Suzy Abd El-Hakeem El-Sherbeni et al /J. Pharm. Sci. & Res. Vol. 12(1), 2020, 138-146

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Assessment of Renoprotective Effect of *Physalis pubescens* L. & *Plumeria acutifolia* Poir: Flavonoids from *P. pubescens* L. Leaves

Suzy Abd El-Hakeem El-Sherbeni[*], Ghada Mohammad Al-Ashmawy[1]

[*]*Pharmacognosy Department, Faculty of Pharmacy, Tanta University, Tanta, El-Gharbia, Egypt.*

[1]*Biochemistry Department, Faculty of Pharmacy, Tanta University,Tanta, El-Gharbia, Egypt.*

Abstract

The research aims are to carry out biologically guided phytochemical study by measuring the renoprotective effect of *Physalis pubescens* L. (Solanaceae) and *Plumeria acutifolia* Poir (Apocynaceae) and to isolate then identify compounds have antioxidant effect from the most effective plant extract. Male rats (50) were divided into 5 groups: control group, gentamycin group, methanol extracts of *P. pubescens* leaves (PPML), *P. acutifolia* leaves (PAML) and *P. pubescens* roots (PPMR) groups. Gentamycin (40 mg/kg) was injected for 9 consecutive days. Rats were administrated the different extracts (250 mg/kg) orally daily concomitantly with gentamycin. The kidney function was evaluated *via* kidney biomarkers, histopathological and immunohistochemical assessment as well as by gene expression of renal iNOS, NF-κB and AMPK-α1 using RT-qPCR. There was no symptoms of systemic toxic effects in any tested group. Serum BUN is decreased by PPML (25 ± 0.5921), PAML (28 ± 0.613), PPMR (28.22 ± 0.587) mg/dL. Serum Creatinine is decreased by PPML (0.562 ± 0.023), PAML (0.737 ± 0.015), PPMR (0.73 ± 0.045) mg/dL. Gene expression of iNOS was downregulated by PPML (0.383 ± 0.02), PAML (0.878 ± 0.027), PPMR (2.078 ± 0.072). NF-κB was decreased by PPML (1.06 ± 0.096), PAML (1.678 ± 0.225), PPMR (1.513 ± 0.226). AMPK-α1 enhanced by PPML (28 ± 0.258), PAML (21.5 ± 0.183), PPMR (17.5 ± 0.408). *Physalis pubescens* L. leaves extract was the most effective. Four flavonoids were isolated for the first time from it. They are identified as 5, 6, 7-trimethoxyflavone (1), myricetin (2), rutin (3) and quercitrin (4).

Keywords: BUN, Creatinine, Flavonoids, Histopathology, Immunohistochemical, RT-qPCR

1. INTRODUCTION:

The increment of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as the decreasing of endogenous antioxidants were accompanied with oxidative stress -related acute kidney injury (AKI). Extracts of medicinal plants can act against oxidative stress-related kidney diseases through decreasing of lipid peroxidation and enhancement of endogenous antioxidants. Therefore, natural products from plants could be good sources of exogenous antioxidants which might be considered the important remedies to decrease pathological alterations in oxidative stress-related acute kidney injury [1].

Gentamycin is a known aminoglycoside antibiotic which is famous to treat gram negative bacterial infections. There were different complications resulted from aminoglycoside toxicity led to common reasons for prolonging hospital stays in the developed world. It was found that about 30% of patients treated with GM for more than 7 days demonstrated some signs of nephrotoxicity. Mechanisms of gentamycin induced nephrotoxicity are tubular, glomerular and vascular. Gentamycin causes tubular damage through necrosis of tubular epithelial cells and alteration of main cellular function, it causes phospholipidosis by binding to membrane phospholipids and alters its function. It is also causing mesangial contraction and reducing glomerular filtration. Besides, it stimulates expression of iNOS and NO production in isolated glomerulus and mesangial cells. The increased vascular resistance by GM toxicity led to decrease in renal blood flow which also decrease glomerular filtration in tubules [2].

The genus *Physalis* contains about 95 species belong to family Solanaceae [3]. They have other names such as gooseberry, ground cherry, little ground cherry and pubescent ground cherry [4].

Physalis pubescens L. is a low-growing, annual herbaceous plant with 5 sepals, united and persistant calyx and has a shallow, fibrous root system [3]. It is native to tropical and subtropical regions. It contains different active constituents of withanolides [5], alkaloids [6], flavonoids and poly phenolic compounds [7]. *Physalis* species have been used in folk medicine for their antispasmodic, diuretic, antiseptic, sedative, and analgesic effects [8]. *P. pubescens* was used in traditional Chinese medicine to treat sore throat, urethritis and orchitis [9, 10]. It has demonstrated antidiabetic properties [11]. It was reported that withanolides exerted anti-inflammatory effect [12].

Plumeria acutifolia Poir is an ornamental shrub or small tree native to central America, leaves are alternate and clustered near ends of branches, flowers have sweet smell with contorted white corolla lobes [3, 4].

2. MATERIALS

2.1. Plant Material: Leaves and roots of *P. pubescens* L. were collected from Shebin el-kanater of Qalyubia governorate. Leaves of *Plumeria acutifolia* Poir were collected from a garden in Tanta, Gharbia governorate. They were collected in May and July, respectively then identified by Prof. Dr. Mohammed Ibrahim Fotoh, professor of ornamental horticulture and landscape design. Voucher specimens (PG00413) and (PG00414) were deposited at Pharmacognosy Department Herbarium of Tanta University. Leaves and roots were

dried to afford two kg powdered leaves, one kg powdered roots of *P. pubescens* L. and one kg *Plumeria acutifolia Poir* leaves. Concentrated methanol extract (95%) was obtained of leaves & roots of *P. pubescens* and leaves of *P. acutifolia*, as three separate extracts. The yield of dry residues of crude extracts was 11.3%, 6.1% and 8.23%, respectively.

2.2. Animals: Carboxy methyl cellulose, methanol 95% and diethyl ether were purchased from Sigma-Aldrich. Gentamicin was obtained from EIPICO (Egypt). Saline was obtained from Al Mottaheddoon Pharma Co. (Egypt). Wistar strain adult male albino rats (150-200 g) were purchased from the animal facility of Giza Institute of Ophthalmology (Cairo, Egypt), fed a standard diet and water *ad libitum* and conditioned to the laboratory conditions for a week before starting the experiment. Temperature was adjusted to 25ºC and humidity of 65 ± 5% with 12 hours of each light & dark cycles. All procedures performed with animals were in compliance with the ethical guidelines of the Research Ethics Committee of Faculty of Pharmacy- Tanta University (REC-FPTU).

