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Original Article

Phenolic content of *Ruprechtia salicifolia* leaf and its immunomodulatory, anti-inflammatory, anticancer and antibacterial activity

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ABSTRACT

Objectives: This work aimed to isolate phenolics from leaves of *Ruprechtia salicifolia* and evaluate its immunomodulatory, anti-inflammatory, anticancer and antibacterial activity.
Methods: 80% MeOH leaf extract was subjected to chromatographic separation, structures of the isolated compounds were established by different chromatographic and spectral techniques UV, MS, ^{1}H and ^{13}C NMR. Immunomodulatory was evaluated through RAW 264.7 macrophages proliferation by MTT assay. Anti-inflammatory was evaluated by inhibition of NO and TNF-α factor generation in LPS-stimulated cells through bicinchoninici acid assay and ELISA assay, respectively. Cytotoxicity estimated through Hep-G2, MCF-7 and HCT-116 cell lines measured by MTT assay. Antibacterial activity tested by agar diffusion method.
Results: Eleven known phenolic compounds were isolated for the first time from this species including five flavonoid glycosides *viz*; Rutin **3**, quercetin 3-*O*-arabinoglucoside **4**, apigenin 7-*O*-β-D-glucoside **5**, quercetin 3-*O*-α-L-arabinofuranoside **6** and isoquercetin **7** along with four aglycones *viz*; kaemferide **8**, apigenin **9**, quercetin **10** and naringenin **11** and two phenolic acids; caffeic **1** and gallic **2**. Compounds **5** showed the most activity increasing macrophage proliferation implying immunomodulatory activity. 80% MeOH extract, **4**, **5** and **11** inhibited nitrite oxide by 68.19%, 52.95%, 20.33% and 15.22%, respectively and TNF-α generation by 70.82%, 29.88%, 13.13% and 6.14%, respectively in LPS-stimulated cells implying anti-inflammatory activity. 80% MeOH leaf extract and the tested compounds **4** and **5** were safe possessing no cytotoxic activity against hepatocellular carcinoma (Hep-G2), breast adenocarcinoma (MCF-7) and colon carcinoma (HCT-116), while Compound **11** had cytotoxicity against only HCT-116 cells (IC_{50} = 27.67 μg/ml). Also 80% MeOH leaf extract showed antibacterial activity against both G +ve and G −ve bacteria, moreover it inhibits growth of *Klebsiella pneumonia* strain, which is resistant to Ciprofloxacin broad-spectrum antibiotic.
Conclusions: *R. salicifolia* contain phenolics of immunomodulatory anti-inflammatory, cytotoxicity and antibacterial activity, giving *R. salicifolia* grate potential as a medicinal natural drug.

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1. Introduction

The family Polygonaceae, derived from the Greek word meaning knees referred to the swollen joints of some species. Family Polygonaceae comprises 800 species occurring in 30–40 genera, which are widely distributed in both cold and worm countries. Several Polygonaceae species are grown for ornamental purpose and a few for food production.[1] Genus *Ruprechtia* reported to have several biological activities as antioxidant, cytotoxic, antimicrobial and anti-inflammatory activities,[2–7] which are attributed to their terpenoid, tannin and flavonoid contents.[8] *Ruprechtia* includes 37 species among, which are three species cultivated in Egypt, the paucity of phytochemical and biological reports on the *R. salicifolia* C.A. Mey species encouraged the authors to undertake this study.

2. Materials and methods

2.1. Apparatus

NMR (^{1}H- and ^{13}C NMR) spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian Mercury 300. The δ-values are reported as ppm relative to TMS in DMSO-d_6 and J-values are in Hz. ESI–MS spectra were measured on mass spectrometer connected to an ESI-II ion source (Finnigan, LC–MS LCQ^{deca} Advantage MAX, Finnigan Surveyor LC pump) (Department of Biological Genetics, NRC, Cairo, Egypt). ELISA reader (BioRad, München, Germany) was used in measuring the absorbance of viable cells in the proliferation assay. Concentration of extracts was done at low temperature under vacuum using Rotatory evaporator (Bűchi G, Switzerland). Shimadzu UV 240 spectrophotometer was used for UV analysis.

2.2. Plant materials

Leaves of *Ruprechtia salicifolia* were collected from El-Orman Garden, Giza, Egypt in April 2010. Identification of the plant was confirmed by Dr. Tearse Labib, Department of Flora and Taxonomy, El-Orman Garden, Cairo, Egypt. Voucher specimen (Reg. no. R.s-7) was kept in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Helwan University, Cairo, Egypt.

