-arabinose 8-glucose	1.00	458.40
Gallic acid	0.53	70.62	Luteolin 6-glucose 8-arabinose	1.14	166.75
ρ- Amino-benzoic acid	0.62	78.85	Apigenin 6-arabinose 8-galactose	1.23	224.60
Protocatchuic acid	0.63	376.78	Apigenin 6-rhamnose 8-glucose	1.27	28.34
Catechin	0.64	218.67	Apigenin 6-glucose 8-rhamnose	1.28	92.27
Catechol	0.68	1011.02	Luteolin 7-O-glucoside	1.30	50.91
Epi catechin	0.72	203.24	Naringin	1.32	86.29
ρ-hydroxybenzoic acid	0.73	1214.76	Hisperidin	1.32	165.31
Caffeine	0.74	343.39	Rutin	1.33	191.75
Chlorogenic acid	0.75	1628.43	Kaempferol 3,7-dirhamnoside	1.41	65.02
Vanillic acid	0.76	714.63	Quercetrin	1.42	57.55
Caffeic acid	0.77	452.67	Rosmarinic	1.52	10.47
ρ- coumaric acid	0.86	368.10	Quercetin	1.57	37.71
Ferulic acid	0.88	755.93	Naringenin	1.59	16.76
Iso-ferulic acid	0.91	139.38	Kaempferol 3-*O*-(2-p-coumaroyl)glucoside	1.61	592.75
α -coumaric	0.98	83.41	Hispertin	1.62	81.27
Benzoic acid	0.99	2554.87	Kaempferol	1.71	20.48
Ellagic acid	1.00	7670.04	Rhamnetin	1.74	10.28
3,4,5-methoxy cinnamic acid	1.05	894.56	Apigenin	1.76	27.95
Coumarin	1.08	212.02	Apigenin 7-O-glucoside	1.82	8.61
Cinnamic acid	1.14	67.76	Acacetin	2.00	72.61
Salycilic acid	1.22	555.42			

RRT[a]: Relative retention time to ellagic cid (Rt = 13.4 min).
RRT[b]: Relative retention time to Luteolin 6-arabinose 8-glucose (Rt = 9.45 min).

identified, within which Kaempferol 3-*O*-(2-*ρ*-coumaroyl) glucoside (592.75 ppm) (**Table 1**) was the major compound.

Characterization and identification of isolated compounds

Promising antioxidant and cytotoxic activities of the aqueous methanol extract of *N. ritchiana* leaves was the basis for further bioassay-guided fractionations using different chromatographic techniques that resulted in identification of eight compounds which elucidated using various spectroscopic methods including UV, MS, ^{1}H NMR, ^{13}C NMR along with 2D NMR and confirmed by comparison of the data with those reported in the literature.

Compound 4 was obtained as yellow amorphous powder (5 mg). **UV**- spectral data λ_{max} (nm) (MeOH): 272, 365. **^{1}H NMR** (500 MHz, DMSO-*d6*) δ: 7.4 (1H, s, H-6′), 6.91 (1H, d, *J* = 1.5 Hz, H-8), 6.67 (1H, d, *J* = 1.5, H-15), 6.52 (1H, d, *J* = 8, H-18), 6.42 (1H, d, *J* = 1.5 Hz, H-6), 6.16 (1H, dd, *J* = 8, 1.5 Hz, H-19), 5.03 (1H, d, *J* = 7 Hz, H-1″), 4.75 (1H, s, H-13), 3.93 (3H, s, MeOH at C-5′), 3.75-3.24 (overlapped sugar protons), 3.65 (3H, s, MeOH at C-16), 3.49 (3H, s, MeOH at C-3′). Due to the overlapping of signals, H-12 and H-11 needed a more detailed analysis which, was achieved by proton spectra recorded in CD_3OD δ: 3.85 (1H, br. d, *J* = 10 Hz, Ha-11), 3.61 (1H, br. d, *J* = 10 Hz, Hb-11), 3.43 (1H, br. d, *J* = 9 Hz, H12). **^{13}C NMR** (125 MHz, CD_3OD) δ: 180.7 (C-4), 163.1 (C-7), 161.4 (C-5), 160 (C-2), 156.8 (C-9), 144.0 (C-4′), 147.5 (C-3′), 147.5 (C-16), 146.3 (C-17), 148.4 (C-5′), 135.1 (C-14), 119.0 (C-2′),119.4 (C-19), 127.5 (C-1′), 114.5 (C-18), 111 (C-3 and C-15), 106.6 (C-10), 102.8 (C-6′), 100.3 (C-1″), 99.6 (C-6), 94.6 (C-8), 77.1 (C-5″), 76.5 (C-3″), 74.3 (C-2″), 70 (C-4″), 61.4 (C-11), 61.2 (C-6″), 59.5 (3′-OCH_3), 55.6 (5′-OCH_3), 54.9 (16 -OCH_3), 41 (C-12), 36 (C-13). Atmospheric pressure chemical ionization **(APCI)**: *m/z*