2.3. Materials & Instruments for isolation and structure elucidation of active constituents: Methanol, methylene chloride (analytical grade), methanol and water (HPLC grade), UV spectra were recorded on Shimadzu UV/Vis spectrophotometer, UV-1800, Japan. Mass spectra were obtained on Thermo Scientific ISQ Single Quadrupole MS, USA and Thermo Scientific TSQ Quantum Access MAX triple quadrupole system, USA. ^{1}H-NMR and ^{13}C-NMR measurements were obtained using NMR Jeol ECA (500 MHz), Japan and Bruker High performance Digital FT-NMR spectrophotometer AvanceIII (400 MHz), Germany, using solvents as CD_3OD. Column chromatography was performed on silica gel G 60 (70-230 mesh, Merck) and ODS-C18 (Merck). TLC was carried out using pre-coated TLC plates of silica gel 60 F_{254} (Merck) and silica gel 60 RP-18 F_{254} sheets (5 x 7.5 cm, Merck).

3. EVALUATION OF RENOPROTECTIVE EFFECT OF DIFFERENT PLANT EXTRACTS AGAINST GM-INDUCED NEPHROTOXICITY

3.1. Experimental design:

Rats (50) were grouped into 5 groups each of 10 rats. Group I injected with CMC 1% in saline intraperitoneally (-ve control group), Group II injected intraperitoneally with Gentamycin 40 mg/kg body weight [13] (+ve Control group), Groups III, IV & V for extracts of leaves *P. pubescens* (PPML), leaves of *P. acutifolia* (PAML) and roots of *P. pubescens* (PPMR), respectively. These groups were injected with 250 mg/kg body weight of corresponding plant extracts for every day of 9 days. Scarification of rats was performed under ether anesthesia and blood samples were withdrawn from the retro-orbital venous plexus and put in clean and dry test tubes. Blood samples left for 10 minutes to clot and centrifuged at 2500× g for 20 min at 4ºC. The supernatant serum was collected and stored at –80ºC for further determination of creatinine and urea-nitrogen concentration. Then the right kidneys were cut off and preserved in 10% formalin for hematoxylin and eosin staining histological studies. The left kidneys are removed and promptly frozen in liquid nitrogen for studying gene expression of iNOS, NF-κB and AMPK-α1 by RT-qPCR.

3.2. Measurement protocol of kidney biomarkers:

Blood urea nitrogen (BUN) and serum creatinine biomarkers levels were assessed using commercial kits obtained from Biodiagnostic Co. (Egypt). Blood urea nitrogen (BUN) level was analysed colorimetrically with the Urease-Berthlot method [14], while serum creatinine level was determined colorimetrically according to a method described by [15].

3.3. Examination of Kidney tissues histopathology:

Preparation of 3-µm-thick kidney sections was done using a microtome (Leica RM 2135, Germany), after that they were deparaffinized and hydrated in a descending series of ethyl alcohol. Sections were stained with hematoxylin (H) and eosin (E) stains, then dehydrated and clarified with xylene [16]. The slides were then inspected for histological changes using a light microscope (Olympus Electron Microscope, Olympus, Japan) and photographed. A minimum of six fields for each kidney section were examined and classified by the severity of changes (brush border loss, tubular degeneration and glomerular atrophy). An observer who was blinded to the whole experiment determined the severity of the changes. The different treatments which were given to the animals were evaluated using grades of (-: not detected), (+: mild), (++: moderate) and (+++: severe).

3.4. Immunohistochemical study of iNOS:

Kidney tissues in paraffin were deparaffinized in xylene, dehydrated in a graded alcohol series. Incubation is carried out in a solution of 3% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase. Then sections of tissues were incubated with primary antibody for iNOS (Lab Vision, Fremont, CA, USA). The antigen-antibody interaction afforded complex which was visualized by a labelled streptavidin-biotin followed by diaminobenzidine as a chromogen. Blind examination of all sections were performed by a pathologist using a photomicroscope (Leica DM 500, Heerbrugg, Switzerland) with an image analysis system under x200 magnification. Semi-quantitation of iNOS was carried out via histo-score or H-score [17].

3.5. Measurement protocol for gene expression of iNOS, NF-κB and AMPK-α1 by RT-qPCR:

Extraction of total RNA from frozen renal tissues was done using a miRNeasy Mini Kit (Qiagen®, USA) according to the manufacturer's instructions. The concentration and purity of the obtained RNA were detected spectrophotometrically at 260/280 nm by a ScanDrop Nano-volume spectrophotometer (Analytik Jena®, Italy). Reverse transcription of the extracted RNA

into complementary DNA (cDNA) was carried out using Applied Biosystems Inc.®, USA. A SensiFAST™ SYBR No-ROX kit (Bioline, USA) was used to amplify the obtained cDNA as described by the manufacturer. The adjustment of PCR program was done by starting with initial activation for 2 min at 94ºC, followed by 45 cycles (94ºC for 5 sec, 62ºC for 10 sec, and 72ºC for 20 sec) using a Pikoreal 5100 RT-qPCR system (Thermo Fisher Scientific Co., Finland). Reference gene was GAPDH, samples were analysed and normalized according to its level, which was expressed as relative copy number (RCN). Calculation of different threshold cycle (Ct) values of the samples as well as the analysis of transcript levels by $2^{-\Delta\Delta Ct}$ method were both done [18].

Sequences of primers (Invitrogen Co., USA) used for RT-qPCR:

	Forward primer	Reverse primer
iNOS	5'-GATGAGAAGCTGAGGCCCAG-3'	5'-TAGCGCTTCCGACTTTCCTG-3'
NF-κB	5'-GAACTTGTGGGGAAGGACTG-3'	5'-GGGGTTATTGTTGGTCTGGA-3'
AMPK-α1	5'-GGTCCTGGTGGTTTCTGTTG-3'	5'-ATGATGTCAGATGGTGAATT-3'
GAPDH	5'-TGGACCACCCAGCCCAGCAAG-3'	5'-GCCCCTCCTGTTGTTATGGGGT-3'

4. PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOL EXTRACT OF *P. ACUTIFOLIA*: Flavonoids ware detected in methanol extract of *P. acutifolia* leaves, method of detection was reported [19].

5. SEPARATION OF FLAVONOIDS FROM *P. PUBESCENS* LEAVES:

5.1. Method of flavonoids isolation: The total methanol extract 95% of *P. pubescens* leaves was fractionated successively with petroleum ether, methylene chloride, ethyl acetate and *n*-butanol. Methylene chloride fraction (3 gm) was chromatographed on silica gel column and eluted with methylene chloride and increasing polarity with methanol, fractions 31-39 were eluted with methylene chloride: methanol (94:6) and re-chromatographed on another silica gel column. Sub-fractions 5-11 eluted by methylene chloride: methanol (95: 5) afforded compound (1). Fractions (66-78) of the first column eluted with methylene chloride: methanol (91:9) were re-chromatographed using another silica gel column to obtain compound (2) at methylene chloride: methanol (92:8) ratio.

Ethyl acetate fraction (3 gm) was chromatographed on column of silica gel using methylene chloride and methanol as gradual eluting system. Fractions (22-39) eluted at (92:8) were chromatographed on reversed phase ODS-18 column starting with water 100% then decreasing polarity with methanol using water : methanol mixtures of different ratios. Compounds (3) & (4) were afforded in a pure state at 25% & 40% (methanol in water), respectively. Identification was done with spectroscopic analysis of UV, EI-MS, ESI-MS, ^{1}H- and ^{13}C-NMR.

