Evaluation of the Anti-diabetic Activity and Lc-esi/ltqorbitrap/ms/ms Profiling of an Aqueous Extract of Pergularia Tomentosa L. Aerial Parts

seyeh Hamzeh Hosseini (Hamze@ujiroft.ac.ir)
University of Jiroft https://orcid.org/0000-0001-5736-3804
Fereshteh Ezzati Ghadi
University of Jiroft
Abdollah Ramzani Ghara
University of Jiroft
Antonietta Cerulli
University of Salerno: Universita degli Studi di Salerno
Sonia Piacente
University of Salerno: Universita degli Studi di Salerno

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Abstract

Background*: Pergularia tomentosa* L. is a milkweed tropical plant. In the Middle Est, the aerial parts of *P. tomentosa* are traditionally used as an anti-diabetic remedy. In order to find scientific evidence for the traditional use of this plant, the effect of an aqueous extract of *P. tomentosa* aerial parts by *in vivo* assay was investigated. Moreover, to achieve deeper insight into the chemical composition of the above-mentioned extract an analytical approach based on LC-ESI/LTQOrbitrap/MS/MS^n was evaluated. Wistar rats were fasted overnights and diabetes mellitus induced using streptozotocin (50 mg/kg body weight). The rats were randomly and equally divided into four groups (n=5): group I (normoglycaemic control), group II (rats treated with streptozotocin 50 mg/kg body weight), group III (*P. tomentosa* extract 200 mg/kg body weight), group IV (normoglycaemic treated with *P. tomentosa* extract, 200 mg/kg body weight). At the end of the treatment period, they were anesthetized under diethylether. Vital organs (kidneys, liver and pancreas) and blood samples were obtained for histopathological, biochemical and haematological analysis.

Results*: P. tomentosa* showed a reasonable reduction in blood glucose level. Probably, *P. tomentosa* effect on hyperglycemic and hyperlipidemic diabetic animals was associated to antioxidant properties, triglyceride levels, as well as the liver enzymes. Furthermore, the metabolite profile of the aqueous extract of *P. tomentosa* obtained by LC-ESI/LTQOrbitrap/MS/MS^n highlighted the presence of double-linked cardenolides along with cardenolides and flavone glycosides.

Conclusion*: P. tomentosa* decreased the glucose level and induced beneficial effect on lipid profile. This study confirms the capability of cardenolide glycosides to decrease the level of glucose in the blood.

Background

Diabetes is a metabolic disorder characterized by persistent hyperglycemia and dysfunctional metabolism of carbohydrates, fats and protein metabolism [1, 2]. World Health Organization (WHO) has classified this pathology as type-1 diabetes (β-cell destruction), type-2 diabetes (insulin resistance with insulin hypo-secretion), gestational diabetes and other specific kinds of diabetes including genetic defect on β-cell-function, disease of the pancreas and chemical/drug-induced diabetes. In particular, in type 2 diabetes mellitus, the body cells do not use insulin effectively and consequently the glucose level goes up. Such a deficiency results in increased concentration of glucose in the blood, which in turn damages many of the body's systems, in particular, the blood vessels and nerves [3].

Diabetic disease could lead to several complications such as oxidative stress, hyperlipidemia and enzymatic glycation of protein [4, 5].

The pharmacological agents, used currently for the treatment of diabetes mellitus include mainly oral anti-diabetic drugs as well as insulin subcutaneous therapy. These treatments are used as mono-therapy or in different combinations to control the diabetic condition. Some issues, however, limit the effectiveness of these options, such as failure to hinder diabetic complications and prominent side

effects [6, 7]. WHO reported that 366 million people would be affected by type 2 diabetes mellitus until 2030 increasing the risk of morbidity and mortality due to cardiovascular disease [8]. Therefore, there is an increasing interest in investigating medicinal plants recognized in Traditional Medicine as a remedy to treat diabetes.

Some plants of Asclepiadaceae family, *Calotropis procera* [9] and *Pergularia daemia* [10], have been reported to exert *in vivo* anti-diabetic activity.