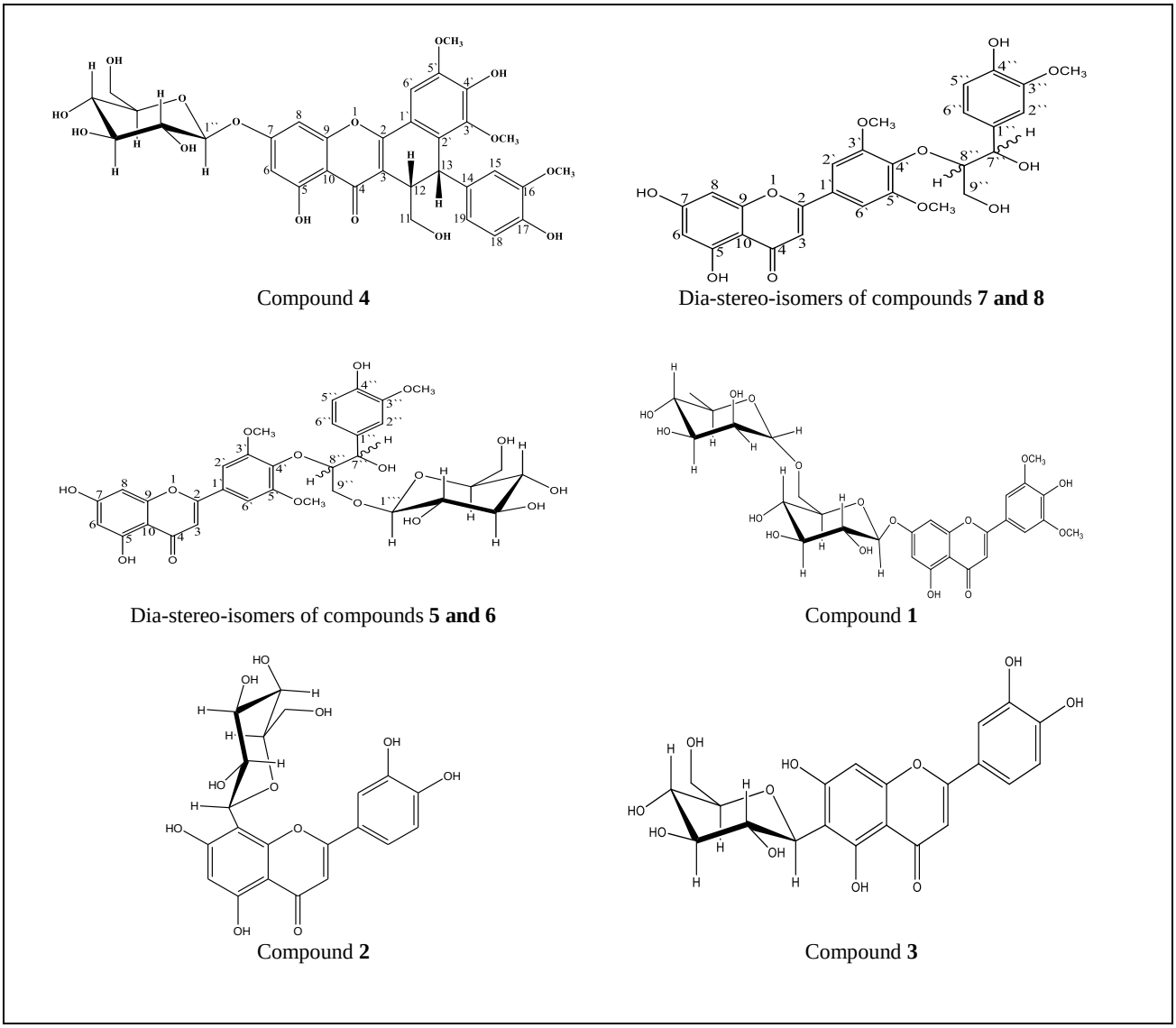

Figure 1. Structures of isolated compounds from *N. ritchiana leaves*

671.2 $[M + H]^+$ and 509 $[A + H]^+$. In ^1H NMR spectrum, the meta doublet signals at δ 6.42 (1H, d, *J* = 1.5 Hz, H-6) and 6.91 (1H, d, *J* = 1.5 Hz, H-8) showed 5, 7-dihydroxysubstituted A ring, the ABX aromatic ring represented by upfield shifted signals at δ 6.16 (1H, dd, *J* = 8.5-1.5 Hz, H-19), 6.52 (1H, d, *J* = 8 Hz, H-18) and 6.67 (1H, d, *J* = 1.5Hz, H-15) revealed its attachment to aliphatic system especially if considering the singlet signal at δ 4.75 (1H, s, H-13). Also, the singlet signal with intensity for one proton at δ 7.4 (1H, s, H-6′) with signals of methoxyl protons at δ 3.93 (3H, s, 5′-OCH_3), 3.49 (3H, s, 3′-OCH_3) and 3.65 (3H, s, 16-OCH_3) suggesting a flavonolignan-type flavonoid[13]. Comprehensive study of ^{13}C NMR showed the presence of aliphatic group at 36 ppm which correlated through HSQC with proton signal at δ 4.75 (1H, s, H-13). Using HMBC, H-13 showed eight long-range C-H correlations (via 3 bonds with C-3, C-15, C-19, C-1′ and C-3′ and via 2 bonds with C-12, C-2′ and C-14), while H-15 correlated with C-13, C-17 and C-19, which proven presence of a hexatomic ring of a C-2, 3, 1′, 2′, 13 and 12[14]. As previously mentioned, the singlet signal of H-13 can be attributed to axial – equatorial orientation between H-12 and H-13. However, presence of anomeric proton at δ 5.03 (1H, d, *J* = 7 Hz, H-1″) and other overlapped sugar protons at δ (3.24 – 3.75) with noticeable downfield shift of H-6 and H-8 supposed the compound to be 7-*O*-glucosylated which was evidenced by ^{13}C NMR analysis. First-order positive ion APCI-MS allowed determination of molecular masses of compounds from the $[M+H]^+$ pseudo-molecular ions. Also, the identification of the aglycone moiety could be

Table 2. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) Data of Compounds 7, 8 and 5, 6.