6. STATISTICAL ANALYSIS:

Data were entered and statistically analyzed using the Statistical Package for Social Sciences (SPSS) version 16. Quantitative data were described as means (SEM). They were tested for normality by Shapiro-Wilk test. In the normally distributed variables, one way ANOVA with LSD post-hoc multiple comparisons was used for comparison between groups. "p value ≤ 0.05" was considered to be statistically significant.

7. DECLARATION OF INTEREST:

The authors declare that there is no conflict of interest.

8. FUNDING:

The current research did not receive any special grant from any funding institution or agency.

9. RESULTS:

9.1. Results of renoprotective study of different plant extracts

9.1.2 Effect of plant extracts on kidney biomarker: Figure 1. demonstrate that blood urea nitrogen (BUN) serum level was increased in GM group to 32.33 ± 2.699 compared to control group. It was decreased due to effects of PPML, PAML, and PPMR to 25 ± 0.5921, 28 ± 0.613 and 28.22 ± 0.587, respectively. The decrease in BUN was significant in PPML treated rats when compared to GM group. Creatinine serum level was of GM group 0.70 ± 0.015 which is more than control group. Treated group with PPML decreased creatinine value to 0.562 ± 0.023, but with PAML, and PPMR groups the level was 0.737 ± 0.015 and 0.73 ± 0.045, respectively. The significant decreasing effect was found only in PPML treated group in comparison with other treated groups.

9.1.3 RT-qPCR of iNOS, NF-κB and AMPK α1-subunit gene expression:

Figure 2A. shows that GM significantly ($p<0.05$) increased iNOS gene expression (2.176 ± 0.588) compared to the control. On the other hand, iNOS gene expression in rats treated with PPML and PAML extracts was significantly ($p<0.05$) decreased at value of 0.383 ± 0.02 and 0.878 ± 0.027, respectively, compared to that of GM-treated rats. Treatment with PPMR reduced the gene expression at value of 2.078 ± 0.072.

Figure 2B. shows that GM significantly ($p<0.05$) upregulated NF-κB gene expression at value of 3.208 ± 0.445, which is compared to the control. On the other hand, NF-κB gene expression in rats treated with PPMR extract was decreased at value of 1.513 ± 0.226, which is significant ($p<0.05$) compared with the control rats. The highest significant ($p<0.05$) downregulation of NF-κB gene expression was found in treated rats with PPML (1.06 ± 0.096) compared to GM-treated rats. Treatment with PAML extract non-significantly changed NF-κB gene expression at value of (1.678 ± 0.225), compared to control and GM groups.

Figure 2 C. shows that GM significantly ($p<0.05$) downregulated AMPK α1-subunit gene expression (0.093 ± 0.008) compared to the control group. On the other hand, AMPK α1-subunit gene expressions in rats treated with PPML, PAML and PPMR were significantly upregulated to 28.0 ± 0.258, 21.5 ± 0.183, 17.5 ± 0.408, respectively compared to that in normal control and GM-treated rats. The highest significant increase was found in PPML group.

9.1.4 Role of plant extracts against kidney histopathologic changes induced with gentamicin:

It was demonstrated from control group kidney sections the normal histological structure (Figure 3A). Kidney sections of gentamicin group showed deformed tissues with molecular cells infiltration and interstitial nephritis (Figure 3B). PAML group exhibited moderate protective effect on the affected renal tubules and glomeruli (Figure 3C). Moderate focal tubular epithelium degeneration was found in PPML treated group (Figure 3D). The PPMR group exerted the least protective effect as its kidney section photomicrograph showed focal interstitial nephritis (Figure 3E). Table (1) listed the semi-quantitative evaluation of the histopathological results of the different treated groups.

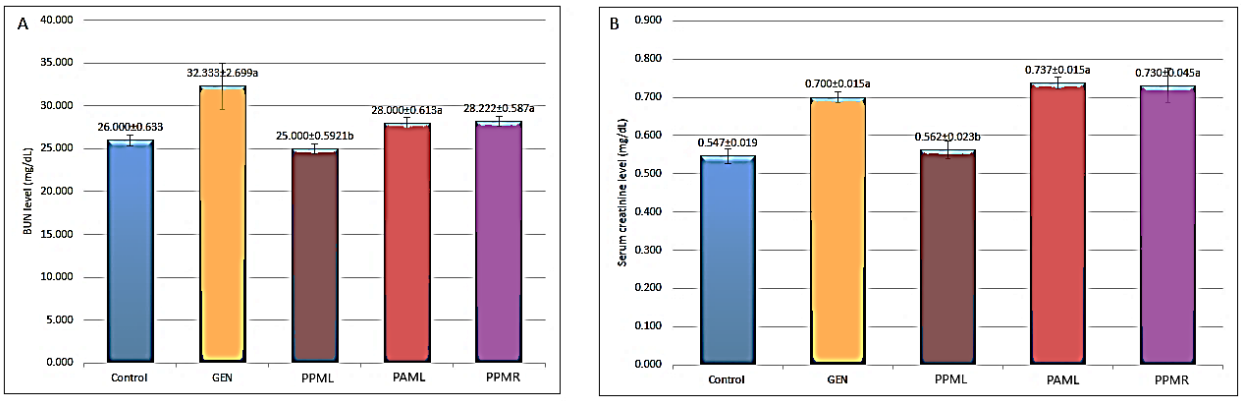

Figure 1. Effect of treatment with methanol extract of *P. pubescens* leaves (PPML), *P. acutifolia* leaves (PAML) and *P. pubescens* roots (PPMR) on (A): Blood urea nitrogen (BUN) and (B): Serum creatinine. Values are mean ± SEM, significance was set at $p<0.05$, $n = 10$ per group. GEN is Gentamicin, a: Significant vs. control group, b: Significant vs. GM group.