Pergularia tomentosa L. is a wild perennial shrub 50 cm high, native species of the Middle East and North of Africa, known in Iran as Labashir or Keshtuk. *Pergularia tomentosa* displays a lot of secondary metabolites responsibles for the biological activities shown by the different parts of the plant. Previous investigations on *P. tomentosa* highlighted the occurrence of cardiac glycosides including desglucouzarin, coroglaucigenin, and uzarigenin in the leaves, [7, 11]; uzarigenin, ghalakinoside, calactin, 6'-hydroxycalactin, 6'-hydroxy-16α-acetoxycalactin, 16α-hydroxycalactin, 12'-dehydroxyghalakinoside, 3-*O*-*β*-glucopyranosiylcalactin, and 6'-dehydroxyghalakinoside in the roots [12, 13]. Moreover, our recent publications reported the isolation and identification of double-linked cardenolides and flavonol glycosides from the aerial parts of *P. tomentosa* [14, 15]. Chemically, cardiac glycosides are compounds characterized by a steroidal nucleus with a lactone moiety at position C-17 which leads to the chemical classification of subfamilies such as cardenolides or bufadienolides with an unsaturated butyrolactone and α-pyrone ring, respectively; the steroidal skeleton links at position 3 a sugar chain. In cardiac glycosides produced by plants of Asclepiadaceae family, the A/B rings are *trans* fused, resulting thus in rather flat structures. The above mentioned fusion gives to the aglycon nucleus of these cardiac glycosides a typical “U” shape, resulting in a markedly more potent binding to Na^+/K^+-ATPase pump (particularly to Na^+/K^+-ATPase α1 subunits) [13]. Different parts of *P. tomentosa* have been reported to exert molluscicidal activity (16), to cause apoptotic cell death of Kaposi’s sarcoma cells [12], to prevent bronchitis, constipation and skin diseases [7, 17, 18], to exert hypoglycemic effects.

In order to evaluate the hypoglycemic activity of an aqueous extract of the aerial parts of *Pergularia tomentosa* L., *in vivo* hypoglycemic assays have been carried out. Moreover, the level of alanine and aspartate transaminase (ALT and AST), and lipid peroxidation (MDA) in the serum of rats as well as histopathological studies have been performed.

With the aim to correlate the hypoglycemic activity to the chemical composition, the metabolite profile of the aqueous extract of *Pergularia tomentosa* aerial parts has been evaluated by high-performance liquid chromatography coupled to electrospray negative ionization Orbitrap multicollisional high resolution mass spectrometry (HPLC-ESIOrbitrap MS) [19, 20]. In particular, LTQ (Orbitrap) MS analyzer was used since it has MS^n capabilities for enhanced levels of structural analysis. By this way, a wide range of cardenolides along with a minor rate of flavonoids, have been obtained.

Material And Methods

Chemicals

Streptozotocin (STZ) was purchased from Sigma, ALT, AST, High Density Lipoproteins (HDL), Low Density Lipoprotein (LDL) test kits were purchased from Pars Azmoon Co., Iran. All other chemicals and reagents used were of analytical grade. Acetonitrile, formic acid, and water for LC-MS were bought Merck (Merck KGaA, Darmstadt, Germany).

General Procedures

HRESIMS spectra were carried out by an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) operating in negative ion mode. The Orbitrap mass analyzer was calibrated according to the manufacturer's directions using a mixture of caffeine, methionine-arginine-phenylalanine-alanine-acetate (MRFA), sodium dodecyl sulfate, sodium taurocholate and Ultramark 1621. Data were collected and analyzed using the software (Xcalibur) provided by the manufacturer [14, 15].

Plant Material

Fresh aerial parts of *P. tomentosa* L. were collected in Kahnouj, Kerman province, Iran, in October 2019 and identified by Mr. Ahmad Pormirzaee. Plant material was air-dried and stored at room temperature. Voucher specimen (no. 8644) was deposited at the Herbarium of the Kerman Agricultural & Natural Resources Research & Education Center, Kerman, Iran.

Extraction

The aerial parts of *P. tomentosa* (500 g) were extracted using distilled water (2.000 L x 24 h x three times) at room temperature. After filtration and evaporation of the solvent to dryness in vacuo, 7.00 g of crude MeOH extract were obtained.

Animals

Experiments were performed on twenty male Wistar rats weighting 180–220 g (Pasteur Institute of Kerman, Iran).

All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines.

