Phytochemical and Biological Investigation of Leaf Extracts of *Podocarpus gracilior* and *Ruprechtia polystachya* Resulted in Isolation of Novel Polyphenolic Compound

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Abstract: Phytochemical investigation of polyphenolic contents of *Podocarpus gracilior* Pilger *and Ruprechtia polystachya* Griseb leaves were resulted in isolation and identification of three and six known polyphenolic compounds respectively. In addition of a new polyphenolic compound isolated for the first time from nature from *R. polystachya* which is identified as 4`-*O*-Galloyl-myricetin-3-*O*-*α*-L-rhamnopyranoside (4`-*O*-galloyl myricetrin). Identification of hydrocarbons in *P. gracilior and R. polystachya* leaves resulted in identification of 19 and 21 compounds respectively. Concerning the composition of fatty acids content in *P. gracilior* it could be concluded that the unsaturated fatty acids (51.16%) represented higher percentage than that of saturated ones (38.87%). In the case of *R. polystachya*, the saturated fatty acids (70.58%) were dominated on the unsaturated ones (27.30%). The tested methanol extracts of *P. gracilior* and *R. polystachya* leaves showed antioxidant, antimicrobial and stimulatory activities to nitric oxide release from macrophage cell line. Methanol extracts of *P. gracilior* leaves had weak cytotoxic effect against MCF-7 cells (breast adenocarcinoma cell line) while methanol extracts of *R. polystachya* leaves did not show cytotoxic activity.
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Key words: *Ruprechtia polystachya*, *Podocarpus gracilior*, polyphenols, antioxidant, cytotoxic, antimicrobial and anti-inflammatory.

1. Introduction:

Plants containing, flavonoids and/or tannins received considerable attention for their biological activities[1-4]. For example some species of *Podocarpus* genus (family Podocarpaceae) and *Ruprechtia* genus (family Polygonaceae) reported to have several biological activities like antioxidant, cytotoxic, anti-inflammatory, antiviral, antimicrobial activities. These biological activities were revealed for their contents of terpenoid, flavonoids and tannins[5-10]. So in this study it was interesting to take an overview of two interesting species growing in Egypt each from one of the previous mentioned families. Like *Podocarpus gracilior* (Family Podocarpaceae) (*Pg*) which was reported as antioxidant and detected to contain Taxol,[6&11] this could be used as anticancer. Another plant is *Ruprechtia polystachya (*family: Polygonaceae) (*Rp*) which has an inhibitory activity on the glucose-6-phosphatase system, so may be used as antidiabetic drug[10].

2. Material and Methods:

Apparatus

JOEL GX-500 (500 and 125 MHz for ^{1}H and ^{13}C NMR), NMR department, National Research Center (NRC), and NMR in Strathclyde Institute of Pharmacy and Biomedical Science, Glasgow, United Kingdom were used. The *δ*-values were reported as ppm relative to TMS in DMSO-d_6 and *J*-values were in Hz. ESI-MS spectra were measured on Walters ACQUITY/TQD triple quadrupole, Center for Applied Research and Advanced Studies, Faculty of Pharmacy Cairo University. The UV analyses for pure samples were recorded on a Shimadzu UV 240 spectrophotometer, separately as solutions in methanol and with different diagnostic UV shift reagents[12&13]. Rotary evaporator (Bűchi, G, Switzerland). Fractionation of the extracts was done by columns chromatography using polyamide 6S (Riedel-De Hän Ag, Seelze Hannover, Germany), isolation and purification of compounds were done on either cellulose (Pharmacia, Uppsala, Sweden) or Sephadex LH-20 (Fluka, Switzerland) columns of different dimensions and eluted with different solvent systems (Figures 1 and 2). Separation processes were followed up by 2D-PC and CoPC using Whatmann No. 1 paper with (S_1) and (S_2) as in table 1. Ultraviolet lamp (VL-215 LC, Marne La Vallee, France): It was used for visualization of spots on paper and thin layer chromatograms and follow up the columns fractionation on columns at 254 and/or 365 nm and also with sprayed Naturstoff reagent[14]. Gas liquid chromatography TRACE GC ULTRA was used for analysis of both total fatty acids (TFA) and unsaponifiable matter (USM) using GC/MS HP 6890 series (Agilent) MSD, Faculty of Agriculture, Cairo University according to the following conditions:

Capillary column HP6890 series (30 m x 0.25 mm i.d. and 0.25 µm film thickness); detector: MSD; carrier gas: Helium, with flow rate: 1 ml/min; injector temperature: 270ºC; detector temperature: 280ºC; initial column temperature: 70ºC, programmed by 8ºC/min up to final temperature 270ºC within 20 min. GLC conditions for total fatty acids analysis: Capillary column: Thermo TR-FAME (70% cyanopropyl polysilphenylene siloxane) (30 m x 0.25 mm i.d. and 0.25 µm film thickness); detector: flame ionization; carrier gas: N_2, with flow rate 30 ml/min; injector temperature: 200°C; detector temperature: 220°C; initial column temperature: 140°C, programmed by 5°C/min up to final temperature 200°C within 12 min. Fluostar Optima microplate ELISA reader and 96 well cell culture microplates were used for pharmacological studies.

Plant material

Leaves of *Ruprechtia polystachya* Griseb were collected from El-Orman garden, Giza, Egypt in April 2010. Leaves of *Podocarpus gracilior* Pilger were collected from El-Zohria garden, Cairo, Egypt in June 2010. Identification of the plants was confirmed by Dr. Trease Labeb, lecturer of Taxonomy, Department of Floral and Taxonomy, El-Orman garden, Cairo, Egypt.

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Sigma-Aldrich Co. (St Louis, MO). All other chemicals, solvents and reagents used in chromatography were of analytical grade. Authentic reference of phenolic compounds were obtained from Phytochemistry Laboratory, Department of Molecular and cell Biology, University of Texas at Austin (Austin, TX) and from Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt. Authentic of fatty acids, hydrocarbons and sterols were obtained from Faculty of Agriculture Research Park, Giza, Egypt.