Carbon No	Dia-stereo-isomers of compounds 7, 8		Dia-stereo-isomers of compounds 5, 6	
	δ H	δ C	δ H	δ C
2		166.0		163.2
3	6.67 s (t), 6.66 s (e)	103.6	6.70 s	105.6
4		182.0		181.9
5		163.8		160.4
6	6.16 d (1.5)	99.2	6.16 br. s	100.6
7		167.0		163.8
8	6.43 d (1.5)	94.0	6.49 br. s	95.1
9		158.2		158.0
10		104.6		103.5
1′		127.0		126.0
2′, 6′	7.21 s (t), 7.18 s (e)	104.5	7.29 s (t), 7.27 s (e)	104.6
3′, 5′		153.3		153.3
4′		140.0		139.8
1″		132.2		133.3
2″	6.99 d (1.8)(t) 6.96 d (1.8)(e)	110.3	6.99 br. s. (t) 6.98 br. s. (e)	111.6
3″		148.0		147.4
4″		147.0		145.9
5″	6.71 d (7.8)(t) 6.68 d (7.8)(e)	114.4	6.72 d (8.0)(t) 6.70 d (8.0)(e)	115.0
6″	6.86 dd (7.8, 1.8)(t) 6.79 dd (7.8,1.8)(e)	119.4	6.87 br. d (8.0)(t) 6.80 br. d (8.0)(e)	119.6
7″	4.99 d (6.4)	73.0	5.07 d (6.8.0) (t) 4.92 d (4.5) (e)	73.9
8″	4.27 m (t) 4.45 m (e)	87.5	4.49 m (t) 4.58 m (e)	84.8
9″ a	3.81 dd (11.9,3.6)(t) 3.89 under methoxy signal (e)	60.6(t)	3.74 dd (12.0,3.6) (t) 3.99 under methoxy signal (e)	68.4
9″ b	3.40 dd (11.9, 4)(t) 3.66 dd (11.9, 4)(e)	60.3(e)	3.36 dd (10.0,4.0) (t) 3.60 dd (12.3, 4.6)(e)	
3′,5′-OMe	3.92 s (t), 3.89 s (e)	55.97	3.91 s (t), 3.87 s (e)	56.8
3″-OMe	3.81 s (t), 3.80 s (e)	54.98	3.81 s (t), 3.78 s (e)	56.0
1‴			4.26 d (8)	104.5
2‴			Overlapped within (3.74-3.11)	74.1
3‴				77.8
4‴				71.5
5‴				77.2
6‴				61.4

δ is chemical shift in ppm, J is coupling constant in Hz, (t) threo isomer, (e) erythro isomer.

supported from the first order positive ion APCI-MS due to the presence of a prominent ion (referred to as the $[A+H]^+$ ion) produced by the loss of the sugar moieties from the pseudo-molecular ion. APCI-MS spectrum exhibited the molecular ion peak at *m/z* 671.2 $[M + H]^+$, which corresponds to the molecular weight of 670 and a molecular formula of $C_{33}H_{34}O_{15}$, it also showed prominent ion at *m/z* 509 $[A + H]^+$ referred to aglycone with molecular mass 508 confirming the structure elucidation of compound **4** as **Indocalatin A [(-)-(5S, 6R)-5, 6-dihydro-3, 8, 10-trihydroxy-5-(4-hydroxy-3-methoxyphenyl)-6-hydoxymethyl-2,4-di-methoxy 7*H* benzo[*c*]xanthen-10-*O*-glucopyranosyl-7-one],** which is a rare flavonolignan that has only been reported only

once from nature, isolated from, the leaves of *Indocalamus latifolius*[15].

Compounds 7 and 8 were dia-stereo-isomers isolated as yellow amorphous powder (25 mg). UV-spectral data λ_{max} (nm) (MeOH): 272, 286, 305sh, 335. ^{1}H NMR spectra (CD_3OD, **Table 2**) showed two meta-coupled signals at δ 6.16 (1H, br s, H-6) and 6.43 (1H, br s, H-8), a sharp singlet at δ 6.67 (1H, s, H-3) along with a sharp singlet with integration of two protons at δ 7.21 (2H, s, H-2′ and H-6′) and singlet signal at δ 3.92 (6H, s, 3′ and 5′-OCH_3) which suggested tricin nucleus. Also, ABX- spin coupling system of three protons with upfield shift at δ 6.99 (1H, d, *J* = 1.8 Hz, H-2″), 6.86 (1H, dd, *J* = 7.8-1.8 Hz, H-6″) and 6.71 (1H, d, *J* = 7.8 Hz, H-5″) and aliphatic region with two signals at δ 4.99 (1H, d, *J* = 6.4, H-7″) and 4.27 (1H, m, H-8″), along with two geminal protons signals at δ 3.81 (1H, dd, *J* = 11.9-3.6, H-9″a) and 3.4 (1H, dd, *J* = 11.9-3.6, H-9″e) and singlet at δ 3.81 (3H, s, 3″-OCH_3) proposed a tricin-based flavonolignan[16,17]. Careful analysis of PENDANT ^{13}C NMR (CD_3OD, **Table 2**) and HMQC showed presence of 27 carbons, including eight aromatic and two oxygenated aliphatic methines, one ketonic group, twelve aromatic quaternary carbons, three methoxyls and one oxygenated methylene, while HMBC studying revealed that aromatic proton H-2″ at δ 6.99 correlated via 3-bonds with C-7″ at δ 73 and proton of this carbon correlated via 2-bonds with C-8″ at δ 83 which coincided with previously published data in which case, the compound is proposed to be **tricin 4′-*O*-(guaiacylglyceryl) ether**. Further confirmation by EI-MS fragmentation pattern revealed fragments at 508 (M-$H_2O)^+$, 490 (M- $2H_2O)^+$ and 330 (a tricin moiety). Moreover, the duplicated signals with small integration in NMR spectra with slight up or downfield shift supported the previous literature of the compound being present in mixture of erythro and threo forms which was attributed to presence of two vicinal protons (H-7″ and H-8″). So, compounds **7** and **8** were **salcolin A, (tricin 4′-*O*-(erythro--guaiacylglyceryl) ether), and** its epimer **sacolin B, (tricin 4′-*O*-(threo--guaiacylglyceryl) ether),** respectivley which are reported here for the second time in family Arecaeae after their first isolation from *Calamus quiquesetinervius*[18,19].