Acute toxicity study

Determination of acute toxicity of the extract was performed by using the OECD (Organization of Economic Co-operation and Development) guidelines for testing chemicals (Acute Oral Toxicity-Sect. 423). To three male Wistar rats, single doses (200 mg/kg body weight) of an aqueous extract of *P. tomentosa* aerial parts were administered orally, after overnight fasting. Rats were observed for symptoms and weight at post-administration interval of 1, 3, and 4h and then twice per day for the subsequent 14 days [21].

Induction of diabetes

Animals were housed with a 12h light-dark cycle with free access to food and water. Intraperitoneal injection of STZ (50 mg/kg body weight) in 0.1 M citrate buffer (pH 4.5) was performed to induce diabetes mellitus (type I). Rats were fasted for 12–14 h before the induction of the experimental diabetes. Diabetes was confirmed by the evaluation of levels of serum glucose after 42 h (above 120 mg/dL) [22]. The animals were treated with *P. tomentosa* extract by oral-gavage for 15 days.

All experimental protocols were approved by University of Jiroft (3818-97-3) Jiroft, Iran.
Animals were randomly and equally divided into four groups (n = 5).

Experimental Design was as follows:

Group I- control rats

Group II- rats treated with STZ

Group III- diabetic rats treated with *P. tomentosa* extract (200 mg/kg body weight).

Group IV- normal rats treated with *P. tomentosa* extract (200 mg/kg body weight)

At the end of the study, blood samples of all fasted rats (12–14 h) were collected for biochemical analysis.

Biochemical parameters

At the end of the treatment period, the rats were anesthetized with diethylether and blood samples were collected by cardiac puncture. The serum levels of ALT, AST, LDL, HDL, TG and blood glucose were measured by automated chemical analyzes (Auto-analyzer, Hitachi 912) and special kits (Pars Azmoon Co., Iran).

Histological examination

Pancreas tissue was fixed in 10 % neutral buffered formalin. After pancreas was sectioned into 5µm thick slices, and stained with hematoxylin/eosin for histological examination.

LC-HRESIMS Analysis

Qualitative LC-MS profile of aqueous extract *P. tomentosa* aerial parts was obtained by LC-ESI/LTQOrbitrap/MS/MS^n. A quaternary Accela 600 pump and an Accela autosampler coupled to a LTQOrbitrap XL (ThermoScientific, San Jose, CA), operating in the negative electrospray ionization mode was performed. The separation was carried out using a C18 reversed-phase (RP) column (2.1 x 250 mm; X-Terra MS C18 5 µm; Waters, Milford, MA) at a flow rate of 0.2 µl/min and coupled to a LTQ-Orbitrap XL mass spectrometer. Linear gradient elution was acquired by using water with 0.1% formic acid as eluent

A and acetonitrile with 0.1% formic acid as B. The HPLC gradient started at 10% B, after 25 min, % B was at 60%, and after 10 min was at 100% holding it for 10 min. before returning back to the starting percentage. The mass range was from 200 to 1200 m/z with a resolution of 30000. The *m/z* of each identified compound was calculated to 4 decimal places and measured with a mass accuracy < 3 ppm. The source voltage was 3.5 kV and capillary voltage − 48 kV, the tube lens offset − 176.5 V and the capillary temperature was set at 280°C, the auxiliary gas was set at 5 (arbitrary units) and the sheath gas at 15 (arbitrary units). In full LC-ESIMS experiments Total Ion Current (TIC) profile was produced by monitoring the intensity of all the ions produced and acquired in every scan during the chromatographic run. In order to get structural information, Data Dependent experiments were performed by acquiring MS2 spectra of the most intense ions produced during the acquisition; a normalization collision energy at 30%, a minimum signal threshold at 250, and an isolation width at 2.0; multiple-stage tandem mass have been used [14]. For MeOH extract of *P. tomentosa* aerial parts, the autosampler was set to inject 2 µl of extract (0.5 mg/mL); for standards solution, the autosampler was set to inject 2 µl of each standard (1.0 mg/mL);

Statistical analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by LSD test of variance homogeneity. A difference in the mean values of $P < 0.05$ was considered to be statistically significant.

Results And Discussion

On the basis of the hypoglycemic activity reported by plants belonging to the Asclepiadaceae family, the anti-diabetic activity of the aqueous extract of *P. tomentosa* aerial parts in rats affected by streptozotocin-induced diabetes has been evaluated. Diabetes mellitus is a complex and progressive metabolic disease characterized by chronic hyperglycemia. Pathogenesis of diabetes mellitus involves the generation of free radicals especially reactive oxygen species (ROS), glucose oxidation and lipid peroxidation. MDA is the well-known indicator of lipid peroxidation and oxidative stress. Moreover, it is well known that high levels of water intake, fasting blood glucose, with loss of body weights and polyuria are important indicators of diabetes [23].