Cell line and culture medium

Human breast adenocarcinoma cell line (MCF-7), purchased from ATCC, USA, and was used to evaluate the cytotoxic effect of the tested samples. Cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 100 units/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained at sub-confluence at 37ºC in humidified air containing 5% CO_2. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%. Tested extracts were dissolved in dimethyl sulphoxide (DMSO), and then diluted thousand times in the assay. All cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark)[15].

Extraction and isolation

Powdered, air-dried leaves of *P. gracilior* (1050 g) *and R. polystachya* (950 g) were separately exhaustively extracted with hot 80% MeOH (5×3 L), under reflux. The dry residues obtained of *P. gracilior* (140 g) and *R. polystachya* (170 g) were extracted with chloroform (3×1 L). The 2D-PC revealed that chloroform soluble portion contained limited polyphenolic contents, while they were concentrated in MeOH soluble portion. The aqueous residues of *P. gracilior* (120 g) and *R. polystachya* (150 g) were fractionated on a polyamide column (Ø 5.5×120 cm) as illustrated in flow charts (Figures 1 and 2). Three compounds were isolated from *P. gracilior* and seven compounds were isolated from *R. polystachya* (Figures 1 and 2). While the chloroform soluble portions concentrated under vacuum (12 g for *P. gracilior* and 15 g for *R. polystachya*) were used for identification of lipoidal matters in both plants. The chloroform extracts were saponified with 10% ethanolic potassium hydroxide under reflux[16]. After evaporation of the ethanol, the aqueous solution was extracted with chloroform. The collected chloroform was washed with water and dried over anhydrous sodium sulphate, evaporated and weighted 2.3 g (19%) and 3.5 g (23%) for *P. gracilior* and *R. polystachya* respectively then kept for further study by GLC (USM). The remaining aqueous layer after extraction with chloroform was acidified with 10% hydrochloric acid and the liberated fatty acids were extracted with chloroform and washed with distilled water, dried over anhydrous sodium sulphate, evaporated then weighted 5 g (42%) and 5.5 g (37%) for *P. gracilior and R. polystachya* respectively and were kept for further study of TFA according to Vogel method[17].

Fig.1: Fractionation and purification of polyphenolic compounds isolated from the leaves of *P. gracilior*

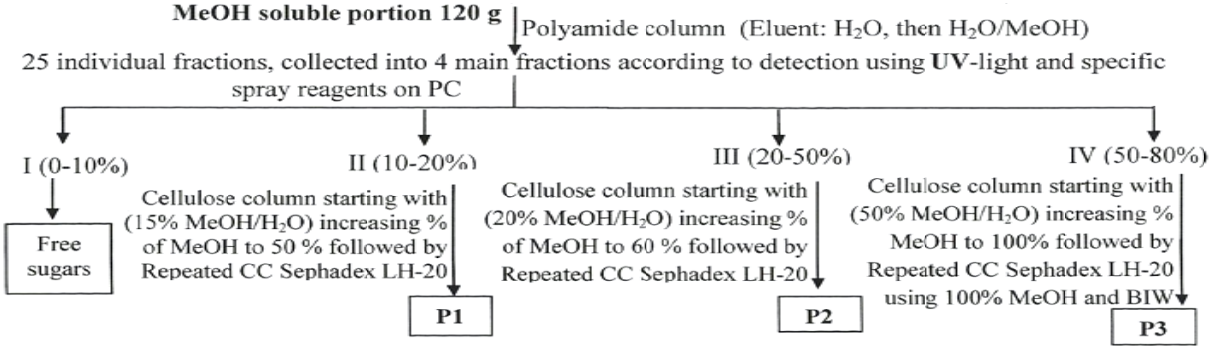
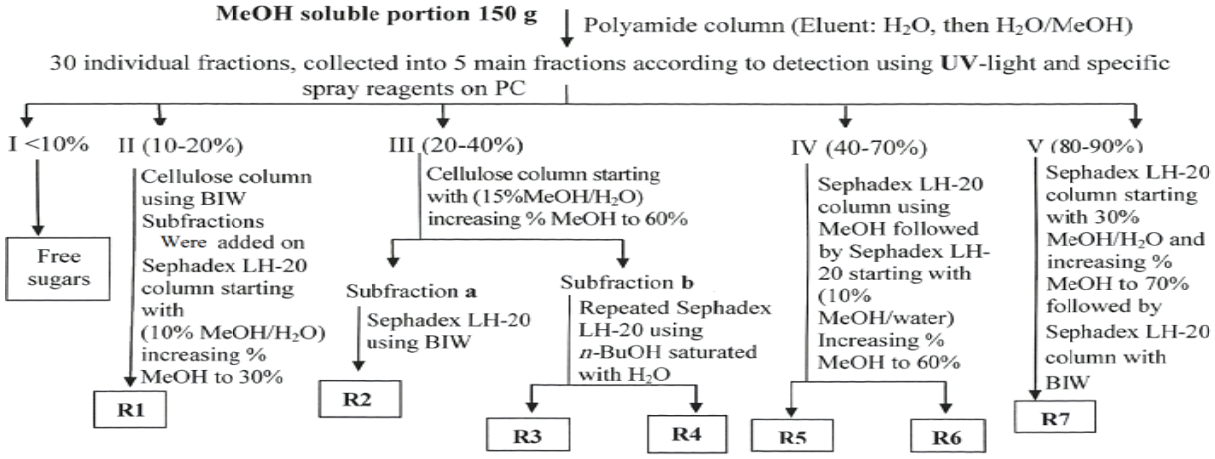


Table 1: Solvent systems

S_1	n-Butanol – Acetic acid –Water (BAW)	(4:1:5 v/v/v, upper layer)
S_2	Acetic acid – Water	(15:85 v/v)
S_3	n-Butanol – Isopropyl alcohol – Water (BIW)	(4:1:5 v/v/v, upper layer)

Fig.2: Fractionation and purification of polyphenolic compounds isolated from the leaves of *R. polystachya*



Anti-tumor activity

Cytotoxic activity for the extracts was measured against MCF-7 cells using the MTT Cell Viability Assay. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay 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, accumulated within healthy cells. Solubilization of the cells results in the liberation and solubilization formazan crystals. 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[15]. Cells (0.5X105 cells/ well), in serum-free media, were plated in a flat bottom 96-well microplate, and treated with 20 µl of different concentrations of the tested samples for 48 hrs at 37ºC, in a humidified 5% CO_2 atmosphere. After incubation, media were removed and 40 µl MTT solution / well were added and incubated for an additional 4 hrs. MTT crystals were dissolved by adding 180 µl of acidified isopropanol/ well and plate was shacked at room temperature, followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader. Triplicate repeats were performed for each concentration and the average was calculated. 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 (Figure 8)[15].