Table 3. Antioxidant activity of methanol extract compared to ascorbic acid as standard (expressed as percentage inhibition ± SD)

Methanol extract		Ascorbic acid (vitamin C)	
Conc. µg/ml	**% Inhibition ± SD**	**Conc. µg/ml**	**% Inhibition ± SD**
10	**14.73 ± 2.75**	**0.5**	**21.93 ± 0.37**
50	**78.04 ± 3.36**	**1.0**	**28.42 ± 1.79**
100	**92.27 ± 0.35**	**1.5**	**44.76 ± 3.08**
		2.5	**63.80 ± 2.57**

Table 4. Antioxidant activity of isolated fractions (expressed as percentage inhibition ± SD)

Tested sample Conc.	% Inhibition ± SD		
	5 µg/ml	**10 µg/ml**	**25 µg/ml**
Fraction **II**	**35.10 ± 2.83**	**75.88 ± 0.89**	**92.64 ± 0.19**
Fraction **III**	**17.20 ± 3.28**	**51.39 ± 2.35**	**93.36 ± 0.5**
Fraction **IV**	**26.03 ± 1.63**	**52.24 ± 1.33**	**93.08 ± 0.12**

Table 5. Antioxidant activity of methanol extract and isolated fractions (expressed as SC_{50} ± SD)

Tested sample	SC_{50} ± SD
Ascorbic acid (vitamin C)	**1.80 ± 0.35 µg/ml**
Methanol extract	**39.40 ± 1.06 µg/ml**
Fraction **II**	**6.10 ± 0.24 µg/ml**
Fraction **III**	**9.73 ± 0.67 µg/ml**
Fraction **IV**	**9.77 ± 1.12 µg/ml**