Influence of an aqueous extract of *P. tomentosa* aerial parts on body weights and blood glucose level

Diabetic rats treated with an aqueous extract of *P. tomentosa* aerial parts showed improvement of body weight if compared to diabetic untreated control. Probably, the improvement of body weight could be associated to positive modification of blood sugar, which enhanced weight gain through successful glucose utilization [24, 25]. In addition, in diabetic rats, oral administration of *P. tomentosa* (Table 1) caused significant declines of the fasting blood glucose compared to untreated diabetic rats. The effects of *P. tomentosa* extract and water intake in diabetic rats on body weight and on blood glucose level are shown in Table 1. A significant difference between diabetic rats and diabetic rats treated with *P.*

tomentosa has been observed. In particular, after injection of STZ, a significant loss ($P < 0.001$) in the final body weight if compared with initial body weight was observed. In diabetic group treated with *P. tomentosa* the final body weight was higher than the initial body weight. Moreover, the administration of *P. tomentosa*, at dose of 200 mg/kg to diabetic rats, caused significant ($P < 0.01$) reduction of blood glucose levels and water intake.

Influence of *P. tomentosa* aqueous extract on lipids blood level

Hypertriglyceridemia has been identified as a major risk factor for cardiovascular complications. In the present study an increase of triglycerides level after injection of STZ, was observed. The obtained results are in agreement with literature in fact the insulin action on lipoprotein metabolism is exerted mainly through the lipolysis increase of triglyceride rich lipoproteins by stimulating lipase and lipolysis prevention of fats stored in tissues by inhibition of hormones sensitive lipase [26]. Diabetic rats treated with the aqueous extract of *P. tomentosa* aerial parts displayed triglycerides levels not significantly different from the normal control. Furthermore a decrease of ALT and AST levels in serum of the diabetic rats treated with *P. tomentosa* extract, probably due to the compounds exerting free radical scavenging activity protecting liver cells against lipid-peroxidation, was observed (Table 2).

Triglyceride level of the diabetic group was significantly higher than the normal control, while the administration of *P. tomentosa* to STZ-treated diabetic rats, caused a significant reduction of blood triglyceride levels. HDL and LDL levels did not show evident significant differences between tested groups. Moreover, the MDA level was increased ($P < 0.001$) in the diabetic group and in diabetic rats treated with *P. tomentosa* if compared to control group (Table 2).

Histological assessment of pancreas, kidney and liver by Eaematoxylin and Eosin staining

To evaluate the effects of *P. tomentosa* aqueous extract on pancreas, kidney and liver of diabetic and control rats, the histological analysis of the above mentioned organs has been evaluated.

The pancreas cells of control rats showed the normal acinar cells which stained strongly and arranged in lobules with prominent nuclei. As shown in Fig. 1-IA, in control rats, the islet cells were embedded within the acinar cells and surrounded by capsule. The pancreas of the diabetic untreated group revealed a high level of cellular damage; in particular, diabetic rats revealed pathological changes of both exocrine and endocrine components. Islet β-cells were almost entirely lost in STZ-treated rats. Islets of Langerhans showed hyaline and necrotic changes (black arrow).

Wider interlobular and intralobular ducts were observed (Fig. 1-IB). *P. tomentosa* administration to diabetic rat demonstrated marked improvement of the cell injure, as evident from the partial restoration of Islets of Langerhans and exocrine components. (Fig. 1-1D). These observations showed that the extract could confer some protective effect on the pancreas, consequently improving glucose metabolism. This could be attributed to the antioxidant effect of some of the phytochemicals in *P. tomentosa* which prevent

streptozotocin-induced free radical destruction of the pancreatic islets. The pancreas of control rats treated with *P. tomentosa* showed normal architecture of the pancreas (Fig. 1-IC).