2.3. Chemicals

Polyamide 6S (Riedel-De Hän Ag, Seelze Hannover, Germany), cellulose (Pharmacia, Uppsala, Sweden) and Sephadex (Fluka, Switzerland) were used in chromatography. Sugars, reagents and solvents of analytical grade were purchased from Sigma–Aldrich Co. (St Louise, Mo, USA). Chemicals used in biological activity; Griess reagent (0.2% naphthylenediamine dihydrochloride + 5% phosphoric acid, dissolved in 1 ml deionized water), used for evaluation of anti-inflammatory activity and MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide), used for cytotoxic activity, were both purchased from Sigma–Aldrich Co. (St. Louise, MO, USA). Tumor necrosis factor-α (TNF-α) commercial kit used in determination of anti-inflammatory activity was purchased from Endogen Inc. (Cambridge, MA, USA). Authentic reference of flavonoid compounds were obtained from Phytochemistry Laboratory, Department of Molecular and Cell Biology, University of Texas at Austin, (Austin, TX, USA).

2.4. Cell line and culture medium

Hepatocellular carcinoma (Hep-G2), breast adenocarcinoma (MCF-7), colon carcinoma (HCT-116), and Raw murine macrophage (RAW 264.7), were purchased from ATCC, (VA, USA). Hep-G2 and MCF-7 cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), while HCT-116 cells were grown in Mc Coy's medium at 37 °C in humidified air containing 5% CO_2 and RAW 264.7 cells were grown in phenol red-free RPMI-1640. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulfate and 250 ng/ml amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment, except for RAW 264.7 cells, which were collected by gentle scraping. The tested compounds were dissolved in dimethyl sulphoxide (DMSO, 99.9%, HPLC grade) and then diluted to 1000-fold during the assay. Compound dilutions were tested before assays for endotoxin-free using Pyrogent Ultra gel clot assay. Cell culture materials were obtained from Cambrex Bio-Science (Copenhagen, Denmark).

2.5. Microorganisms

Both Gram positive bacteria; *Staphylococcus aurous* and *Streptococcus pyogenes* and Gram negative bacteria; *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Proteus mirabilis* strains were all available in the Department of Microbiology, Research Institute of Ophthalmology, Cairo, Egypt.

2.6. Extraction and isolation of phenolics

Powdered air-dried leaves of *R. salicifolia* (1 kg) was extracted with hot 80% aqueous methanol under reflux (70 °C) (4 × 3 L), then the dried residue (150 g) was fractionated on polyamide 6S column (Ø 5.5 × 120 cm) and was eluted with water followed by H_2O/MeOH mixtures with decreasing polarity affording six collective fractions (I-VI). Separation processes were followed by 2D-PC and CoPC using Whatmann No. 1 paper with (S_1) *n*-BuOH–AcOH–H_2O (4:1:5, top layer) and (S_2) 15% aqueous AcOH as solvent systems,[9] visualized with UV lamp and sprayed with $FeCl_3$ and Naturstoff reagent (Diphenylboryl oxyethylamine in MeOH/5% polyethylene glycol 400 in EtOH).[10] Purification of compounds was done by successive column chromatography on cellulose and Sephadex using different solvent systems of H_2O/MeOH mixtures and (S_3) *n*-butanol–isopropanol–water (BIW) (4:1:5, v/v upper layer)[9] as shown in the flow chart (Fig. 1).

2.7. Acid hydrolysis for glycosides

Complete acid hydrolysis was carried out by treating 4–5 mg of each compound with 1.5 N HCl in aqueous methanol (50%)