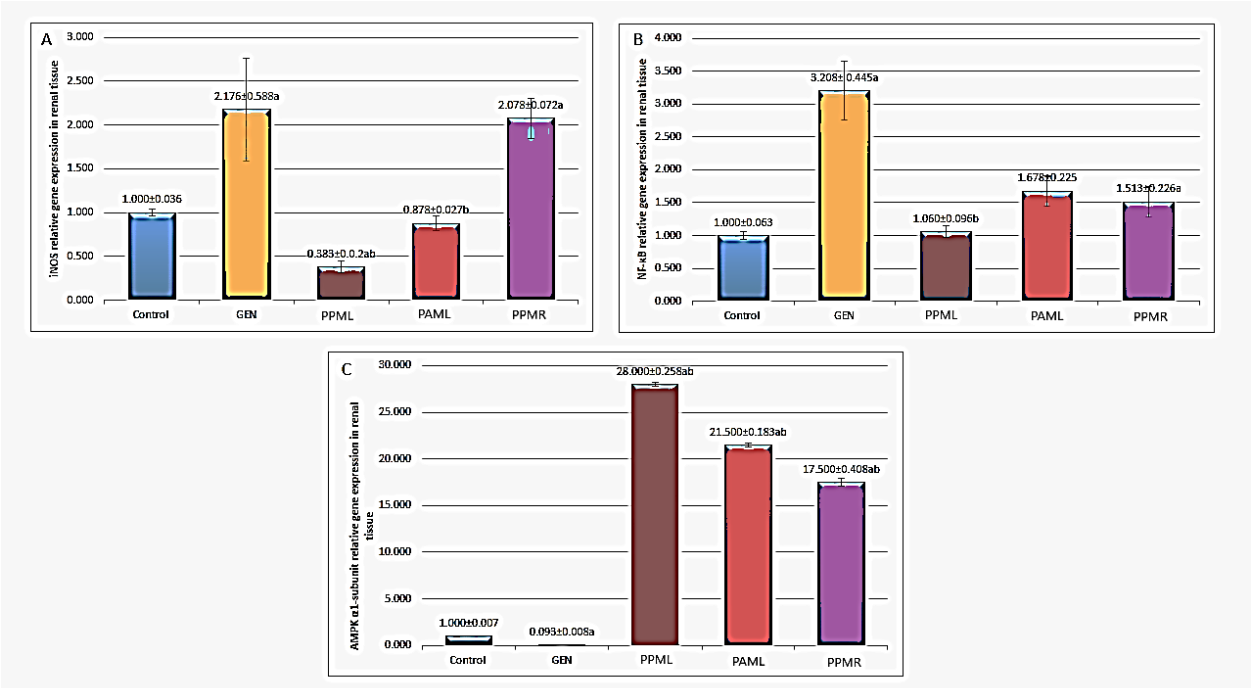

Figure 2. Effect of treatment with leaves of *P. pubescens* (PPML), leaves of *P. acutifolia* (PAML) and roots of *P. pubescens* (PPMR) extracts on (A): Renal iNOS, (B): NF-κB, (C): AMPK α1-subunit gene expression. Values are mean ± SEM, significance was set at $p<0.05$, $n = 10$ per group. GEN is Gentamicin. a: Significant vs. control group, b: Significant vs. GM group.

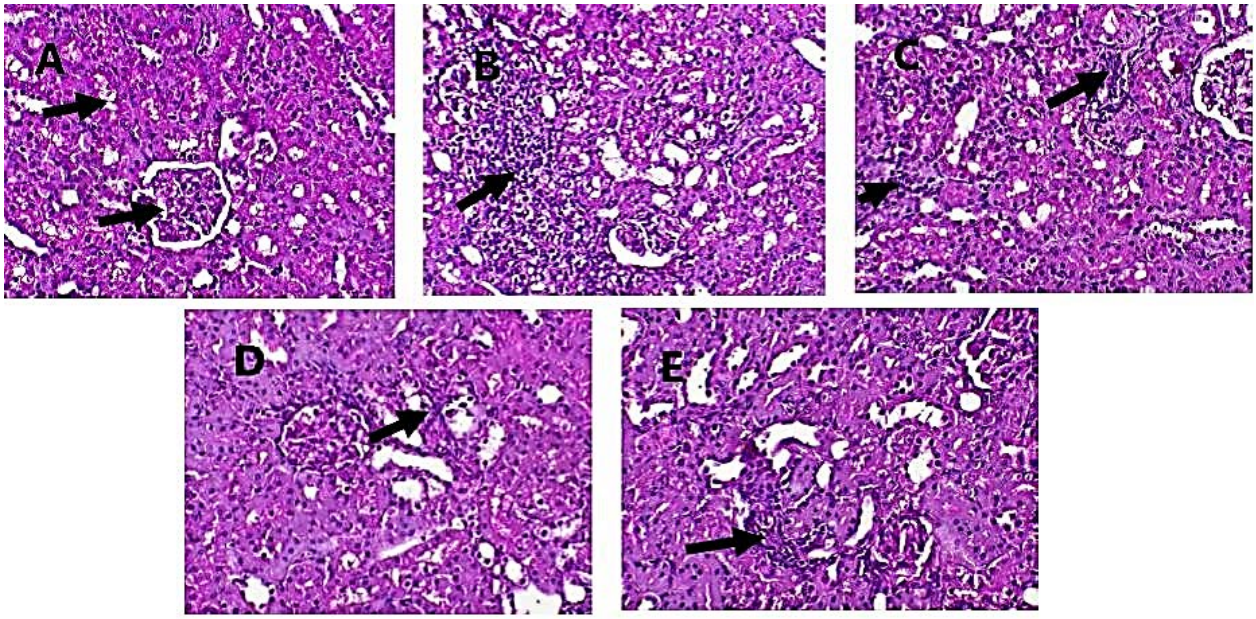

Figure 3. Micrographs of hematoxylin and eosin (H&E) magnified at x200 A. Kidney of control animal showing normal renal glomeruli and tubules (arrow), B. Kidney of diseased animal (not treated) showing interstitial nephritis (arrow indicates mononuclear cells infiltration). C. Kidney of diseased animal treated with *P. acutifolia* leaves extract showing moderate focal interstitial nephritis (arrow) with fibroblastic cell proliferation (arrow). D. Kidney of diseased animal treated with *P. pubescens* leaves extract showing moderate focal renal tubular epithelium degeneration (arrow). E. Kidney of diseased animal treated with *P. pubescens* roots extract showing focal interstitial nephritis (arrow).