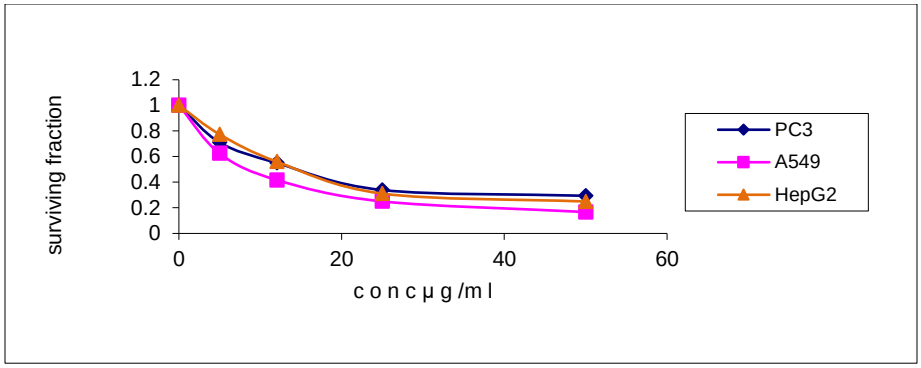

Figure 2. Cytotoxic activity of methanol extract against human cancer cell lines

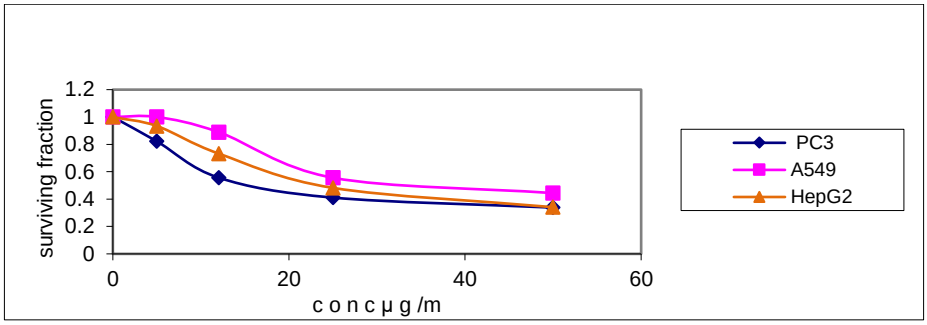

Figure 3. Cytotoxic activity of fraction II against human cancer cell lines

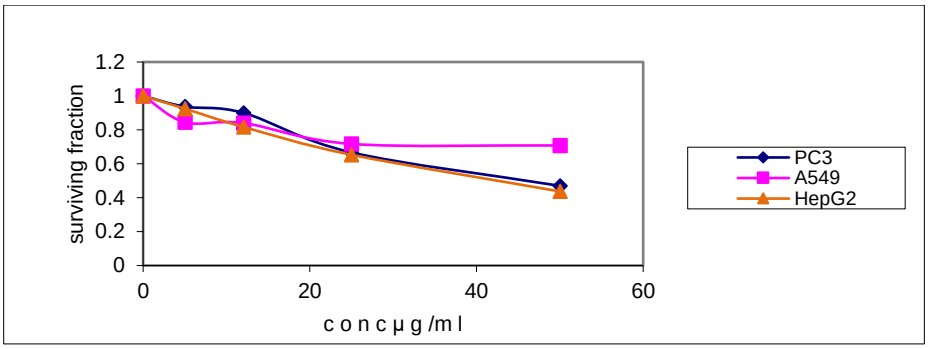

Figure 4. Cytotoxic activity of fraction III against human cancer cell lines

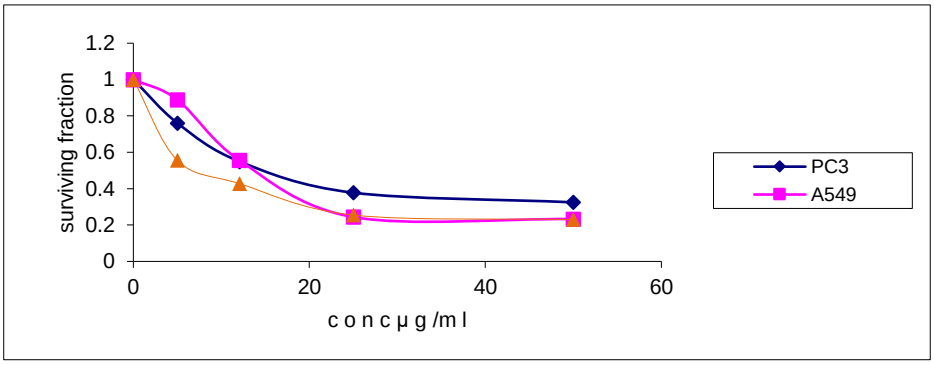

Figure 5. Cytotoxic activity of fraction IV against human cancer cell lines

Table 6. Cytotoxic activity of the methanol extract and fractions (II - IV) of *N. ritchiana* leaves compared to Doxorubicin against Hep-G2, PC3 and A549 cancer cell lines (expressed as IC_{50} mean values)

Tested Samples	IC 50 (µg/mL)				
	Extract	Fraction II	Fraction III	Fraction IV	Doxorubicin
A549	9.5 ± 1.98	37 ± 1.59	14.7 ± 2.60	105 ± 3.12	3.9 ± 0.41
HepG2	15.6 ± 1.30	24.3 ± 1.42	8.15 ± 1.30	42.8 ± 0.63	4.85 ± 0.52
PC3	15 ± 1.77	16.7 ± 1.18	15.6 ± 0.56	45.7 ± 4.10	2.93 ± 0.34

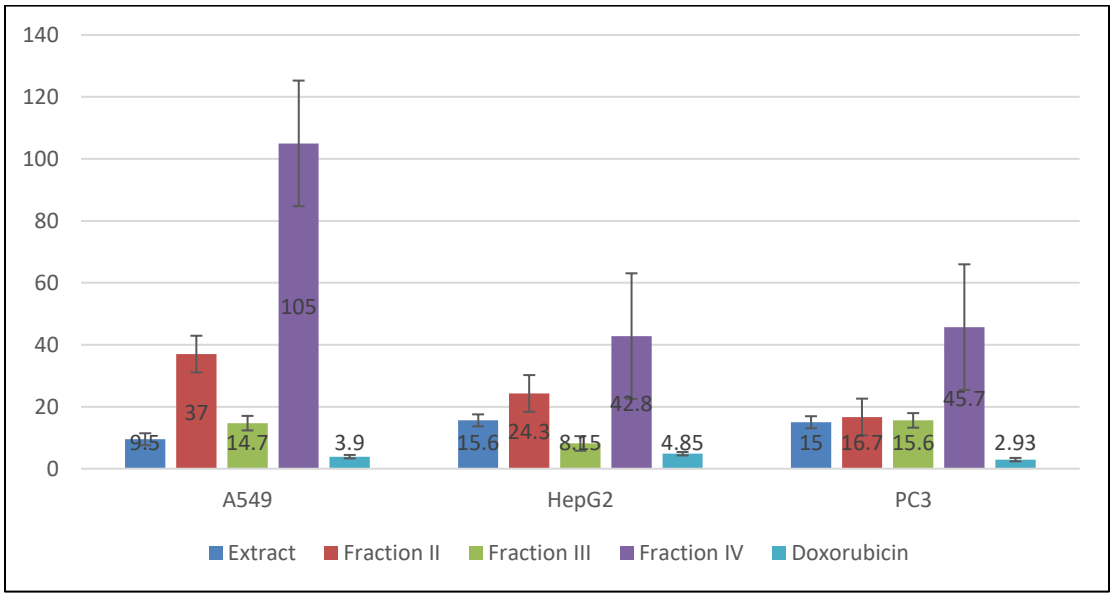

Figure 6. Cytotoxic activity of the methanol extract and fractions (II - IV) of *N. ritchiana* leaves compared to Doxorubicin against Hep-G2, PC3 and A549 cancer cell lines (expressed as IC_{50} mean values)

Compounds 5 and 6 were dia-stereo-isomers isolated as yellow amorphous powder (7 mg). UV-spectral data λ_{max} (nm) (MeOH): 270, 287, 335. Studying ^{1}H NMR and APT ^{13}C NMR (DMSO-*d6*, **Table 2**) along with HMBC spectrum showed that the chemical shift values and coupling constants were almost similar to those of **7 and 8** (**Table 2**). However, presence of sugar moiety assigned by presence of anomeric proton at δ 4.26 (1H, d, *J* = 8, H-1‴) and the rest sugar protons overlapped within δ (3.74-3.11), with downfield shift in C-9″ to 68.4 suggested *O*-glucosylation at this carbon. The molecular weight was deduced to be 688.2 from ion peak recorded at *m/z* 687.19 $[M-H]^-$ by ESI-MS in negative mode which agreed with those previously reported. Thus compounds **5 and 6** were confirmed to be a mixture of **9″-*O*-glucopyranosyl salcolin A and 9″-*O*-glucopyranosyl salcolin B** which are reported for the first time from *N. ritchian*, and as a second time from nature after their first separation from *Oryza sativa*[20].