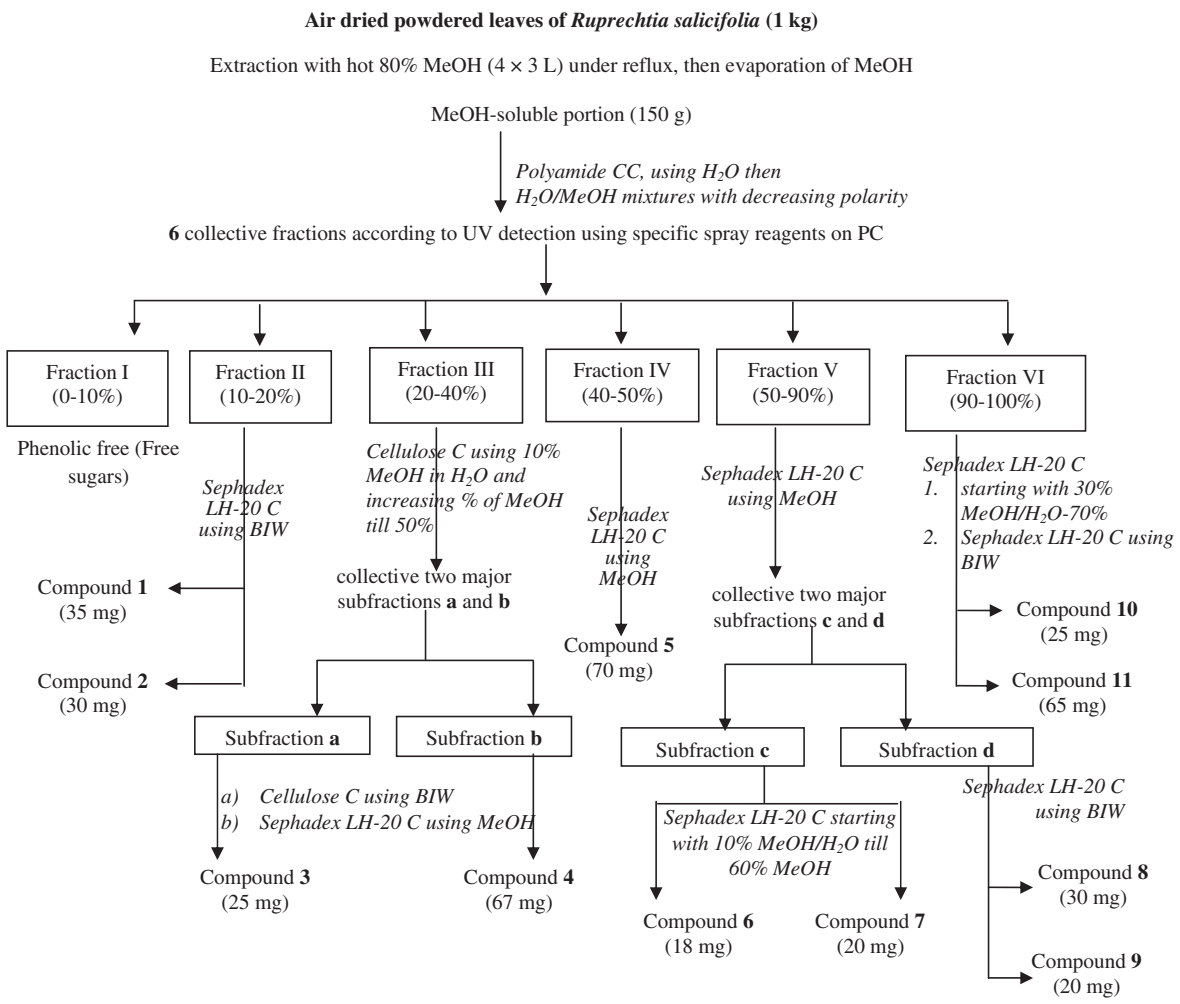


Fig. 1 – Flow chart of extraction, fractionation and purification of phenolic compounds from the leaves of *Ruprechtia salicifolia*.

for 2 h at 100 °C. Each hydrolyzate was then extracted with ethyl acetate and the extract was subjected to CoPC investigation alongside with authentic aglycones. The mother liquor was neutralized with sodium carbonate and used for the identification of the sugars by CoPC against standard sugars.[9]

2.8. *Proliferation of immune cells*

The effect of the synthesized compounds on the growth of Raw macrophage 264.7 was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.[11] The yellow tetrazolium salt of MTT is reduced by mitochondrial dehydrogenases in metabolically active cells to form insoluble purple formazan crystals, which are solubilized by the addition of a detergent. Cells (5×10^4 cells/well) were incubated with various concentrations of the compounds at 37 °C in an FBS-free medium, before submitted to MTT assay. The absorbance was measured with an ELISA reader at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data were expressed as the mean percentage of viable cells as compared to DMSO-treated cells. Treatment of macrophage with 1000 U/ml recombinant macrophage colony-stimulating factor (M-CSF, Pierce, USA) was used as positive control.

2.9. *Nitrite oxide assay*

The accumulation of nitrite, an indicator of NO synthesis, was measured by Griess reagent[12] using a microplate assay, based on two-step diazotization reaction, in which acidified nitrite generates a nitrosating agent that reacts with sulfanilic acid to form diazonium ion, which coupled with the reagent N-(1-naphthyl) ethylenediamine to produce the chromophoric pink azo-derivative that was determined spectrophotometrically at 540 nm. RAW 264.7 cells were incubated for 24 h with LPS (1 μg/ml) in presence or absence of different tested compounds (10 μg/ml). Fifty microliter of cell culture supernatant were mixed with 50 μl of freshly prepared Griess reagent and incubated for 10 min. The absorbance was measured spectrophotometrically at 540 nm. A standard curve was plotted using serial concentrations of sodium nitrite. The nitrite content was normalized to the cellular protein content as measured by bicinchoninic acid assay.[13,14] The NO inhibition

percentage was calculated by submitting the nitrite contents of cell supernatant of cultures treated with DMSO (control), LPS, or LPS/tested compounds according to the following equation:

$$(\text{Nitrites}_{\text{compound}} - \text{Nitrites}_{\text{control}})/(\text{Nitrites}_{\text{LPS}} - \text{Nitrites}_{\text{control}}) \times 100$$