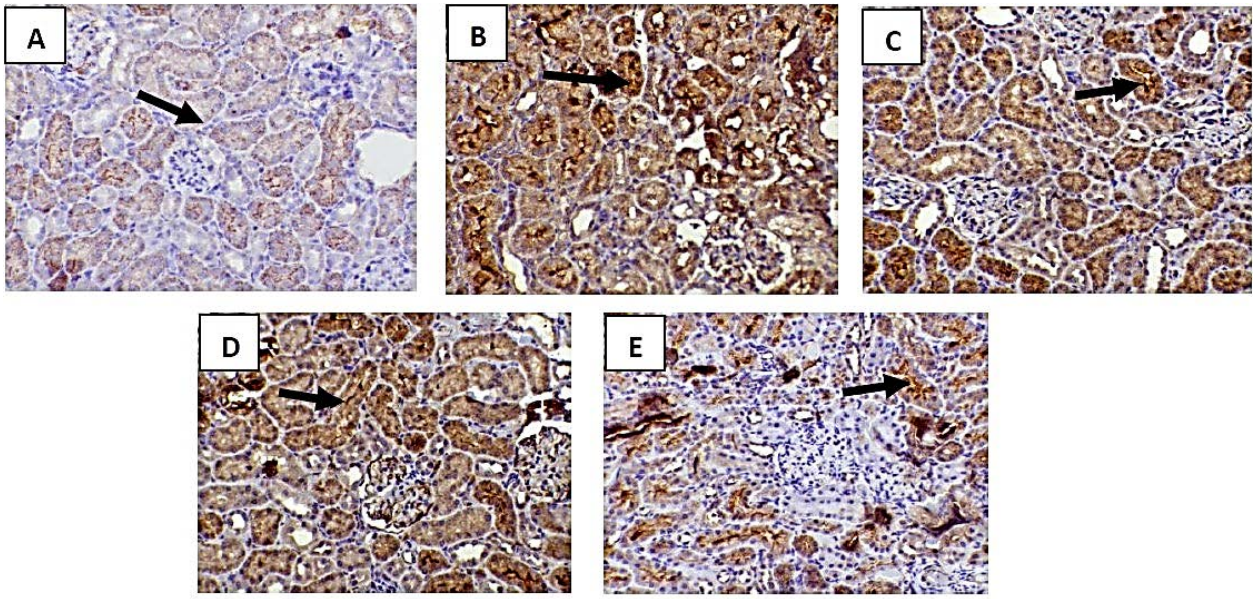

Figure 4. Micrographs of renal sections with immunohistochemical staining of iNOS (magnified at x200). A. Kidney of control animal showing mild expression of iNOS within the renal tubules (arrow). B. Kidney of diseased animal (not treated) showing marked expression of iNOS within the renal tubules (arrow). C. Kidney of diseased animal was treated with *P. acutifolia* leaves showing decrease the expression of iNOS within the renal tubules (arrow). D. Kidney of diseased animal *P. pubescens* leaves extract showing decrease in the expression of iNOS within the renal tubules (arrow). E. Kidney of diseased animal *P. pubescens* roots extract showing decrease in the expression of iNOS within the renal tubules (arrow).

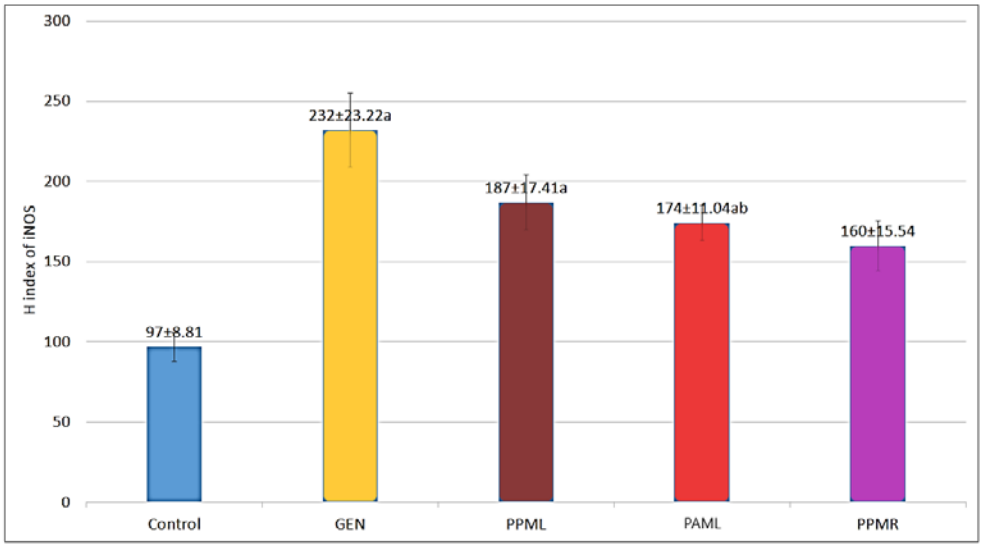

Figure 5. Semiquantitative determination of histochemical score of iNOS of different treated groups. GEN=gentamicin with increased H-score than control group. PPML=group was treated with *P. pubescens* leaves extract, PAML=group was treated with *P. acutifolia* leaves extract and PPMR= group was treated with *P. pubescens* roots extract. a: Significant *vs* control group, b: Significant *vs* GEN group.

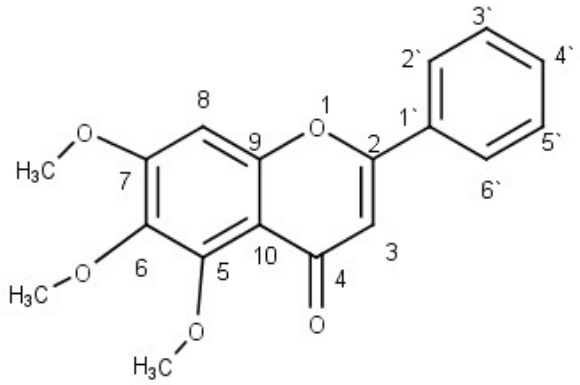

Figure 6. (5, 6, 7-trimethoxy flavone)

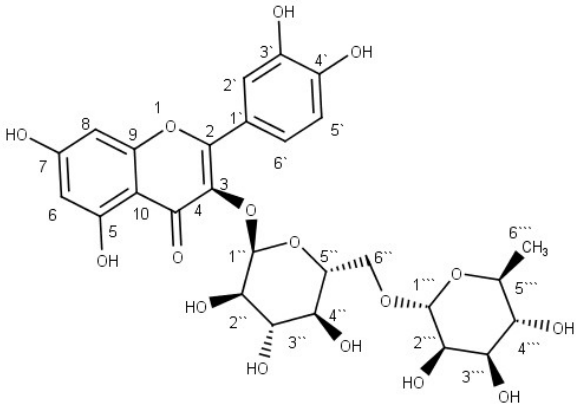

Figure 8. (Rutin)

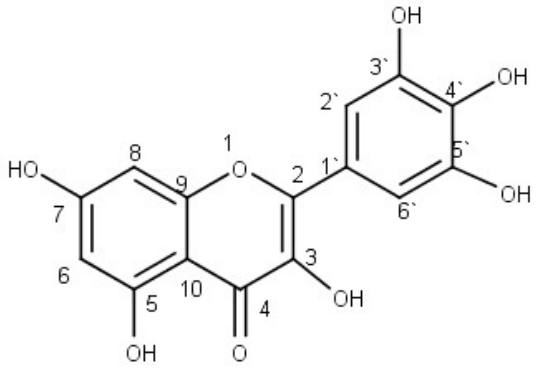

Figure 7. (Myricetin)

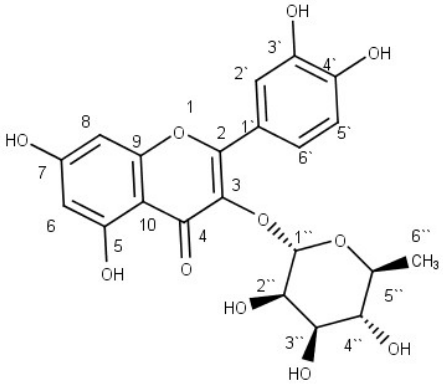

Figure 9. (Quercitrin)

Table 1. Semiquantitative analysis of morphological changes in different groups:

Groups	Negative control group	Gentamycin group	*P. pubescens* leaves	*P. acutifolia* leaves	*P. pubescens* roots
Renal degeneration& necrosis	-	+++	++	++	++
Tubular regeneration	-	+	++	++	++
Inflammation	-	++++	++	++	++
Fibrosis	-	+++	++	++	++
Extent of lesions	-	+++	++	++	++

(-) means no detectable lesions; (+) indicates mild lesions; (++) indicates moderate lesions; (+++) indicates severe focal lesions; (++++) indicates severe diffuse lesions.