Compound 1 was isolated as yellow powder (42 mg); UV- spectral data λ_{max} (nm) (MeOH): 249, 269, 350; (+NaOMe): 259, 301, 414; (+NaOAc): 259, 266sh, 364, 412; (+NaOAc/H_3BO_3 acid): 256, 352; (+$AlCl_3$): 275, 299sh, 329, 400; (+$AlCl_3$/HCl): 275, 295sh, 357, 389; EI-MS (negative mode) m\z 637 $[M-H]^-$, 329 [aglycon-H]$^-$; ^{1}H-NMR (400 MHz, DMSO-*d6*) δ: 7.36 (2H, s, H-2′, H-6′), 7.06 (1H, s, H-3), 6.87 (1H, d, *J* = 2 Hz, H-8),6.49 (1H, d, *J* = 2 Hz, H-6), 5.08 (1H, d, *J* = 8 Hz, H-1″), 4.55 (1H, br. s, H-1‴), 3.9 (6H, s, -OCH3 at C-3′ and C-5′), 3.12-3.86 (further glycosidic protons), 1.07 (3H, d, *J* = 6.06 Hz, H-6‴). APT ^{13}C NMR (100 MHz, DMSO-*d6*) δ: 182.4 (C-4), 164.6 (C-2), 163.3 (C-7), 161.7 (C-5), 157.3 (C-9), 148.7 (C-3′, C-5′), 140.8 (C-4′), 121 (C-1′), 105.8 (C-10), 105 (C-2′, C-6′), 104.1 (C-

3), 101 (C-1″), 100.3 (C-1‴), 99.8 (C-6), 95.7 (C-8), 76.8 (C-3″), 76.1 (C-5″), 73.6 (C-2″), 72.5 (C-4‴), 71.2 (C-2‴), 70.8 (C-3‴), 70.1 (C-4″), 68.8 (C-5‴), 66.5 (C-6″), 56.9 (3′, 5′-OCH3), 18.3 (C-6‴). Comparing obtained data with structurally related compounds[21,22], compound **1** was proved to be **Tricin-7-*O*-rutinoside**

Compound 2 was isolated as yellow amorphous powder (28 mg). UV- spectral data λmax (nm) (MeOH): 262, 350; (+NaOMe): 278, 377; (+NaOAc): 278, 330sh, 385; (+NaOAc/ H_3BO_3 acid): 267, 377, 425sh; (+$AlCl_3$): 278, 301sh, 353sh, 409; (+$AlCl_3$/HCl): 278, 301sh, 350sh, 387; ^{1}H NMR (400 MHz, CD_3OD) δ: 13.05 (1H, s, 5-OH), 7.49 (1H, br d, *J* = 7.8 Hz, H-6′), 7.37 (1H, br s, H-2′), 6.88 (1H, d, *J* = 8.2 Hz, H-5′), 6.5 (1H, s, H-3), 6.24 (1H, s, H-6), 4.96 (1H, d, *J* = 9.6 Hz, H-1″), 4.11 (1H, t, *J* = 9.6-9.3 H-2″), 3.96 (1H, br d, *J* = 11.6 Hz, Hb-6″),3.84 (1H, dd, *J* = 12.0, 5.5 Hz, Ha-6″), 3.69 (1H, t, *J* = 9.6-9.3, H-3″),3.51 (2H, m, H-4″, H-5″). ^{13}C NMR (100 MHz, CD_3OD) δ: 182.7 (C-4), 165.2 (C-2), 165.3 (C-7), 161.3 (C-5), 157.3 (C-9), 149.5 (C-4′), 145.6 (C-3′), 122.6 (C-1′),119.5 (C-6′), 115.3 (C-5′), 113.6 (C-2′), 98 (C-6),104.4 (C-10), 102.3 (C-3), 103.7 (C-8), 81.6 (C-5″), 78.9 (C-3″), 75 (C-1″), 70.9 (C-2″), 70.8 (C-4″), 61.8 (C-6″).

Compound 3 was isolated as yellow amorphous powder (15 mg). UV- spectral data λ_{max} (nm) (MeOH): 269, 295sh, 348; (+NaOMe): 278, 327, 403; (+NaOAc): 278, 320, 385; (+NaOAc/H_3BO_3 acid): 269, 375, 430; (+$AlCl_3$): 278, 325sh, 415; (+$AlCl_3$/HCl): 278, 298sh, 350sh, 385; ^{1}H NMR (400 MHz, CD_3OD) δ: 13.05 (1H, s, 5-OH), 7.34 (2H, m, H-6′, H2′), 6.86 (1H, d, *J* = 7.8 Hz, H-5′), 6.53 (1H, s, H-3), 6.46 (1H, s, H-8), 4.6 (1H, d, *J* = 9.6 Hz, H-1″), 4.11 (1H, t, *J* = 9.6-9.3 H-2″), 3.96 (1H, br d, *J* = 11.6 Hz, Hb-6″), 3.84 (1H, dd, *J* = 12.0, 5.5 Hz, Ha-6″), 3.69 (1H, t, *J* = 9.6-9.3, H-3″),3.51 (2H, m, H-4″, H-5″). ^{13}C NMR (100 MHz, CD_3OD) δ: 180.4 (C-4), 164.7(C-2), 165.7(C-7), 161.7 (C-5), 157.3 (C-9), 149 (C-4′), 147 (C-3′), 121 (C-1′),118.9 (C-6′), 115.4 (C-5′), 112.7 (C-2′), 110 (C-6), 105.8 (C-10), 102.4 (C-3), 93.9 (C-8), 81.2 (C-5″), 78.7 (C-3″), 73 (C-1″), 71.2 (C-2″), 71.2 (C-4″), 61.5 (C-6″). Analysis of the above data of both compounds **2** and **3** and comparing it with similar compounds[23,24], revealed the elucidated compounds to be **Orientin** and **Isoorientin,** respectively.