Antioxidant activity

Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. The method of scavenging DPPH free radicals can be used to evaluate the antioxidant activity of methanol extracts obtained from the leaves of *P. gracilior* and *R. polystachya*. Ethanolic DPPH

(1,1-diphenyl-2-picrylhydrazyl): 0.1 mM DPPH/absolute ethanol. In the presence of an antioxidant radical scavenger, which donates an electron to DPPH, the deep violet color decolorize to the pale yellow non-radical form are which monitored spectrophotometrically at 515 nm[18]. In a flat bottom 96 well-micro plates, a total test volume of 200 µl was used. In each well, 20 µl of different concentrations (0-40 µg/ml final concentration) of the tested samples were mixed with 180 µl of ethanolic DPPH and incubated for 30 min at 37ºC. Triplicate wells were prepared for each concentration and the average was calculated. Then the absorbance at 515 nm was determined photometrically by micro plate ELISA reader. Concentrations ranging from 0-25 µg/ml standard ascorbic acid solutions were used for plotting a standard calibration curve[18&19].

Evaluation of anti-inflammatory activity

Nitrite accumulation was used as an indicator of NO production using a micro plate assay based on the Griess reaction. The Griess reaction is based on a two-step diazotization reaction in which acidified nitrites generate a nitrosating agent that reacts, with sulfanilic acid to form diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to produce the chromophoric pink azo-derivative that can be determined spectrophotometrically at 540 nm[20]. 40 mg Griess reagent (0.2% naphthylenediamine dihydrochloride, and 2% sulphanilamide in 5% phosphoric acid) was dissolved in 1 ml deionized water. In each well of a flat bottom 96 well- micro plate, 40 µl freshly prepared Griess reagent was mixed with 40 µl cell supernatant after cell treatment with LPS (lipopolysaccharides). 25 µg/ml of the methanol extracts of both *P. gracilior* leaves and *R. polystachya* leaves were separately added and incubated for 48 hrs. The plates were incubated for 10 min in the dark then the absorbances of the mixtures at 540 nm were determined using the micro plate ELISA reader. A standard curve relating NO in µM to the absorbance was constructed (Figure 9), from which the NO level in the cell supernatant was computed by interpolation.

Antimicrobial study

Gram positive bacteria (*Staphylococcus aureus* ATCC12600), Gram negative bacteria (*E- coli* ATCC117750), Yeast (*Candida albicans* ATCC26555) and Fungi (*Aspergillus flavus*) were used to test the antimicrobial activity of *P. gracilior* and *R. polystachya* methanolic extracts. The antimicrobial study of the tested methanol extracts was determined by applying modified disc diffusion method[21]. The extract residue was diluted with DMSO at concentration 1:5 w/v, then 20 µl was aseptically transferred onto sterile discs of Whatmann filter paper (5 mm diameter). Standard discs of tetracycline and amphotericin B served as positive controls for antimicrobial activity but filter discs impregnated with 20 µl of solvent (DMSO) were used as negative control. The diameters of the inhibition zones were measured in millimeters by the use of slipping calipers (Table 4).

3. Results and discussion:

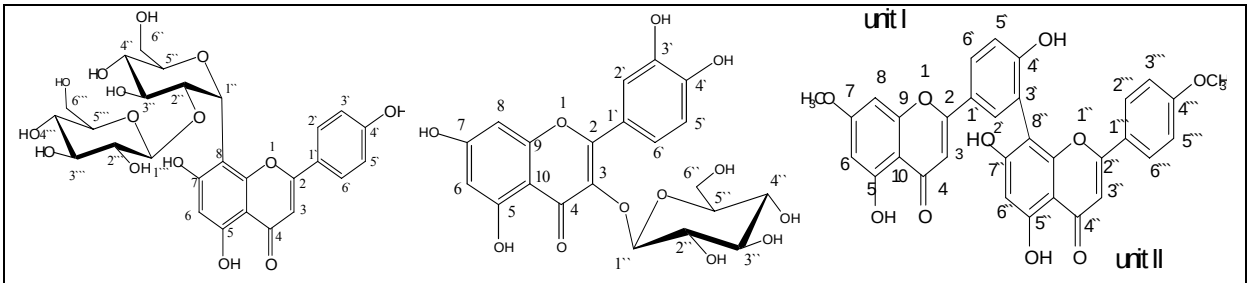
Investigation of polyphenolic contents

Methanol extracts of *P. gracilior* and *R. polystachya* leaves were separately fractionated on a polyamide column followed by successive separation on cellulose and Sephadex LH-20 columns yielded three and seven compounds in *P. gracilior* and *R. polystachya* leaves extracts respectively (Figures 1 and 2). The isolated pure compounds were identified on the basis of acid hydrolysis, comparative PC, UV, ESI-MS, ^{1}H-, ^{13}C-NMR and in some cases 2D-NMR spectroscopic analyses and comparing with previous reported data[10,12&22-34]. The known isolated compounds from *P. gracilior* leaves are identified (Figure 3) as P_1: Apigenin 8-*C*-*β*-D-glucopyranosyl-(1```→2``)-*O*-*β*-D-glucopyranoside (Vitexin 2``- *O*-*β*-D-glucopyranoside) which is isolated for the first time from genus *Podocarpus*. P_2: Quercetin 3-*O*-*β*-D-glucopyranoside (Isoquercetin) which is isolated for the first time from *P. gracilior* but isolated before from *P. fasciculus* bark[35] and P_3: II-4```, I-7-dimethoxy amentoflavone (Podocarpusflavone B) which is isolated for the first time from *P. gracilior* but was isolated before from *P. neriifolius, P. fasciculus, P. fleuryi, P. elongatus*[35-40]. In the case of *R. polystachya* leaves extract we have isolated seven compounds (Figure 4) which are identified as R_2: Gallic acid, which is isolated for the first time from genus *Ruprechtia*, while R_3: Myricetin-3-*O*-*α*-L-rhamnopyranoside (Myricetrin), R_4: Quercetin-3-*O*-*α*-L-rhamnopyranoside (Quercetrin) R_5: Quercetin-3-*O*-*α*-L-arabinofuranoside, were isolated before from *R. polystachya*[10] as well as R_6: Quercetin 3-*O*-*β*-D-glucopyranoside (Isoquercetin) and R_7: Quercetin were isolated for the first time from genus *Ruprechtia*. In addition, the new compound R_1: 4`-*O*-Galloyl-myricetin-3-*O*-*α*-L-rhamnopyranoside was found in *R. polystachya*, this new compound was identified according to the following description (Figure 5).