The kidney section of control untreated group and *P. tomentosa* treated group showed normal architecture of glomerular capillary (blue arrow), glomerular tubule and urinary space (black arrow), with normal basement membrane and capillaries (Fig. 1-IIA and IIC). In diabetic rats (Fig. 1-IIB), kidney sections showed mild thickening of the basement membrane, atrophy of glomerular capillaries (blue arrow), with increased Bowman's space (urinary space, black arrow). Diabetic rats treated with *P. tomentosa* showed features of healing, i.e., normal structure of basal membrane and glomerulus. Moreover, Bowman's space was improved towards normal condition after treatment with *P. tomentosa* (Fig. 1-IID). Therefore, the kidneys of diabetic rats treated with *P. tomentosa* showed an improvement if compared to those of the diabetic untreated group. In detail, the extract slowed down the renal impairment associated with diabetes mellitus.

From the histopathology of the liver an improvement of *P. tomentosa* treated diabetic rats was observed. The liver of the diabetic untreated group showed evidence of congestion, inflammation and necrosis. These observations suggest steatosis of the liver and are evident of the toxic effects of STZ on the liver of rats [27]. In detail, treatment with *P. tomentosa* extract improved the hepatic architecture; moreover, as evidenced by the liver biochemical parameters, a hepatoprotective effect at *P. tomentosa* extract could be inferred. Liver of control rats (Fig. 1-IIIA) showed a preserved architecture with central vine (black arrow), hepatocytes and sinusoids (blue arrow). Moreover, normal sinusoids with flatted endothelial cells were seen (Fig. 1-IIIA and IIIC). The histology of liver sections obtained from diabetic rats showed loss of the normal architecture with congested central vein (black arrow), disarranged sinusoids (blue arrow), binucleated hepatocytes (red arrow) and more Kupffer cells (green arrow) (Fig. 1-IIIB). Liver sections of diabetic rats treated with *P. tomentosa* (Fig. 3D) showed almost normal liver histology with slight dilated in sinusoids, lesser degree of inflammation

LC-MS qualitative profile of aqueous extract of P. tomentosa aerial parts

In order to correlate the hypoglycemic activity to the chemical composition, the aqueous extract of *P. tomentosa* aerial parts, has been investigated by an analytical approach based on LC-ESI/LTQOrbitrap/MS/MS^n, operating in the same conditions reported previously [14]. The analysis of LC-HRMS spectra allowed to assign both accurate molecular mass and molecular formula to the $[M-H]^-$ pseudomolecular ions occurring in the LC-MS profile. Identification of compounds has been performed on the basis of the retention times, the accurate masses and characteristic fragmentation patterns, and by comparison with literature data on *P. tomentosa*.

By this way, 23 metabolites could be identified. In particular, the LC-MS analysis of *P. tomentosa* extract suggested the occurrence of cardenolides (**4**, **8**, **16**–**17**, **21**) duble-linked cardenolides (**1**–**3**, **5**, **7**, **12**–**15**, **18**–**20**, **22**–**23**), and flavone glycosides (**6**, **9**–**11**) (Fig. 2 and Table 3).

LC-MS analysis showed for some compounds the same pseudomolecular ions; this is the case of compounds **2**, **7**, **12** and **13** with a pseudomolecular ion $[(M + HCOOH)-H]^-$ at m/z 593, compounds **5** and **14** with a pseudomolecular ion $[(M + HCOOH)-H]^-$ at m/z 595, as well as compounds **19** and **20** with a pseudomolecular ion $[(M + HCOOH)-H]^-$ at m/z 577 (Table 3).

In order to unambiguously establish the molecular structure of compounds **1**–**23**, and to discriminate among structural isomers or stereoisomers, a LC/MS analysis of naturally occurring standards, previously isolated from the aerial parts and roots of *P. tomentosa*, has been carried out.

LC-MS analysis highlighted the occurrence of different classes of compounds, mainly cardiac glycosides. These compounds represent a group of secondary metabolites that share the capacity to bind the extracellular surface of the main ion transport protein in the cell, the membrane inserted sodium pump (Na^+/K^+-ATPase) [13]. Earlier studies showed the hypoglycemic effect exerted by ouabain, a cardiac glycoside present in plants belonging to the Asclepiadaceae family; in particular this compound displayed a significant decrease in glucose and glycerol concentrations [28, 29].