2.10. Determination of tumor necrosis factor-α (TNF-α)

TNF-α, an indicator of inflammation, was measured by ELISA kit of the supernatant of RAW 264.7 incubated for 24 h with LPS in presence and absence of tested compounds (10 μg/ml), where the concentration of TNF-α in samples was calculated from a plotted standard curve using the recombinant TNF-α, measured by the supplied ELISA kit, and then normalized to the protein concentration in each sample (data was expressed as ng/mg protein). The inhibition percentages of LPS-induced TNF-α generation are an indicator for anti-inflammatory activity of the tested samples.[15]

2.11. Cytotoxicity assay

Cytotoxicity of tested extract was measured against Hep-G2, MCF-7 and HCT-116 cells using MTT cell viability assay,[11] which is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals which is largely impermeable to cell membranes, resulting in its accumulation within healthy cells. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm using microplate ELISA reader. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by <100% relative viability. Then the half maximal growth inhibitory concentration (IC_{50}) was calculated from the equation of the dose-dependent response curve and percentage of relative viability was calculated using the following equation:

$$[\text{Absorbance of treated cells}/\text{Absorbance of control cells}] \times 100$$

2.12. Agar diffusion method

Tested samples were evaluated for antibacterial activity against six different bacterial strains using the agar diffusion method.[16] A loopful of the test organisms was inoculated into 5.0 ml of nutrient broth and incubated at 37 °C for 24 h, and then 0.2 ml from the 24-hour culture of the organism was dispensed into 20 ml sterile nutrient broth and incubated for 3–5 h to standardize the culture to 10^6 cfu/ml. One ml of the tested organisms was added to 19 ml of nutrient agar. A sterile cork borer (7 mm) was used to make ditches in each plate for the tested sample. The base of each ditch was filled with molten nutrient agar to seal the bottom and allowed to gel. Half ml of the reconstituted tested sample with the concentration of 20 μg/ml was dispensed into each ditch. The plates were left to allow for diffusion of the tested sample before incubation at 37 °C for 24 h. Then the zones of clearance produced around the ditches were measured in mm.

2.13. Statistical analysis

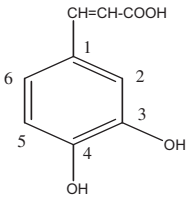
MTT assay data were analyzed by using two-factorial analysis of variance (ANOVA), including first-order interactions (two-way ANOVA), followed by the Tukey's post hoc test for multiple comparisons. $P < 0.05$ indicated statistical significance.

3. Results and discussion

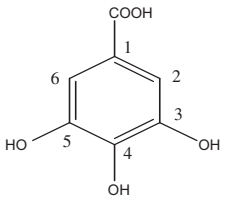
Chromatographic separation of 80% MeOH leaf extract of *R. salicifolia* has resulted in eleven compounds (Fig. 2), which were isolated for the first time from this species. They were identified by different spectral techniques UV, ^1H, ^{13}C NMR and MS also by CoPC against standard sugars and authentic aglycones after complete acid hydrolysis.

UV spectra of compounds **3**, **4**, **7** and **10** showed peaks of absorption characteristic for 3′ and 4′ disubstituted flavonoids, confirmed by the bathochromic shift in band I after addition of boric acid to NaOAc cuvette referring the presence of an *ortho* dihydroxyl groups.[9] ^1H NMR spectra showed an ABX system confirming the disubstitution of ring B at positions 3′ and 4′ by the appearance of H-6′ signal as a doublet of doublet (dd) at δ 7.54 ppm ($J = 8.5$ & 2.0 Hz) and H-2′ signal as a doublet (d) at δ 7.56 ppm ($J = 8.5$ Hz), while H-5′ proton appeared as a doublet at δ 6.85 ppm ($J = 2.0$ Hz).[9] A doublet signal at δ 4.10 ppm ($J = 6.5$ Hz) refers to the anomeric proton of arabinose in compound **4**, a doublet signals at δ 5.34 ppm ($J = 7.4$ Hz), δ 5.29 ppm ($J = 7.3$ Hz) and at δ 5.05 ppm ($J = 7.4$ Hz) refer to the anomeric protons of glucose β-configuration attached to position 3 in the compounds **3**, **4** and **7**, respectively, while its absence in compound **10** confirming its free aglycone structure. The appearance of doublet signals at δ 4.39 ppm ($J = 1.7$ Hz) of anomeric proton for a characteristic terminal α-rhamnose and at δ 1.08 ($J = 6.23$ Hz) of its methyl protons in compound **3**, which was confirmed by ^{13}C NMR spectrum signals at δ 102.2 (C-1‴) and 17.9 (CH_3) ppm. ^{13}C NMR spectra showed typical carbon signals characteristic for quercetin nucleus in compounds **3**, **4**, **7** and **10** in addition to the characteristic signals of the anomeric carbons at δ 100.7 and 101.2 ppm of glucose and rhamnose, respectively, confirming the presence of rutinosyl group in compound **3**, and at δ 101.0 and 103.0 ppm of glucose and arabinose, respectively in compound **4** and δ 101.62 ppm of glucose in compound **7** The upfield shift of C-3 at δ 133.5 ppm when compared to that of unsubstituted flavonol (138.9 ppm) of compound **10** confirmed the glycosylation of compounds **3**, **4** and **7**.[16] Negative ESI–MS m/z 609 $[M-H]^-$, m/z 595 $[M-H]^-$ m/z 431 $[M-H]^-$ of compounds **3**, **4** and **7** confirming their structures as rutin, quercetin-3-*O*-arabinoglucoside and isoquercetin, respectively, together with their aglycone peak of quercetin at m/z 301 $[\text{quercetin-H}]^-$, which is also of compound **10**.[17]