Characterization and identification of compound R_1 which is isolated from *R. polystachya*:

Yellow amorphous powder (35 mg). Chromatographic properties, R_f value: 0.40 (S_1), 0.62 (S_2); dark purple spot under UV-light turned to

yellow fluorescence on exposure to ammonia vapors, it gave bluish green color and deep red fluorescence with $FeCl_3$ and Naturstoff spray reagents, respectively. UV-spectral data λ_{max} (nm) (MeOH): 268, 291 (sh), 352; (+NaOMe): 275, 325, 390; (+NaOAC): 278, 329, 387; (+$AlCl_3$): 277, 427; (+$AlCl_3$/HCl): 277, 418. Complete acid hydrolysis gave rhamnose in aqueous phase, myricetin and gallic acid in the organic phase (CoPC). ^{1}HNMR spectrum (500 MHz, DMSO-d_6): δ ppm 6.88 (2H, s, H-2```/6```), 6.87 (2H, s, H-2`/6`), 6.33 (1H, d, *J*=1.8 Hz, H-8), 6.16 (1H, d, *J*=1.8 Hz, H-6), 5.15 (1H, br s, H-1``), 3.94 (1H, br s, H-2``), 3.47-3.13 (H_s- 3``, 4``, 5``, hidden by $H_2O$ signal), 0.80 (3H, d, *J*=6.0 Hz, H-6``). ^{13}CNMR (125 MHz, DMSO-d_6): δ ppm 178.27 (C-4), 170.83 (C-7```), 164.74 (C-7), 161.79 (C-5), 158.02 (C-9), 156.93 (C-2), 146.24 (C-3`/5`), 145.87 (C-3```/5```), 138.48 (C-4```), 136.98 (C-4`), 134.77 (C-3), 123.24 (C-1```), 120.12 (C-1`), 109.23 (C-2```/6```)108.43 (C-2`/6`), 104.53 (C-10), 102.39 (C-1``), 99.22 (C-6), 94.09 (C-8), 71.75 (C-4``), 70.87 (C-3``), 70.86 (C-2``), 70.52 (C-5``), 17.99 ($CH_3$-6``). Negative ESI-MS: *m/z* 615.29 [M-H]$^-$, 463.17 [M-galloyl]$^-$, 317.06 [Myricetin-H]$^-$. The UV-spectral data of compound R_1 showed in the MeOH spectrum two characteristic absorption maxima at λ_{max} 268 nm (band II) and 352 nm (band I), for flavonols. The enhanced UV absorbance of band II at 268 nm in MeOH in comparison with compound R_3 gave the suggestion of the presence of gallic acid moiety in the structure[12&41]. The bathochromic shift and decrease in intensity of band I observed with NaOMe indicated 4`-*O*-substituted compound, while the bathochromic shift in band II with NaOAc was indicative of free 7-OH group. In addition to the strong bathochromic shift in band I remaining after addition of HCl to $AlCl_3$ indicated the presence of free 5-OH group and the absence of a free 3-OH group[12]. Based on the chromatographic properties and the UV- spectral data, compound R_1 was expected to be Myricetin 3-*O*-galloyl glycoside. On complete acid hydrolysis, R_1 gave L- rhamnose in aqueous phase, myricetin and gallic acid in organic phase when compared with standards using PC (CoPC) suggesting myricetin 3-*O*-galloyl rhamnoside. A supporting evidence achieved from Negative ESI-MS spectrum that showed molecular ion peak [M-H]$^-$ at *m/z* 615 corresponding to M.wt. 616 and molecular formula $C_{28}H_{24}O_{16}$ together with the fragment ion peaks at *m/z* 463 (after the loss of galloyl moiety) and at *m/z* 317 for [Myricetin-H]$^-$ aglycone. ^{1}HNMR spectra showed the characteristic three proton resonances of H-2`/6` at δ ppm 6.87, H-8 at δ ppm 6.33 and H-6 at δ ppm 6.16 for myricetin aglycone. The presence of galloyl moiety in the structure was concluded from the proton resonance at δ ppm 6.88 (2H,s) assignable to H-2```/6```. The sugar moiety was simply identified as *α*-rhamnose from the anomeric proton resonance at 5.15 and doublet signal of CH_3-6`` at 0.80 (6.0 Hz)[12]. The structure of compound R_1 was finally confirmed by ^{13}CNMR spectrum that showed the characteristic signals at δ ppm 178.27, 146.24, 108.43 and 134.77 for C-4, C-3`/5`, C-2`/6` and C-3 respectively for 3-*O*- substituted myricetin compound. The presence of galloyl moiety was confirmed from the carbon resonance at δ ppm 170.83 for the carbonyl carbon (C-7```) and the typical four carbon resonances for the rest of galloyl carbons, the position of esterification by gallic acid was confirmed at OH-4` (not at 3` or 5`) from the slight up-field shift C-4` in comparison with compound R_3, remaining of symmetrical structure in ring-B and finally from the decreasing of intensity of band I with NaOMe reagent as described before in the UV-spectral results. The ^{13}CNMR also showed the typical ^{13}C-resonance for rhamnose moiety characterized by anomeric carbon at δ ppm 102.39 and CH_3-6`` at 17.99. Stereo-structure of the rhamnoside moiety was established as *α*-$^1C_4$-pyranose depending on δ and *J*-values in ^{1}H and ^{13}C NMR spectra. Finally compound R_1 was assigned by comparison with the corresponding published data of structural related compounds[12,23,41&42]. Thus compound R_1 was identified as 4`-*O*-galloyl myricetin-3-*O*-*α*-L-rhamnopyranoside (4`-*O*-galloyl myricetrin) which has been identified as a new natural compound.