9.1.5 Effect of plant extracts on immunohistochemical staining of iNOS:

It was noticed the normal iNOS staining of the control group (Figure 4A) against the increased expression in sections of GM treated group (Figure 4B). Degree of staining of kidney sections of PAML, PPML and PPMR groups showed decreased iNOS expression in Figures 4C, 4D and 4E, respectively. Figure 5 shows diagram of histochemical-score of iNOS. It was demonstrated that treatment with PPML, PAML and PPMR showed decreased values at 187 ± 17.41, 174 ± 11.04 and 160 ± 15.54, respectively compared to GM group (232 ± 23.22). PPML and PAML showed significant decrease compared to control group.

9.2 Results of structure elucidation of isolated compounds

9.2.1 Compound 1

White powder; UV λ_{max} (MeOH): 286 nm; +NaOH: 239 and 279 nm; EI-MS *m/z* 312.33 $[M]^+$; ^{1}H-NMR (400 MHz, CD_3OD): *δ*H 6.73 (1H, s, H-8), 7.13 (1H, s, H-3), 7.58 (3H, m, H-3',4', 5'), 8.018 (2H, dd, *J* = 7.4 Hz, 1.6 Hz, H-2`, H-6`), 3.88 (3H, s, 5-O-CH_3), 3.94 (3H, s, 6- O-CH_3), 4.03 (3H, s, 7- O-CH_3); ^{13}C-NMR (100 MHz, CD_3OD): *δ*C 162.2 (2), 111.8 (3), 178.1 (4), 151.9 (5), 158.7 (6), 154.8 (7), 96.5 (8), 140.5 (9), 106.9 (10), 131.1 (1`), 125.8 (2`, 6`), 128.8 (3`, 5`), 131.4 (4`), 55.6 (5-O-CH_3), 60.4 (6-O- CH_3), 61.2 (7-O- CH_3).

9.2.2 Compound 2

Yellow powder; UV λ_{max} (MeOH): 254, 373 nm; EI-MS displayed $[M]^+$ at *m/z* 319.24, ^{1}H-NMR (400 MHz, CD_3OD): *δ*H 7.36 (2H, s, H-2′, 6′), 6.39 (1H, d, *J* = 2 Hz, H-8), 6.19 (1H, d, *J* = 2 Hz, H-6); ^{13}C-NMR: (100 MHz, CD_3OD): *δ*C 146.6 (2), 135.9 (3), 175.8 (4), 161.0 (5), 97.8 (6), 164.2 (7), 92.9 (8), 156.8 (9), 103.0 (10), 121.6 (1`), 107.1 (2`,6`), 145.3 (3`,5`), 135.5 (4`).

9.2.3 Compound 3

Yellow powder, UV λ_{max} (MeOH): 227, 258, 359 nm positive ESI-MS displayed $[M+H]^+$ at *m/z* 611.1, ^{1}H-NMR (400 MHz, CD_3OD): *δ*H 6.22 (1H, d, brs, H-6), 6.41 (1H, d, brs, H-8), 7.68 (1H, d, brs, H-2′), 6.88 (1H, d, *J* = 8.4 Hz, H-5′), 7.662 (1H, dd, brs, *J*= 8.4 Hz, H-6′), 5.13 (1H, d, *J* = 7.6 Hz, Glc-H-1″), 4.54 (1H, brs, Rha-H-1″), 1.13 (3H, d, *J* = 6 Hz, Rha-CH_3); ^{13}C-NMR (100 MHz, CD_3OD): δC 157.0 (2), 134.2 (3), 178.0 (4), 161.5 (5), 98.5 (6), 164.6 (7), 93.4 (8), 157.9 (9), 104.2 (10), 122.1 (1`), 114.6 (2`), 144.4 (3`), 148.4 (4`), 116.3 (5`), 121.7 (6`), 101.0 (1``), 74.3 (2``), 76.7 (3``), 72.5 (4``), 75.8 (5``), 67.1 (6``), 103.3 (1```), 70.0 (2```), 70.7 (3```), 70.8 (4```), 68.3 (5```) , 16.4 (6```).

9.2.4 Compound 4

Yellow powder; UV λ_{max} (MeOH): 228, 260, 357 nm; positive ESI-MS displayed $[M+H]^+$ at *m/z* 449.07, ^{1}H-NMR (500 MHz, CD_3OD): *δ*H 6.18 (1H, d, *J*= 1.8 Hz, H-6), 6.36 (1H, d, *J*= 1.8 Hz, H-8), 7.32 (1H, s, *J*=2.2 Hz, H-2'), 6.87 (1H, d, *J*= 8.3Hz, H-5`), 7.29 (1H, dd, *J*=8.3, 2.2 Hz, H-6`), 5.32 (brs, H-1``), 4.18 (1H, s, *J*=3 Hz, H-2``) 3.70 (1H, dd, *J*=9.2, 3 Hz, H-3``), 3.41 (m, H-4``), 3.36 (m, H-5``), 0.91 (3H, d, *J*=6 Hz, H-6``); ^{13}C-NMR (125 MHz, CD_3OD): δC 157.1(2), 134.2 (3), 178.1 (4), 161.2 (5), 98.5 (6), 164.0 (7), 93.4 (8), 157.0 (9), 104.1 (10), 120.0 (1`), 115.1 (2`),145.0 (3`), 148.1 (4`), 115.5 (5`), 121.6 (6`), 102.0 (1``), 70.3 (2``), 70.5 (3``), 71.9 (4``), 70.5 (5``), 16.0 (6``).

10. DISCUSSION

This study focused on the ability of plant extracts to ameliorate the acute nephrotoxic effect of gentamycin toxicity in rats and using the biologically guided phytochemical screening to determine the most effective extract, from which separation of effective natural products was carried out.

It was found from the renoprotective evaluation of different extracts that *P. pubescens* leaves extract exerted the best effect at all levels of assessments with moderate effect of *P. acutifolia* leaves extract. The biomarker study of BUN and serum creatinine levels showed a significant decrease of their levels by PPML extract according to negative control group.