Conclusions

This is the first report on the evaluation of the hypoglycemic activity of *P. tomentosa* by *in vivo* assays. This study led to highlight the capability of an aqueous extract of *P. tomentosa* aerial parts to decrease the glucose level and induce beneficial effects on lipid profile in STZ- induced diabetic rats. In particular, *P. tomentosa* effects on hyperglycemia and hyperlipidemia have been displayed. These effects could be due to antioxidant properties, decrease of the glucose level and triglyceride level as well as to effects on the liver enzymes. Furthermore, in order to correlate the hypoglycemic activity to the chemical composition of *P. tomentosa*, an analytical approach based on the acquisition of a metabolite profile by LC-ESI/HRMS/MS^n analysis has been carried out. By this way, the identification of cardenolides, along with flavonoids, has been accomplished.

Therefore a synergism among secondary metabolites e.g. cardenolides able to inhibit Na^+/K^+-ATPase pump and flavonol glycosides exerting antioxidant activity, occurring in the aqueous extract of *P. tomentosa* aerial parts, could be supposed to explain the hypoglycemic effects exerted by *P. tomentosa*.

Abbreviations

WHO: World Health Organization

MRFA: Methionine-arginine-phenylalanine-alanine-acetate

ALT: Aalanine transaminase

AST: Aspartate transaminase

MDA: Lipid peroxidation

STZ: Streptozotocin

HDL: High Density Lipoproteins

LDL: Low Density Lipoprotein

OECD: Organization of Economic Co-operation and Development

Declarations

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AUTHORS' CONTRIBUTION

SHH designed the work, analyzed the data and drafted the manuscript. SP contributed the revising the draft manuscript. ARQ and FEG participate in the study design, the data collection and manuscript correction.AC participated in the methodology and correction of manuscript. All authors read and approved the manuscript.

AUTORS' INFORMATION

The authors have doctoral qualification in Medicinal plants, Plant systematic, Biology, and Pharmaceutical Biotechnology. This work is based on the research project of SH, which granted by the University of Jiroft.

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AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this survey are included in this article.

ETHICS APPROVAL AND CONSSENT TO PARTICIPATE

This study was reviewed and approved by the Research Deputy at the University of Jiroft. The organization of the institute does not involve an Ethics Committee, therefore there is no specific ethics code assigned to this study. However, each research proposal, like the one corresponding to the current study, is comprehensively reviewed by the University until an approval code is granted (No: 3818-97-3).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

All the authors declare no conflict of interest regarding this work.

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Tables

TABLE 1 Effect of *P. tomentosa* extract on body weight and on blood glucose of STZ induced diabetes in rats.

Group	BODY WEIGHT			BLOOD GLUCOSE LEVEL	
	Initial body weight	Second body weight	Final body weight	Blood Glucose (mg/dl)	Water intake (mL)
Control	221.50 ± 26.43	269.75 ± 15.58	271.575 ± 16.2	111.00 ± 18.85	180.00 ± 24.49
Diabetic untreated group	217± 11.94	227 ± 10.42^{c}	207.93 ± 17.11^{c}	518.50 ± 118.40^{c}	617.50 ± 62.38 ^{c}
Diabetic treated (*P. tomentosa*) group	228 ± 5.88	264 ± 7.02^{z}	249.00 ± 14.63^{y}	126.00 ± 43.87^{z}	257.50 ± 28.72 $^{a, z}$
Treated (*P. tomentosa*) group	215.75 ± 4.34	269 ± 15.52	265.92 ± 18.78	95.25 ± 10.11	187.50 ± 25.00

Results are presented as mean ± S.D. N=5. ^{C}P<0.001 represents the statistical significant difference between control and all treated groups. ^{y}P< 0.001 and ^{z}P< 0.001 also show the significant difference between diabetic and diabetic group treated with *P. tomentosa*.

TABLE 2

Effect of *P. tomentosa* extract on triglyceride, HDL LDL, AST, ALT and serum lipid peroxidation of STZ-induced diabetes in rats.