compound P_1 compound P_2 compound P_3

Fig. 3: Structures of known isolated polyphenolic compounds of *P. gracilior*

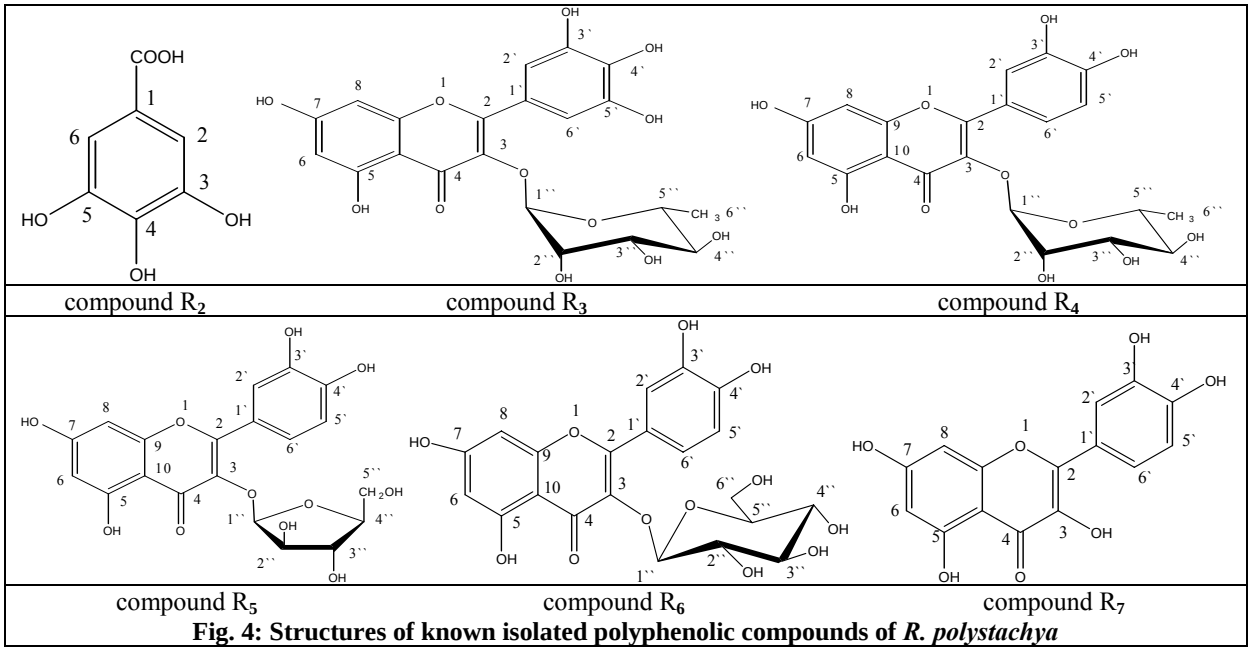


Fig. 4: Structures of known isolated polyphenolic compounds of *R. polystachya*

Investigation of Lipoidal Matter (USM and TFA)

Identification of hydrocarbons in *P. gracilior and R. polystachya* leaves resulted in identification of 19 and 21 compounds respectively (Table 2). n-Nonane (38.91%) and n-Tetracosane (28.74%) represent the major hydrocarbons in *P. gracilior and R. polystachya* respectively. *β*-sitosterol and stigmasterol represent the two identified sterols in both *P. gracilior and R. polystachya*. As shown in table 3 concerning the composition of fatty acids content in *P. gracilior* it could be concluded that the unsaturated fatty acids (51.16%) represented higher percentage than that of saturated ones (38.87%). In the case of *R. polystachya* the saturated fatty acids (70.58%) represented higher percentage than that of unsaturated ones (27.3 %) (Table 3).

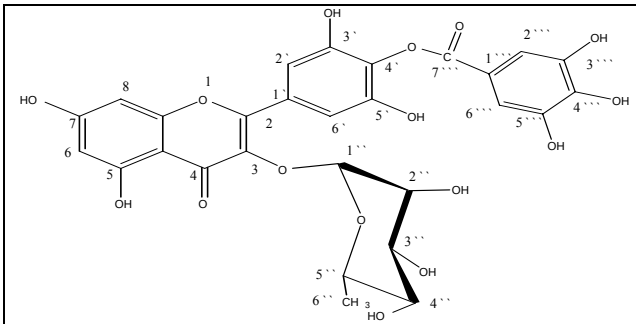


Fig. 5: Compound R_1, 4`-*O*-galloyl myricetrin (4`-*O*-Galloyl-myricetin-3-*o*-*α*-L-rhamnopyranoside)

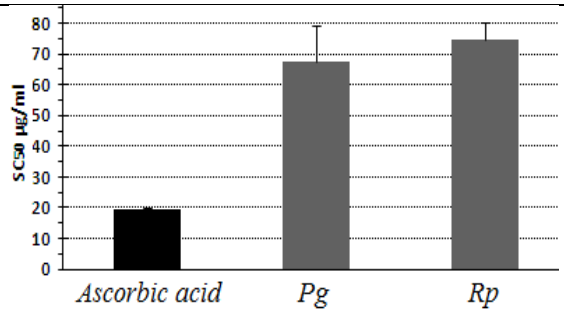


Fig. 6: Calculated SC_{50} of methanol extracts from *Pg and Rp leaves* using DPPH radicals

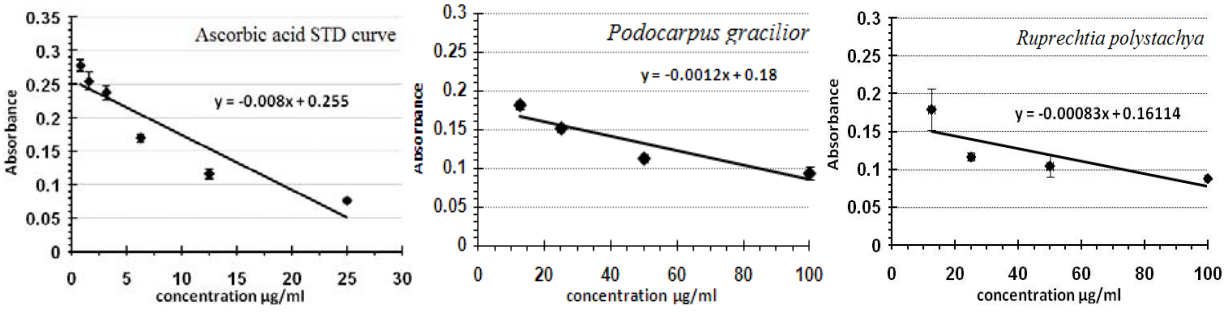


Fig. 7: Absorbance of various drugs versus concentrations showing different antioxidant activities.