The histopathological findings exhibited normal kidney tissues in control group, damaged tissues with gentamycin treatment (inflammatory cell infiltration, degeneration and necrosis) and lesser degree of kidney damage in case of treated groups with plant extracts. The study of kidney histopathology of control group demonstrated normal renal glomeruli with intact bowman capsule and normal brush bordered epithelium cells were lining the proximal and distal convoluted tubules. Gentamycin group showed cells degeneration, granular deposits in lumens of renal tubules, mononuclear cells infiltration, interstitial nephritis and necrosis. Plant extracts of *P. pubescens* leaves and *P. acutifolia* leaves could protect kidney tissues of the treated groups against gentamycin damage by ameliorating the previously mentioned effects. The treated groups exerted

moderate focal interstitial nephritis with fibroblastic cell proliferation and moderate focal renal tubular epithelium degeneration. *P. pubescens* roots extract almost had not protective effect on kidney tissues showing focal interstitial nephritis.

Inducible nitric oxide synthase (iNOS) is used to measure degree of oxidative stress in kidney tissues from the brown color produced due to positive staining of it. The immunohistochemical study of iNOS level in kidney tissues of different groups revealed normal mild expression of iNOS in renal tubules of control group, increased expression of iNOS in GM group and decreased iNOS expression of treated groups with plant extracts. These effects was demonstrated with H-score of iNOS in different treated, control, gentamicin groups.

Kidney gene expression of iNOS, NF-κB and AMPK-α1, using RT-qPCR in different groups which were normalized to reference gene level GAPDH, was performed. Calculation of different threshold cycle (Ct) values of the samples as well as the analysis of transcript levels by $2^{-\Delta\Delta Ct}$ method were both done which revealed that *P. pubescens* leaves exhibit significant effect which was the best effect.

P. pubescens leaves showed the most significant renoprotective effect that make it the plant of choice to carry out phytochemical investigation for its active constituents which led to separation of flavonoids. Flavonoids are natural products with antioxidant effect which can act against oxidative stress exerted by gentamycin. Separation and structural elucidation of these flavonoids were performed.

Compound 1: The UV analysis of compound (I) showed single peak at λ_{max} 286 nm in methanol. NaOH was added to compound (1) and only two peaks at λ_{max} 239 and 279 nm were recorded. It didn't exhibit UV shift with different UV shift reagent: $AlCl_3$, $AlCl_3$/HCl and NaOAc-NaOAc/boric acid mixture. As well as there was no bathochromic shift occurred with NaOH, which indicates the lack of free OH group.

^{1}H-NMR spectral analysis revealed presence of multiplet signal assigned at 7.58 ppm of aromatic protons (H-3`, 4` & 5`). The doublet of douplet signals of meta coupled aromatic protons (H-2`, 6`) resonated at 8.01 ppm with *J*=7.4 of ortho coupled (H-2`, 3` and 6`, 5`) and 1.6 Hz of meta coupled (H-2`, 4` and 6`, 4`). The signals resonating at 6.73 & 7.13 ppm as singlets of (H-8 and 3), respectively. Signals of O-CH_3 protons were resonating as singlets at 3.88, 3.94 and 4.03 ppm. ^{13}C-NMR spectrum provide us with more structural information. The signal resonating at 178.1 ppm is ascribed to carbonyl group at C-4. Signals observed at 151.9, 158.7 and 154.8 ppm can be assigned to C- 5, 6 and 7 respectively, due to deshielding posed by methoxyl groups on the carbons. The carbons of aromatic rings were traced at 96.59, 131.1, 125.8, 128.8 and 133.4 ppm which ascribed for C-8, 1`, 2`, 3` and 4`, respectively due to anisotropic effect of aromatic ring. C-2`, 6` are chemically equivalent as well as 3`, 5` were resonating at 125.8 and 128.8 ppm, respectively. Carbons of 5, 6, and 7-O-CH_3 resonated at 55.67, 60.41 and 61.21, respectively.

EI-MS spectrum of compound (1) showed $[M]^+$ peak at m/z= 312.33, which further confirmed the proposed structure. Based on the above mentioned data which were compared with the published spectral data compound (1) was identified as 5,6,7-trimethoxy-2-phenyl-4H-chromen-4-one or Baicalein 5,6,7-trimethyl ether (Figure 6) [20, 21, 22]. This is the first report to isolate this compound from *P. pubescens*.

Compound 2: It introduced a simple ^{1}H-NMR spectrum. Signal resonating at 7.36 ppm for H-2` and 6` as singlet and other signals in aromatic region of chemical shift resonating at 6.19 and 6.39 ppm for H-6 and 8, respectively, with absence of any other signals in this region.

^{13}C-NMR spectrum showed signal at δ 175.8 ppm assigned to carbonyl carbon at C-4, and downfield signals at 161.0, 164.2, 135.5, 145.3, 146.6, 156.8 ppm assigned to C-5, 7, 4`, 3`, 5`, 2, 9, respectively, which attached to oxygen atom. The chemically equivalent carbons of 2` and 6` gave rise to a signal at 107.1 ppm also 3` and 5` are resonating as one signal at 145.3 ppm. The structure was finally confirmed by $[M]^+$ peak at m/z 319.24 in EI-MS. The aforementioned data for compound (2) was identical with those reported for myricetin; Cannabiscetin; 3,5,7-Trihydroxy-2-(3`,4`,5`-trihydroxyphenyl)-4H-chromen-4-one (Figure 7) [23]

Compound 3: The ^{1}H-NMR data showed meta coupled douplets at 6.22 and 6.41 ppm each integrated for 1 proton, assigned to H-6 and H-8 respectively. Signals at 7.68 and 7.66 ppm integrated for two protons was assigned to H-2`, 6` confirming 3`, 4` dihydroxylation on ring B. Anomeric protons resonating at 5.13, 4.54 ppm for H-1 of glucose and rhamnose, respectively. ^{13}C-NMR spectral analysis showed a characteristic downfield signal at 178.0 ppm for C-4 (carbonyl group), it also showed downfield signals due to conjugation, electronegative and anisotropic effects at 164.6, 157.9, 161.5 and 157.0 ppm for aromatic carbons of C-7, 9, 5 and 2 respectively. The upfield signal at 16.48 ppm of C-6``` of rhamnose was noticed with signals at 101.0 and 103.3 ppm for acetal carbon of C-1`` of glucose and C-1``` of rhamnose. From the above mentioned data compound (3) was identified as 3,3`,4`,5,7-pentahydroxyflavone 3-rutinoside. The positive ESI-MS demonstrated $[M+H]^+$ peak at m/z 611.1. Structure confirmation was done by comparing these data to those published in literature. This compound is 3,3`,4`,5,7-pentahydroxyflavone 3-rutinoside (rutin) (Figure 8) [24].

Compound 4: Spectroscopic data including ^{1}H-NMR showed signal resonating at 7.29 ppm for H-6` as doublet of doublet due to meta coupling with H-2` (*J*= 2.2 Hz) and ortho coupling with H-5` (*J*= 8.3 Hz). There were two doublets resonating at (7.32 and 6.87 ppm) for H-2` and 5`, respectively with *J*= 2.2 Hz for meta coupling in case of H-2` and *J*= 8.3 Hz for ortho coupling in case of H-5`. The meta coupled protons H-6, 8 had their doublet signals at 6.18 and 6.36 ppm, respectively. The anomeric proton of rhamnose resonating at 5.32, while the methyl group at C-6 appeared as a doublet signal at 0.91 ppm.