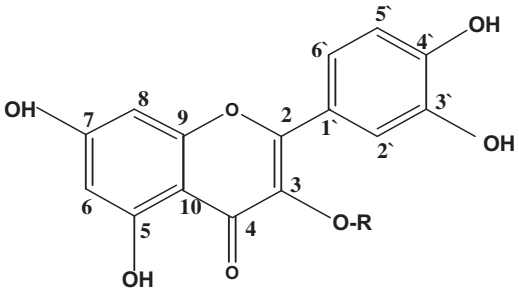
Compound **6** was obtained as yellow amorphous powder (18 mg), chromatographic properties: R_f values; 0.38 (S_1), 0.44 (S_2); dark purple spot under UV-light, turned to yellow fluorescence on exposure to ammonia vapors. It gave deep green color and orange fluorescence with $FeCl_3$ and Naturstoff



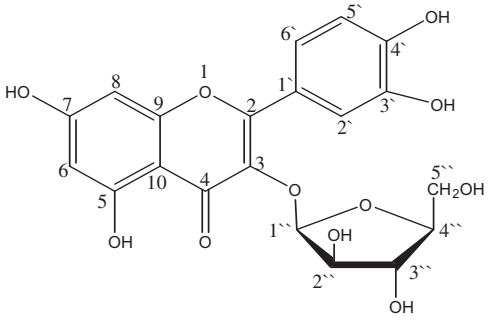
Compound **1**



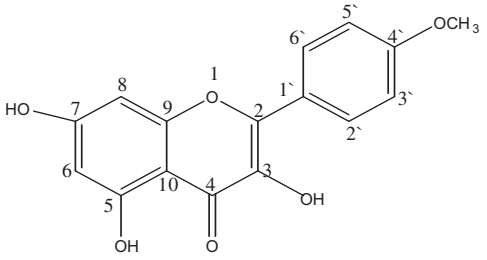
Compound **2**



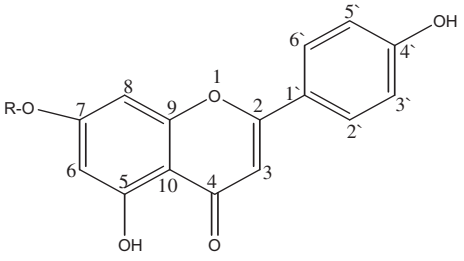
R= glucose – rhamnose = Compound **3**
R= glucose – arabinose = Compound **4**
R= glucose = Compound **7**
R= H = Compound **10**



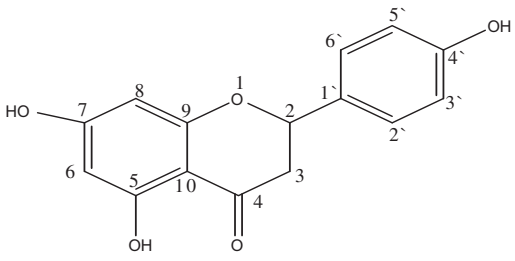
Compound **6**



Compound **8**



R= glucose = Compound **5**
R= H = Compound **9**



Compound **11**

Fig. 2 – Structures of compounds isolated from the leaves of *Ruprechtia salicifolia*.

spray reagents, respectively. It showed λ_{max} (nm) (MeOH): 257, 356; (+NaOMe): 272, 326 (sh), 404; (+NaOAC): 273, 323 (sh), 373; (+$AlCl_3$): 275, 433; (+$AlCl_3$/HCl): 270, 360 (sh), 404. Complete acid hydrolysis resulted in L-arabinose in aqueous phase and quercetin in organic phase (CoPC). ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm 12.54 (1H, s, H-bonded OH-5), 7.50 (1H, dd, J = 8.4, 2.5 Hz, H-6′), 7.48 (1H, d, J = 2.5 Hz, H-2′), 6.82 (1H, d, J = 8.4 Hz, H-5′), 6.38 (1H, d, J = 2.4 Hz, H-8), 6.16 (1H, d, J = 2.4 Hz, H-6), 5.50 (1H, d, J = 1.3 Hz, H-1″), 4.11 (1H, br s, H-2″). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 178.11 (C-4), 164.77 (C-7), 161.63 (C-5), 156.88 (C-2/9), 148.95 (C-4′), 145.52 (C-3′), 133.84 (C-3), 122.23 (C-6′), 121.44 (C-1′), 116.10 (C-5′/2′), 108.34 (C-1″), 104.42 (C-10), 99.26 (C-6), 94.20 (C-8), 86.32 (C-4″), 82.55 (C-2″), 77.43 (C-3″), 61.13 (C-5″). On the basis of its chromatographic properties and UV-spectral data, as the previous explained compounds, compound **6** was expected to be quercetin 3-O-glycoside.