Table 2: GLC analysis of USM of *Pg* and *Rp* leaves (as % of total USM)

Identified compounds	*Pg*	*Rp*
n-Nonane (C9)	38.91	1.50
n-Decane (C10)	0.71	0.53
n-Henedecane (C11)	0.84	0.30
n-Dodecane (C12)	2.90	13.57
n-Tridecane (C13)	1.23	1.36
n-Tetradecane (C14)	2.15	2.62
n-Pentadecane (C15)	0.89	7.15
n-Hexadecane (C16)	0.85	1.01
n-Heptadecane(C17)	0.88	0.69
n-Octadecane (C18)	1.51	1.11
n-Nonadecane (C19)	3.17	10.07
n-Eicosane (C20)	2.02	3.35
n-heneicosane (C21)	10.83	0.51
n-docosane (C22)	0.83	0.40
n-Tetracosane (C24)	1.57	28.74
n-pentacosane (C25)	-	0.79
n-hexacosane (C26)	3.67	2.04
n-heptacosane (C27)	1.47	0.64
n-octacosane (C28)	2.92	0.83
n-Nonacosane (C29)	0.37	0.56
n-triacontane (C30)	-	8.07
Stigmasterol	0.79	1.54
β –sitosterol	1.05	1.31
Total hydrocarbons	77.71	85.85
Total sterols	1.84	2.85
Unidentified compounds	20.45	11.3

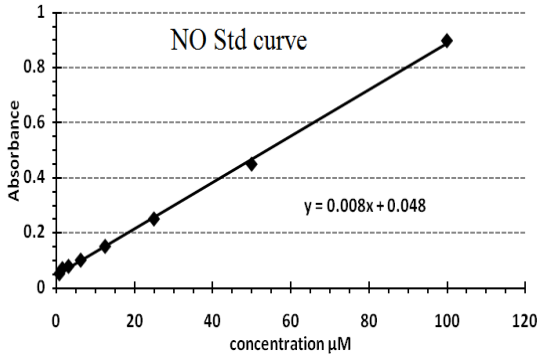


Fig. 9: A standard curve of sodium nitrite

Table 3: GLC analysis of fatty acid methyl ester of *Pg* and *Rp* leaves (% of fatty acids)

Identified compounds	*Pg*	*Rp*
Isocapric (6:0)	-	9.60
Caprylic (8:0)	-	3.48
Capric (10:0)	1.05	-
Lauric (12:0)	0.35	1.06
Myristic (14:0)	0.43	21.54
Palmitic (16:0)	30.75	25
Hexadecenoic acid (16:1)	0.2	3.40
Margaric (17:0)	0.85	1.32
Stearic (18:0)	4.71	6.30
Octadecenoic acid (18:1)	15.96	7.07
Linoleic (18:2)	-	2.13
Linoelaidic (18:2)	14.44	13.37
Octadecatrienoic acid (18:3)	20.83	1.33
Arachidic (20:0)	0.73	2.30
Saturated fatty acids	38.87	70.60
Unsaturated fatty acids	51.16	27.30
Unidentified compounds	9.97	2.10

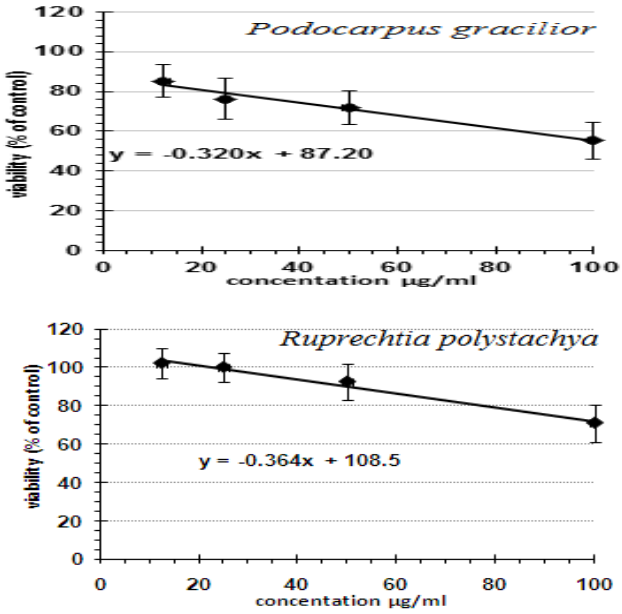


Fig. 8: Cytotoxic effect of different samples against MCF-7 cells using MTT assay (n=4), data expressed as the mean value of cell viability (% of control) ± S.E.

Biological study

Antioxidant activity

The methanol extracts of both *P. gracilior* and *R. polystachya* leaves were proved to exhibit antioxidant scavenging affinity against DPPH as concluded from its SC_{50} value 67.4 µg/ml and 74.8 µg/ml respectively when compared with the standard antioxidant activity of vitamin C; ascorbic acid (SC_{50} 20 µg/ml), as shown in figures 6 and 7.

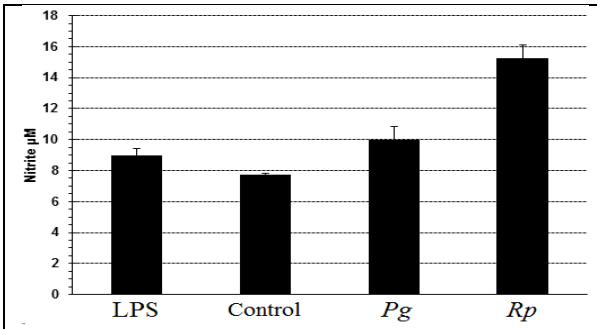


Fig. 10: The level of Nitric oxide in RAW 264.7 cells supernatant after the treatment with the samples (25 µg/ml) for 48 hours compared with LPS-treated cells, as measured by Griess assay.