Group	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	AST (IU/L)	ALT (IU/L)	Lipid peroxidation (nmoles of MDA/ min/ mg protein)
Control	89.33 ± 13.05	52.00 ± 1.41	7.66 ± 1.52	85.50 ± 9.19	61.00 ± 5.65	0.185 ± 0.005
Diabetic untreated group	142.25 ± 27.03[a]	37.33 ± 3.78[a]	6.00 ± 1.00	103.25 ± 1.89[c]	99.75 ± 15.10[b]	0.366 ± 0.017[c]
Diabetic treated (*P. tomentosa*) group	91.00 ± 13.45[x]	44.00 ± 2.64 [a,x]	8.00 ± 1.00	95.00 ± 0.00 [b,x]	67.66 ± 18.58[x]	0.340 ± 0.017[c,x]
Treated (*P. tomentosa*) group	93.75 ± 7.08	44.25 ± 4.031	7.66 ± 1.52	76.00 ± 2.00	63.00 ± 9.64	0.187 ± 0.006

Results are presented as mean ± S.D. N=5. [a]*P*<0.01, [b]*P*< 0.05, [C]*P*<0.001represented the statistical significant difference between control and all treated groups. [x]*P*< 0.01 also showed the significant difference between diabetic and diabetic group treated with *P. tomentosa*.

TABLE 3

Retention times (R_t), molecular formula, Δ ppm, $[M-H]^-$, $[(M+FA)-H]^-$ and MS/MS values, of compounds occurring in aqueous extract of *P. tomentosa* aerial parts identified by LC-ESI/LTQOrbitrap/MS/MS (negative ion mode).

n	R_t* (min)	molecular formula	Δ ppm	[(M+FA)-H]$^-$	[M-H]$^-$	MS/MS	Compound
1	9.67	$C_{30}H_{44}O_{13}$	1.81	611.2687		564, 467, 421	Ghalakinoside
2	11.07	$C_{30}H_{42}O_{12}$	-2.04	593.2580		547, 529, 419	6'-hydroxycalactin
3	11.70	$C_{29}H_{39}O_{11}$	-1.86		563.2476	519, 347	12 β ,6'-dihydroxycalotropin.
4	11.99	$C_{24}H_{36}O_8$	-1.85	451.2312		405	12 β-hydroxycoroglaucigenin
5	12.25	$C_{30}H_{44}O_{12}$	-1.75	595.2738		549, 387	12'-dehydroxyghalakinoside
6	12.35	$C_{21}H_{20}O_{12}$	-1.69		463.0859	301	quercetin 3-*O*-β-D-galactopyranoside
7	12.41	$C_{30}H_{42}O_{12}$	-1.61	593.2283		547, 419	16α-hydroxycalotropin
8	12.61	$C_{30}H_{46}O_{12}$	-1.86	597.2884		551, 373	glucocoroglaucigenin
9	13.03	$C_{21}H_{20}O_{11}$	1.69		447.0914	285, 255, 227	kaempferol-3-*O*-β-D-galactopyranoside*
10	13.39	$C_{21}H_{20}O_{11}$	1.69		447.0914	285, 255, 227	kaempferol-3-*O*-β-D-glucopyranoside*
11	13.69	$C_{22}H_{22}O_{12}$	-1.41		477.1021	357, 315	isorhamnetin-3-*O*-β-D-glucopyranoside
12	14.10	$C_{30}H_{42}O_{12}$	-1.71	593.2582		529, 419, 401	6'β-hydroxycalotropin
13	14.21	$C_{30}H_{42}O_{12}$	-1.40	593.2584		547, 419	12β-hydroxycalactin
14	14.47	$C_{30}H_{44}O_{12}$	-1.60	595.2740		549	6'-dehydroxyghalakinoside
15	14.67	$C_{35}H_{50}O_{14}$	-1.19	739.3163		693	3-*O*-*β*-glucopyranosiylcalactin
16	15.19	$C_{30}H_{46}O_{11}$	-1.65	581.2947		535, 391,373	Desglucouzarin
17	16.02	$C_{24}H_{36}O_7$	-1.63	435.2370		389	Coroglaucigenin
18	17.04	$C_{32}H_{44}O_{13}$	-2.09	635.2685		589, 571	16α-acetoxycalotropin
19	17.50	$C_{30}H_{42}O_{11}$	-1.61	577.2634		531, 403, 373,	Calotropin

20	18.80	$C_{30}H_{42}O_{11}$	-1.66	577.2634		531, 403, 373, 271	Calactin
21	20.05	$C_{39}H_{52}O_{12}$	-2.55		711.3357	696, 571, 373	6'-*O*-feruloyl-desglucouzarin
22	21.69	$C_{34}H_{46}O_{14}$	-2.32	677.2788		631, 571, 461	16α-acetoxyasclepin
23	22.46	$C_{32}H_{44}O_{12}$	-2.48	619.2734		573, 531	Asclepin

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