The acid hydrolysis of **6** afforded quercetin as an aglycone and the sugar moiety was identified as arabinose by CoPC. Negative ESI-MS spectrum exhibited a molecular ion peak at *m/z* 433.56 $[M-H]^-$, corresponding to molecular weight 434 and molecular formula $C_{20}H_{18}O_{11}$ for quercetin pentoside, this was further supported by the fragment ions at *m/z* 867.12 $[2M-H]^-$, for the dimeric adduct ion and at 301.30 $[quercetin-H]^-$, for quercetin aglycone. ^{1}H NMR spectrum showed a douplet at δ ppm 5.50 with $J = 1.3$ Hz was characteristic for the anomeric proton of α-L-arabinofuranoside moiety.[18] ^{13}C NMR spectrum showed in addition to 15 carbon resonances for 3-O-glycosyl-quercetin,[18] three highly downfield shifted peaks at 108.34, 86.32, 82.55 assignable to C-1″, C-4″, and C-2″ of an arabinofuranoside moiety by compared to data.[17,19,20] Accordingly compound **6** was identified as Quercetin 3-O-α-L-arabinofuranoside, which was isolated before from *R. polystachya*[3] but first time from this species.

Compounds **5** and **9** showed UV spectra of two major absorption bands in methanol at λ_{max} 268 nm (band II) and at λ_{max} 333 nm (band I) indicating its flavonoid nature giving the chromatographic properties of the characteristic apigenin nucleus.[9] The batho-chromic shift in band II upon the addition of NaOAc reagent in compound **9** indicated its free C7–OH, while its absence in compound **5** indicated the substitution of C7–OH. The batho-chromic shift in band I in both compounds confirmed free 4′ OH. This evidence supported by complete acid hydrolysis yielding glucose in the aqueous layer of compound **5** only and apigenin was detected in the organic layer in both compounds (CoPC).[9] ^{1}H NMR spectrum showing an AX system exhibiting two ortho doublets each integrated for two protons of H-2′/6′, and of H-3′/5′ indicating 1′,4′-disubstituted B-ring of both compounds. The down-field shift of both H-6 and H-8 to 6.43 and 6.74 meta doublet and the anomeric proton signal at δ 5.22 ppm gave evidence for the presence of β-glycosidic moiety at 7-position in compounds **5**.[18] ^{13}C NMR spectra showed the carbon signals characteristic of apigenin nucleus and its glycosidation at 7-OH in compound **5** was indicated by slight up-field shift of C-7. The structure of the compounds was also confirmed by negative ESI-MS molecular ion peak of compound **9** as a free apigenin aglycone at *m/z* 269 $[M-H]^-$ and of compounds **5** at *m/z* 431 $[M-H]^-$ as apigenin glucoside and was compared with published data.[9,17,21]

^{1}H NMR spectra of compound **11** showed flavanone structure indicated by the appearance of dd signal at δ 5.47 ppm integrated for one proton of two *J* values ($J = 12.8$ and 2.8 Hz), assigned for H-2 and the dd of dd signal at δ 2.71 ppm, (1H, $J = 17.0$, 12.8 and 2.8 Hz, H-3). Negative ESI-MS of compound **11** at *m/z* 301 $[M-H]^-$ indicated its structure as naringenin.[17,22]

Compound **8** was obtained as yellow amorphous powder (30 mg), showed UV spectra of two major absorption bands in methanol at λ_{max} 265 nm (band II) and at λ_{max} 366 nm (band I), chromatographic properties: R_f values; 0.68 (S_1), 0.14 (S_2); dull yellow spot under UV-light with no change on exposure to ammonia vapors, it gave greenish yellow color with $FeCl_3$ and Naturstoff spray reagents. Negative ESI-MS spectrum exhibited a molecular ion peak at *m/z* 299 $[M-H]^-$. ^{1}H NMR (300 MHz, DMSO-d_6): δ ppm; 12.60 (1H, s, OH-5), 7.80 (2H, d, $J = 8.7$ Hz, H-2′/6′), 7.34 (2H, d, $J = 8.7$ Hz, H-3′/5′), 6.40 (1H, d, $J = 1.8$ Hz, H-8), 6.20 (1H, d, $J = 1.8$ Hz, H-6), 3.81 (3H, s, OCH_3-4′). ^{13}C NMR (75 MHz, DMSO-d_6): δ ppm 176.39 (C-4), 164.50 (C-7), 161.30 (C-5), 159.20 (C-4′), 156.68 (C-9), 147.35 (C-2), 136.28 (C-3), 130.10 (C-2′/6′), 120.53 (C-1′), 116.90 (C-3′/5′), 104.22 (C-10), 98.75 (C-6), 93.91 (C-8), 56.40 (OCH_3-4′). The methylation of the hydroxyl group at 4′ was evident by the downfield shift of 3′/5′ protons (δ 7.34 ppm) and their carbons (δ 116.90 ppm), compared to that of kaempferol (δ 6.85 and 115.0 ppm, respectively) and the slight upfield shift of carbon of C-4 (δ 159.20 ppm) compared to that of kaempferol (δ 160.0 ppm).[18,23] Thus compound **8** was identified as kaempferol 4′-O-methyl ether (kaempferide),[23,24] which was obtained here for the first time from Genus *Ruprechtia*.