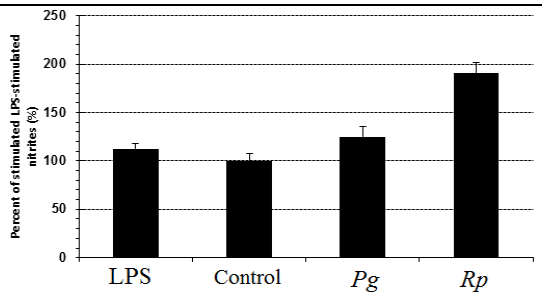


Fig. 11: The percentage of stimulation of Nitric oxide in LPS-stimulated RAW 264.7 cells supernatant after the treatment with the samples (25 µg/ml) for 48 hours compared with LPS treated cells, as measured by Griess assay.

Anti-tumor activity

Cytotoxic activity of methanol extracts obtained from the leaves of *P. gracilior* and *R. polystachya* were examined using MCF-7 (breast adenocarcinoma cell line). Activity was reported in terms of an IC_{50}, which is the concentration (µg/ml) necessary to produce 50% inhibition. Using MTT assay, the effect of both plant extracts on the proliferation of MCF-7 cells were studied after 48 hrs of incubation at 37°C. As shown in figure 8 the treatment of MCF-7 cells with the methanol extract from *P. gracilior* showed weak cytotoxic effect against MCF-7 as its calculated IC_{50} was 116.3 µg/ml while the methanol extract obtained from *R. polystachya* leaves did not show cytotoxic effect as its calculated IC_{50} was 160.7 µg/ml[15].

Evaluation of anti-inflammatory activity using estimation of nitric oxide method

The results indicated that LPS induced nitric oxide production up to 12.5% of the control, and that both of the tested samples (25 µg/ml) possessed a highly significant stimulatory activity against LPS- induced nitric oxide (P<0.001) to the extent 25 and 90% for both *P. gracilior* and *R. polystachya* extracts respectively compared to the control level, as shown in figures 9-11. Such stimulatory samples may be used in immune-compromised patients to improve their immunity against various infections.

The antimicrobial activity

As shown in table 4, the methanol extracts of the leaves of *P. gracilior* and *R. polystachya* exerted marked effect against Gram –ve and Gram +ve bacteria. It showed that the activity on Gram -ve higher than Gram +ve bacteria. Methanol extracts of the leaves of *P. gracilior* and *R. polystachya* showed no antifungal activity.

Table.4: Antimicrobial screening of the methanol extracts of the leaves of *Pg* and *Rp* (diameter of inhibition zone measured by mm)

Microorganism	*Pg*	*Rp*	TC	AMP
E. coli (G-ve)	15 (50%)	13 (43.3%)	30 (100%)	-
S. aureus (G+ve)	14 (48.3%)	11 (40%)	29 (100%)	-
A. flavus (fungus)	0.0	0.0	-	17
C. albicans (fungus)	0.0	0.0	-	19

TC: Tetracycline, AMP: Amphotericin B, -: No inhibition zone

4. Conclusion:

In conclusion, the methanol extracts of the leaves of *P. gracilior* and *R. polystachya* contain various polyphenolic compounds. Their extracts have significant antioxidant and antimicrobial properties. The tested methanol extracts of *P. gracilior* and *R. polystachya* leaves have stimulatory activity to nitric oxide release from macrophage cell line (so may be used in immune-compromised patients to improve their immunity against various infections) thus have great potential as a source for natural health products.