Antioxidant activity

The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized and this can be quantitatively measured from the changes in absorbance. The methanol extract and its fractions tested for scavenging activity relative to ascorbic acid showed promising activity (**Tables 3-5**), especially for isolated fractions. The methanol extract showed good antioxidant activity with SC_{50} 39.4 ± 1.06 µg/ml and more free radical scavenging activity appeared interestingly with fractions **III** and **IV** that has scavenged 50% of DPPH radicals with values of 9.73 ± 0.67 and 9.77 ± 1.12 µg/ml, respectively. Fraction **II** showed the most significant antioxidant activity with SC_{50} 6.1 ± 0.24 µg/ml in comparison with ascorbic acid that exhibited SC_{50} 1.8 ± 0.35 µg/ml. This significant activity of fraction **II** can be attributed to high concentration of flavonoids especially tricin 7-*O*-rutinoside which was the major isolated compound in this fraction. Similarly alike, it is worth mentioning that the flavonolignan present in fractions **III** and **IV** can explain the noticeable activity of both fractions.

Cytotoxic activity

Surviving fraction calculated from optical density values was plotted against drug concentration to get the survival curve for each tumor cell line after treatment with tested samples (Figures 2-6), from which IC_{50} could be calculated and expressed as IC_{50} ± SD where samples showed mortality more than 50% are considered to be cytotoxic. The methanol extract and fraction **III** showed promising cytotoxic activity against selected cell lines especially the A549 and HepG2, respectively. The methanol extract exhibited significant cytotoxic activity against A549 with IC_{50} values of 9.5 ± 1.98 µg/ml and good activity against PC3 and HepG2 with IC_{50} values of 15 ± 1.77 and 15.6 ± 1.3 µg/ml, respectively. Also, fraction **II** showed good activity against PC3 with IC_{50} value of 16.7 ± 1.18 µg/ml despite showing moderate activity against HepG2 and A549 cell lines with IC_{50} values of 24.3 ± 1.42 and 37 ± 1.59 µg/ml, respectively. Fraction **III** significantly reduced the growth of HepG2 cell line in a concentration dependent manner with IC_{50} value of 8.15 ± 1.3 µg/ml, while, it showed moderate cytotoxic activity against A549 and PC3 with IC_{50} values of 14.7 ± 2.6 and 15.6 ± 0.56 µg/ml, respectively. However, fraction **IV** showed mild cytotoxic activity against HepG2 and PC3 cancer cells with IC_{50} values of 42.8 ± 0.63 and 45.7 ± 4.1 µg/ml, respectively while its effect on A549 cell line was lesser compared with doxorubicin (Table 6).

CONCLUSION

According to the obtained results, *N. ritchiana* leaf methanol extract fractions **II**, **III** and **IV** are rich in phenolic compounds that show significant antioxidant activity and promising cytotoxic activity especially the methanol extract against human alveolar adenocarcinoma (A549) and fraction III against human hepatocellular liver carcinoma (HepG2) cell line. Thus *Nannorrhops ritchiana* Jriff. leaves could be considered as a new natural source of flavonolignan with valuable biological activity.

Conflict of Interest

The authors declare that they don't have any conflict of interest.