In addition to the isolated flavonoid compounds two known phenolic acids; caffeic **1** and gallic **2** acids, were isolated here for the first time from this species, which were identified by CoPC against authentic samples and with published data.[18,19,25]

3.1. *Immunomodulatory activity*

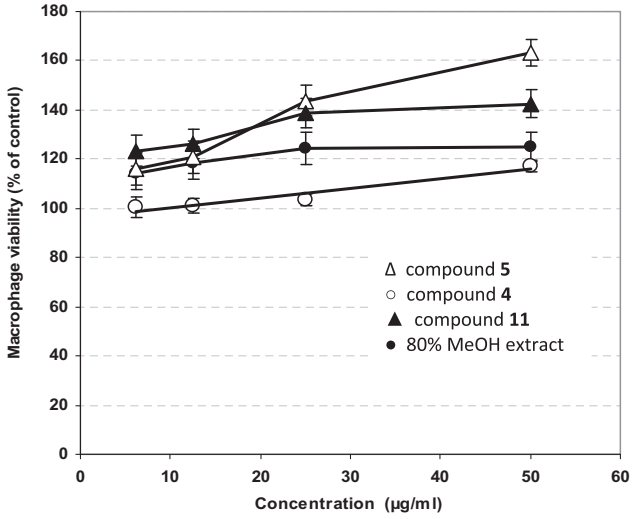
3.1.1. *Proliferation of immune cells*

Results indicated that the incubation of macrophages with compounds **5** and **4** resulted in a highly significant increase ($P < 0.05$) in the cells proliferation at the highest tested dose and that this dose-dependent increase started from the lower tested dose and reached 1.63- and 1.42- fold of the control, respectively, at the highest tested dose, indicating immunomodulatory activity.[11] Treatment of macrophages with the extract and compound **11** showed a non-significant increase ($P > 0.05$) in the macrophage proliferation at any of the tested dose (Fig. 3).

3.2. *Anti-inflammatory activity*

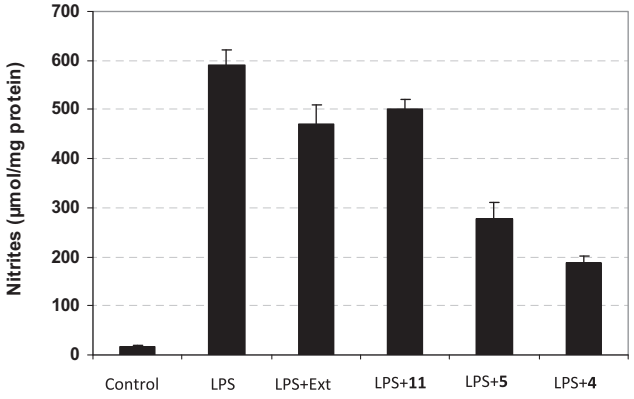
3.2.1. *Nitrite oxide (NO) assay*

Results of the anti-inflammatory activity of the tested samples (80% MeOH leaf extract, compounds **4**, **5** and **11**), evaluated by Griess assay showed inhibitory effect on NO generation in



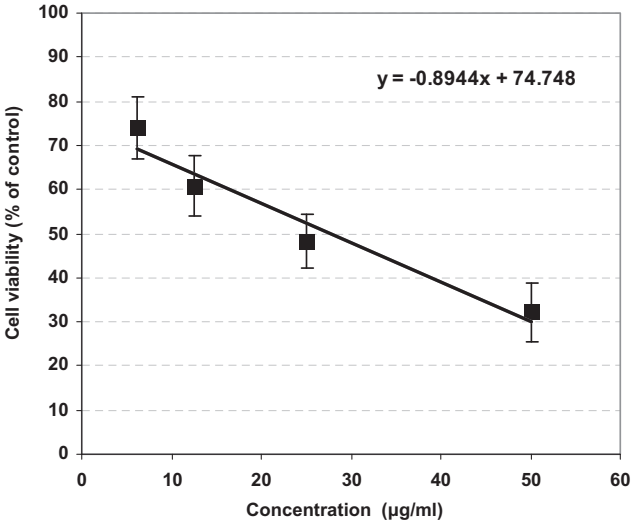
Viability results are represented as percentage of control cells (Mean ± S.E., n = 4)

Fig. 3 – The effect of different doses of 80% MeOH leaf extract of *R. salicifolia*, compounds 4, 5 and 11 on the growth of RAW 264.7 macrophages, as measured by MTT assay.