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Reference

1- Zdunczyka Z, Frejnagela S, Wróblewskaa M, Juśkiewicza J, Oszmiańskib J, Estrellac I. (2002). Biological activity of polyphenol extracts from different plant sources. *Food Research International*, 35: 183-6.
2- Middleton E, Kandaswami C, Theoharides TC. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev*., 52: 673-751.
3- Havsteen B. (1983). Flavonoids, a class of natural products of high pharmacological potency. *Biochem Pharmacol*., 32: 1141-8.
4- Mota M, Thomas G, Barbosa JM. (1985). Anti-inflammatory actions of tannins isolated from the bark of *Anacardwm occidentale*. *J. Ethnopharmacology*, 13: 289-300.
5- Abdillahi HS, Stafford GI, Finnie JF, and Van Staden J. (2008). Antimicrobial activity of South African *Podocarpus* species. *J. Ethnopharmacology*, 119: 191-4.
6- Abdillahi HS, Finnie JF, and Van Staden J. (2011). Anti-inflammatory, antioxidant, anti-tyrosinase and phenolic contents of four *Podocarpus* species used in traditional medicine in South Africa. *J. Ethnopharmacology*, 136: 496-503.
7- Carpinella MC, Andrione DG, Ruiz G, and Palacios SM. (2010). Screening for acetylcholinesterase inhibitory activity in plant extracts from Argentina. *Phytother. Res*., 24: 259-63.
8- Kubo I, Himejima M, and Ying bai P. (1991). An antifungal norditerpene dilactone from *Podocarpus nagi*. *Phytochemistry*, 30: 1467-9.
9- Woldemichael GM, Franzblau SG, Zhang F, WangY, and Timmermann BN. (2003). Inhibitory effect of sterols from *Ruprechtia triflora* and diterpenes from *Calceolaria pinnifolia* on the growth of *Mycobacterium tuberculosis*. Planta. Med. 69: 628-31.
10- Abreu MB, Temraz A, Malafronte N, Mujica FG, Duque S and Braca A. (2011). Phenolic derivatives from *Ruprechtia polystachya* and their inhibitory activities on the glucose-6-phosphatase system. *Chem. Biodivers*., 8: 2126-34.
11- Stahlhut R, Park G, Petersen R, Ma W and Hylands P. (1999). The occurrence of the anti-cancer diterpene taxol in *Podocarpus gracilior* Pilger (Podocarpaceae). *Biochem. Syst. Ecol*. 27: 613-22.
12- Mabry TJ, Markham KR and Thomas MB. (1970). "The systematic identification of flavonoids, Springer Verlag". New York, p. 4-35.
13- Stahl E, (1969). "Thin layer chromatography", 2nd ed., Springer Verlag, Berlin, Heidelberg, New York.
14- Brasseur T.; Angenot L.(1986). Le mélange diphenylborate d´aminoéthanol-PEG 400. Un interessant réactif de révélation des flavonoides. *J. Chromatography* 351, 351-5.
15- Hansen MB, Nielsen SE and Berg K. (1985). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods*. 119: 203-10.
16- British Pharmacopea (1993). Her Majesty's Stationary Office, London.
17- Vogel, A. I., (1961). A Text-Book of Practical organic chemistry. Longmans Green and Co.Ltd., London, UK.
18- Ratty, A.K., J. Sunamoto and N.P. Das, (1988). Interaction of flavonols with 1.1-diphenyl-2-picrylhydrazyl free radical. Liposomal membranes and soybean lipoxygenase-1. *Biochem. Pharmacol*. 37: 989-95.
19- ChuYH., Chang CL, and Hsu HF. (2000). Flavonoid content of several vegetables and their antioxidant activity. *J. Sci. Food. Agr*., 80: 561-6.
20- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JK,and Tannenbaum SR. (1982). Analysis of nitrate, nitrite and 15N in biological fluids. *Anal. Biochem*., 126:131-6.
21- National Committee for Clinical Laboratory Standards. (1997). Antimicrobial Susceptibility of Flavobacteria, vol. 41.
22- Barakat HH, Nawwar MA, Buddrus J and Linscheid M, (1987). A phenolic glyceride and two phenolic aldehydes from roots of *Tamarix nilotia*. *Phytochemistry*, 26: 1837-8.
23- Mahmoud II, Marzouk MSA, Moharram FA, El-Gindi MR and Hassan AMK. (2001). Acylated flavonol glycosides from *Eugenia jambolana* leaves. *Phytochemistry*, 58:1239-44.
24- Markham KR, Terani B, Stanley R, Gieger H and Mebry TJ. (1978). Carbon-13 NMR studies of flavonoid glycosides and their acylated derivatives. *Tetrahedron*, 34: 1389-97.
25- Agrawal PK and Bansal MC (1989). Flavonoid glycosides. In: studies in organic chemistry 39, ^{13}CNMR of flavonoids. Agrawal, P.K. Elsevier: New York, p. 283-364.
26- Metwally AM, Omar AA, Harraz FM and Sohafy SM. (2010). Phytochemical and antimicrobial

activity of *Psidium guajava* L. leaves. *Pharmacogn.*, 6: 212-8.
27- Harborne J.B. (1984). Phytochemical methods: A guide to modern technique of plant analysis, 2nd ed, Champan and Hall Ltd, London, UK, p. 37-99.
28- Marzouk MSA, Moharram FA, Haggag EG, Ibrahim MT and Badary OA. (2006). Antioxidant flavonol glycosides from *Schinus molle*. *Phytother. Res.,* 20: 200-5.
29- Kim I, Chin Y, Lim SW, Kim YC and Kim J. (2004). Norisoprenoids and Hepatoprotective flavones glycosides from the Aerial parts of *Beta vulgaris* var. cicla. *Arch. Pharm. Res*., 27: 600-3.
30-Bahia MV, Dos Santos JB, David JP and David JM. (2005). Biflavonoids and other Phenolics from *Caesalpinia pyramidalis* (Fabaceae), *J. Braz. Chem. Soc.,* 16: 1402-5.
31- De Oliveira MCC, De Carvalho Mg, Da Silva CJ and Werle AA. (2002). New Biflavonoid and other constituents from *Luxemburgia nobilis* (EICHL). *J. Braz. Chem. Soc.,* 13: 119-23.
32- Vazquez E, Martinez EM, Cogordan JA and Delgado G. (2001). Triterpenes, phenols, and other constituents from the leaves of *Ochroma pyramidala* (Balsa wood, Bombacaceae). Preferred confirmations of *O-C*-β-D-glucopyranosyl-apigenin (Vitexin). *Rev. Soc. Quím, Méx*., 45: 254-8.
33- Yaming X,and Sheng ding F. (1991). The chemical constiuents from *Podocarpus nagi*. *Acta. Bot. Sin.,* 33: 406-8.
34- Zheng J, Wang N, Fan M, Chen H, Liu H and Yao X. (2007). A new biflavonoid from *Selaginella uncinata*, *Asian J. of Traditional Medicines*, 2: 92-7.
35- Kuo YJ, Hwang SY, Wu MD, Liao CC, Liang YH, Kuo YH, and Ho HO. (2008). Cytotoxic constituents from *Podocarpus fasciculus*. *Chem. Pharm. Bull.,* 56: 585-8.
36- Faiellab L, Temraz A, Siciliano T, De Tommasi N, and Braca A. (2012). Diterpenes, ionol-derived, and flavone glycosides from *Podocarpus elongates*. *Phytochem. Lett*., 76: 172-7.
37- Rizvi SHM, Rahman W, Okigawa M, and Kawano N. (1974). Biflavones from *Podocarpus neriifolius*. *Phytochemistry*, 13: 1990.
38- Xu L, Chen Z, and Sun N. (1993). Chemical composition of *Podocarpus neriifolius*. *Acta. Bot. Sin.,* 35: 138-43.
39- Xua Y, Fang S and HE Q. (1990). The chemical conistituents from *Podocarpus fleuryi*. *Acta. Bot. Sin.,* 32: 302-6.
40- Xub Y and Fang S. (1990). The structure of a new biflavone from *Podocarpus fleuryi*. *Acta. Bot. Sin.,*33: 162-3.
41- Nair AGR, Krishnan S, Ravikrishna C and Madhusudanan KP. (1999). New and rare flavonol glycosides from leaves of *Syzgium samaragense*. *Fitoterapia*, 70: 148-51.
42- Marzouk MSA, Soliman FA, Shehta IA, Rabie M and Fawzy GA. (2004). Biologically active hydrolysable tannins from *Jassiaea repens* L. *Bull. Fac. Pharm. Cairo univ*., 42: 119-31.