REFERENCES

1. Williams, C. A.; Harborne, J. B.; Clifford, H. T. Negatively charged flavones and tricin as chemosystematic markers in the Palmae. *Phytochemistry* **1973**, *12*, 2417-2430.
2. Mosti, S.; Raffaelli, M.; Tardelli, M. A contribution to the flora of Wadi Abdur (Dhofar, Southern Oman). *Webbia: J. Plant Taxon. Geog.* **2006,** *61*, 253-260.
3. Marwat, S. K.; Rehman, F. U.; Usman, K.; Khakwani, A. Z.; Ghulam, S.; Anwar, N.; Sadiq, M.; Khan, S. J. Medico-ethnobotanical studies of edible wild fruit plants species from the flora of north western Pakistan (D.I. Khan district). *J. Med. Plant. Res*. **2011,** *5* (16), 3679.
4. Sadaf, N.; Shahid, N.; Erum, B.; Khuala, S.; Shamim, S. A. Biogeochemical evaluation of *Nannorrhops ritchiana*: A Mg-flora from Khuzdar, Balochistan, Pakistan. *Chinese J. Geochem*. **2005**, *24*, 327.
5. Murad, W.; Ahmad, A.; Gilani, S. A.; Khan, M. A. Indigenous knowledge and folk use of medicinal plants by the tribal communities of Hazar Nao Forest, Malakand District, North Pakistan. *J. Med. Plants Res***. 2011,** 5 (7), 1072.
6. Rashid, R.; Mukhtar, F.; Khan, A. Antifungal and cytotoxic activities of *Nannorrhops ritchiana* roots extract. *Acta Poloniae Pharmaceutica in Drug Research* **2014**, *71* (5), 789-793.
7. Perveen, S.; Khan, A. M.; Anees, M.; Nurulain, S. M.; Yasmin, T. Antifungal and antibacterial potential of the leaves extracts of *Nannorrhops ritchiana* (Griff), *Phoenix sylvestris* (Linn.) and *Olea ferruginea* Royle. *Bull. Env. Pharmacol. Life Sci.* **2016**, *5* (12), 35-43.
8. Mahmood, A.; Sharif, M.; Ahmad, Q. U.; Mahmood, R.; Riaz, S.; Zafar, M. Phytochemical analysis and comprehensive evaluation of antimicrobial activity of *Nannorhops ritchiana* leaves (Mazari palm). *World J. Pharm. Pharmaceut. Sci.* **2017**, *6* (6), 173-189.
9. Goupy, P.; Hugues, M.; Boivin, P.; Amiot, M. J. Antioxidant and activity of barley (*Hordeum vulgare*) and malt extracts and of isolated phenolic compound. *J. Sci. Food Agric*. **1999,** *79*, 1625–1634.
10. Mattila, P.; Astola, J.; Kumpulainen, J. Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections. *J. Agric. Food Chem*. **2000**, *48*, 5834–5841.
11. Bozin, B.; Mimica-Dukic, M.; Simin, N.; Anackov, G. Characterization of the volatile composition of essential oils of some Lamiaceae species and the antimicrobial and antioxidant activities of the entire oils. *J. Agric. Food Chem.* **2006**, *54*, 1822–1828.
12. Skehan, P.; Storeng, R. D.; Scudiero, A.; Monks, M. C.; Mahon, J. D.; Vistica, J. T.; Warren, H.; Bokesch, S. K.; Boyd, M. R. New colorimetric cytotoxicity assay for anticancer drug screening. *J. Natl. Cancer Inst.* **1990**, *82*, 1107-1112.
13. Wenzig, E.; Kunert, O.; Ferreira, D.; Schmid, M.; Schuhly, W.; Bauer, R.; Hiermann, A. Flavonolignans from *Avena sativa*. *J. Nat. Prod.* **2005**, *68*, 289-292.
14. Liu, Q.; Wu, C. H.; Peng, A.; Gao, K.; Chen, J.; Li, Y.; Fu, H. Flavonolignans from *Elymus natans* L. and Phytotoxic Activities. *J. Agric. Food Chem.* **2017**, *65*, 1320−1327.
15. Sun, J.; Yu, J.; Xun, H.; Yue, Y. D.; Feng, T.; Guo, X. F. Two new compounds from the leaves of Indocalamus latifolius. *J. Asian Nat. Prod. Res*. **2015**, *18(4)*, 360-365
16. Syrchina, A. I.; Gorshkov, A. G.; Shcherbakov, V. V.; Zinchenko, S. V.; Vereshchagin, A. L.; Zaikov, K. L.; Semenov, A. A. Flavonolignans of *Salsola collina*. *Khimiya Prirodnykh Soedinenii*. **1992**, *28*, 155-158. (English translation by Chemistry of Natural Compounds).
17. Bouaziz, M.; Veitch, N. C.; Grayer, R. J.; Simmonds, M. S.; Damak, M. Flavonolignans from *Hyparrhenia hirta*. *Phytochemistry* **2002**, *60*, 515–520.
18. Chang, C. L.; Wang, G. J.; Zhang, L. J.; Tsai, W. J.; Chen, R. Y.; Wu, Y. C.; Kuo, Y. H. Cardiovascular protective flavonolignans and flavonoids from *Calamus quiquesetinervius*. *Phytochemistry* **2010a**, *71* (2-3), 271-279.
19. Chang, C. L.; Zhang, L. J.; Chen, R. Y.; Wu, C. C.; Huang, H. C.; Roy, M. C.; Huang, J. P.; Wu, Y. C.; Kuo, Y. H. Quiquelignan A-H, eight new lignoids from the rattan palm *Calamus quiquesetinervius* and their antiradical, anti-inflammatory and antiplatelet aggregation activities. *Bioorg. Med. Chem.* **2010b**, *18*, 518–525.
20. Jeong, R. H.; Lee, D. Y.; Cho, J. G.; Seo, K. H.; Lee, J. W.; Lee, M. H.; Seo, W. D.; Kang, H. C.; Kim, G. S.; Noh, H. J.; Lee, Y. H.; Baek, N. I. New flavonolignan glucoside from the aerial parts of *Oryza sativa*. *Chem. Nat. Comp.* **2014**, *49* (6), 1003-1005.
21. Hirai, Y.; Sanada, S.; Ida, Y.; Shoji, J. Studies on the constituents of Palmae plants. III. The constituents of *Chamaerops humilis* L. and *Trachycarpus wagnerianus* Becc. *Chem. Pharm. Bull.* **1986**, *34*, 82-87.

22. Zhang, W. K.; Xu, J. K.; Zhang, L.; Du, G. H. Flavonoids from the bran of *Avena sativa. Chin. J. Nat. Med.* **2012**, *10*, 110-114.
23. Nassar, M. I.; Gaara, A. H.; Marzouk, M. S.; El-khrisy, E. A. M. A new gentisic acid glycoside and C-glycosyl flavones from *Erythrina indica* with the antioxidant activity evaluation. *Bulletin of Faculty of Pharmacy, Cairo University*, **2003**, *41* (1), 207-209.
24. El-Toumy, S. A.; Omara, E. A.; Nada, S. A.; Bermejo, J. Flavone C-glycosides from *Montanoa bipinnatifida* stems and evaluation of hepatoprotective activity of extract. *J. Med. Plants Res.* **2011**, *5* (8), 1291-1296.