Results are represented as nitrites content, µmol/mg protein (Mean ± S.E., n = 4)

Fig. 4 – The percentage of inhibition of nitrite oxide in LPS-stimulated RAW 264.7 cells supernatant after treatment with 80% MeOH leaf extract of *R. salicifolia* compounds 4, 5 and 11 (25 µg/ml, each) for 48 h compared to LPS treated cells and non-treated (control) cells, as measured by Griess assay.



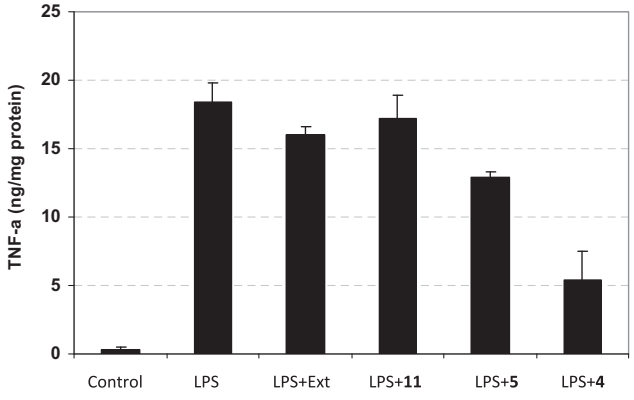
Viability percentage of HCT-116 cells, values are means ± SE, n = 4

Fig. 6 – Cytotoxicity of compound 11 against HCT-116 cells measured by MTT assay.

the supernatant of lipopolysaccharide (LPS) – stimulated RAW 264.7 macrophage cells as the following order: compound **4** > **5**> extract >**11** as indicated from the inhibition percentages: 68.19%, 52.95%, 20.33%, and 15.22%, respectively, where quercetin-3-O-arabinoglucoside (compound **4**) was the most effective inhibitor of LPS-induced NO generation ($P < 0.01$), implying enhanced anti-inflammatory activity[26] (Fig. 4).

3.2.2. *Tumor necrosis factor-alpha (TNF-α) assay*

Results showed that the tested samples revealed an inhibitory effect on TNF-α secretion to a variable extent, as the following order: compound **4** > **5** >extract > **11** as indicated from the inhibition percentages: 70.82%, 29.88%, 13.13%, and 6.14%, respectively, ($P < 0.01$), where quercetin-3-O-arabinoglucoside (compound **4**) was the most effective inhibitor indicating anti-inflammatory activity[15] (Fig. 5).



Results are represented as TNF-α concentration, ng/mg protein (Mean ± S.E., n = 4)

Fig. 5 – The inhibitory effect of 80% MeOH leaf extract of *R. salicifolia*, compounds 4, 5 and 11 on LPS-induced TNF-α generation from RAW 264.7 macrophages, as measured by ELISA assay.

3.3. *Anticancer activity*

3.3.1. *Cytotoxicity*

Results indicated that the treatment of Hep-G2, MCF-7 and HCT-116 cells with the different tested samples was safe and possessed a non cytotoxic effect against different cell types with IC_{50} values >50 µg/ml,[8] except for compound **11**, which was cytotoxic only against HCT-116 cells, as indicated in the dose response curve (Fig. 6) and the low IC_{50} value of 27.67 µg/ml.

3.4. *Antibacterial activity*

The 80% MeOH leaf extract, was evaluated for antibacterial activity using Ciprofloxacin, broad spectrum antibiotic as a positive control and 80% methanol solvent as a negative control, results showed that the leaf extract had significant effect against *S. aurous*, *S. pyogenes*, *E. coli*, *P. aeruginosa*, *K. pneumonia* and *P. mirabilis* with inhibition zone Ø values of 18, 20, 15, 20, 15 and 17 mm, respectively. Moreover it inhibits the growth of *K. pneumonia* strain which is a sensitive strain resistant to Ciprofloxacin antibiotic.

4. Conclusion

In conclusion, the methanol leaf extract of *R. salicifolia* contain phenolic compounds that are isolated for the first time from this species some of which showed immunomodulatory and inhibitory effect against both nitrite oxide and TNF-α release from macrophage cell line indicating anti-inflammatory activity, "so may be used in immune compromised patients to improve their immunity against various infections" thus *R. salicifolia* needs to be evaluated in animal models to determine its potential as natural health care product.

Conflicts of interest

All authors have none to declare.

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