^{13}C-NMR spectrum showed signal at 178.1 ppm assigned to carbonyl carbon at C-4, and downfield signals at δ 145.0, 148.1, 157.1, 157.0, 161.2 and 164.0 ppm, which were assigned to carbons attached to oxygen atom of C-3`, 4`, 2, 9, 5, 7, respectively. The anomeric carbon of sugar moiety was resonating at δ 102.0 ppm, and the other signals of C- 2``, 3``, 4``, 5``, 6`` were all consistent with rhamnose moiety with the signal at 16.0 ppm assigned for methyl group of the deoxy sugar.

The structure was finally confirmed by $[M+H]^+$ peak at m/z 449.07 in +ESI-MS. The aforementioned physical, chemical and spectral data for compound (4) was identical with those reported for quercetin-3-*O*-α-L-rhamnoside (quercitrin) (Figure 9) [25]. Compounds (1-4) were isolated for the first time from *Physalis pubscens* leaves.

Flavonoid compounds consist of large family of natural products with very important role in our lives as they are excellent antioxidant agents and can act against reactive oxygen species (ROS) *in vivo* and *in vitro*. They could be considered as nutraceuticals to offer protection against different diseases. It was reported that quercitrin is a bioflavonoid which has protective effect with antioxidant and scavenger role and could inhibit lipid peroxidation [25]. Myricetin was studied in the past years extensively to show various protective effects, besides it exerts a strong antioxidant, anti-inflammatory anticancer and antidiabetic also it prevents lipid peroxidation [26]. Rutin is a vital phytochemical composed of poly hydroxy flavonoidal glycoside with important biological effects and wide distribution in plants that are used medicinally and as food. It showed potent antioxidant effect which is the answer key for its multiple pharmacological effects [27, 28]. ‘]

11. CONCLUSION:

It was found that the *P. pubescens* leaves extract exerts the best significant nephroprotective effect against gentamycin induced nephrotoxicity in rats. Natural products as flavonoids could ameliorate this toxic effect which were separated from this plant for the first time. They were identified as 5, 6, 7-trimethoxy flavone, myricetin, rutin and quercitrin. On the other hand *P. acutifolia* leaves extract showed moderate nephroprotective effect while roots extract of *P. pubescens* demonstrated weak effect.

REFERENCES:

[1] Palipoch, S., *Afr. J. Tradit. Complement. Altern. Med.* 2013, *10*, 88–93.

[2] Randjelovi, P., Veljkovic, S., Stojiljkovic, N., Sokolovic, D., Ilic, I., *EXCLI J.* 2017, *16* 388–399

[3] Singh, G., *Plant Systematics: An Integrated Approach*, Third ed. Sciense Publishers, Enfield, NH, USA. 2010.

[4] Quattrocchi, U., *CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms and Etymology.* Boca Raton, London, New York.: CRC press. 2012.

[5] Xia, G., Li, Y., Sun, J., Wang, L., Tang, X., Lin, B., Kang, N., Huang, J., Chen, L., Qiu, F., *Steroids.* 2016, *115*,136–146.

[6] Basey, K., Mcgaw, B. A., Woolley, J. G., *Phytohemistry.* 1992, *31*, 4173-4176.

[7] Luo L. P., Cheng F. Q., Ji L., Yu, H. Y., *Zhongguo Zhong Yao Za Zhi*. *40*, 2015, 4424–7.

[8] Perry, L. M., *Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses*. The MIT Press, Cambridge, Ma., 1980.

[9] Yang, M., Chen, Z., Li, X.-R., Xu, Q.-M., Yang S.-L., *Chin. Trad. Herbal Drugs*, *44*, 2013, 253–256.

[10] Jia, Y. -M., Chen, Z., Xu, Q.-M., Li, X.-R., Yang, S.-L., *Chin. Trad. Herbal Drugs. 44*, 2013, 1086–1090.

[11] Hassan, A. I., Ghoneim, M. A. M. , *World Appl. Sci. J. 21*, 2013, 681–688.

[12] Yang, B. Y., Guo, R., Li, T., Wu, J. J., Zhang, J., Liu, Y., Wang, Q. H., Kuang, H. X., *Steroids*. *87*, 2014, 26–34.

[13] El-Ashmawy, N. E., Khedr, N. F., El-Bahrawy, H. A., Helal, S. A., *Biomed. Pharmacother. 99*, 2018, 504–510.

[14] J. K. Fawcett, J. K., Scott, J. E., *J. Clin. Pathol. 13*, 1960, 156–159.

[15] Slot, C., *Scand. J. Clin. Lab. Invest., 17*, 1965, 381–387.

[16] Anan, H. H., Zidan, R. A., Shaheen, M. A., Abd-El Fattah, E. A., *Cytotherapy*, *18*, 2016, 970–84.

[17] Samaka, R. M., Bakry, O. A., Shoeib, M. A. E. M., Zaaza, M. M., *Anal Quant Cytol. 36*, 2014, 245–257.

[18] Rao, X., Huang, X., Zhou, Z., Lin, X., *Biostat. Bioinforma. Biomath. 3*, 2013, 71–85.

[19] Giessman, T. A., *The Chemistry of Flavonoid Compounds*. New York: The Macmillan Company, 1962.

[20] Koh, L. L., Ng, A. S., *Acta Cryst.* C*49*, 1993,105-107.

[21] Wei, X.-H., Yang, S.-J., Liang, N., Hu, D.-Y., Jin, L.-H., Xue, W., Yang, S., *Molecules. 18*, 2013, 1325–1336.

[22] Pegg, R. B., Amarowicz, R., Oszmiañski, J., *POl. J. FOOD Nutr. Sci. Pol. J. Food Nutr. Sci*, 55, 2005, 43–50

[23] Yang, M. H., Kong, L. Y., *Chem. Nat. Compd. 44*, 2008, 98–99.

[24] SU, C., Yang, W.-Q, Jiang, D., Zhang, X., Zheng, J., Shi, S., Tu, P.-F., *Chin. Tradit. Herbal Drugs. 46*, 2015, 2034–2039.

[25] Wagner, C., Fachinetto, R., Dalla Corte, C. L., Brito, V. B., Severo, D., de Oliveira Costa Dias, G., , Morel, A. F., Nogueira, C. W., Rocha, J. B., *Brain Res. 1107*, 2006, 192–198.

[26] Semwal, D. K., Semwal, R. B., Combrinck, S., Viljoen, A., *Nutrients*, *8*, 2016, 1–31.

[27] Ganeshpurkar, A., Saluja, A. K., *Saudi Pharm. J.*, *25*, 2017, 149–164.

[28] Wang, B., Liu, D., Zhu, Q. H., Li, M., Chen, H., Guo, Y., Fan, L. P., Yue, L. S., Li, L. Y., Zhao, M., *Int. Immunopharmacol*., *35*, 2016, 77–84.