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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

In vivo antioxidative activity of 5,7,4'-trihydroxyflavone-7-*O*-(6''-*O*-*[E]*-coumaroyl)-*β*-glucopyranoside isolated from *Panzeria alaschanica*

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Our objective was to assess *in vivo* antioxidative potential of 5,7,4'-trihydroxyflavone-7-*O*-(6''-*O*-*[E]*-coumaroyl)-*β*-glucopyranoside (TFGN) isolated from *Panzeria alaschanica* in a diabetic rat model. The diabetes and the following oxidative stress were induced by intraperitoneal administration of streptozotocin. The effects of TPGN (60 mg/kg) on the plasma concentration of malondialdehyde (MDA) and plasma fat-soluble antioxidants (co-enzyme Q_9, α-and γ-tocopherol) were measured as a parameter of oxidative damage and markers of antioxidant defence, respectively. The level of MDA in plasma was reduced to the same level as in healthy control animals. A significant decrease was observed in the plasma α-tocopherol level in the oxidative stress group compared to the healthy controls.

Key words: *Panzeria alaschanica*, diabetes mellitus model, antioxidant, 5,7,4'-trihydroxyflavone-7-*O*-(6''-*O*-*[E]*-coumaroyl)-*β*-glucopyranoside.

INTRODUCTION

Panzeria alaschanica, family Labiate, is a widely distributed plant in Eerduosi of Inner Mongolia, China. *P. alaschanica* (aerial parts) are used as a characteristic medicine in Mongolian folk to treat pelvic inflammation and chronic pelvic inflammation (Li et al., 2011; Zhang et al., 2001). In previously conducted work, it was found that the highest dose (400 mg/kg) of EtOAc extract from *P. alaschanica* produced significant anti-inflammatory activity (Wang et al., 2015a), and phenylethanoid and acylatedflavone glycosides were isolated from *P. alaschanica* (Shao et al., 2015; Wang et al., 2015b). In addition, a high performance liquid chromatography (HPLC) method for the quantification of the flavonoids in this plant was established (Wang et al., 2015c).

Acylatedflavone glycosides exhibited a wide range of biological activities including anti-inflammatory, antioxidant, and hepatoprotective effects (Julião et al., 2010; Albach et al., 2003; Peng et al., 2003). Every barber knows that the beneficial to health of acylatedflavone glycosides are due to their antioxidative and anti-inflammatory activities (Hasan et al., 2012). The EtOAc extract of *P. alaschanica* and the isolated compounds have widely been measured in various in vivo anti-inflammatory test systems (Wang et al., 2015a, b, d).

The 5,7,4'-trihydroxyflavone-7-*O*-(6''-*O*-*[E]*-coumaroyl)-

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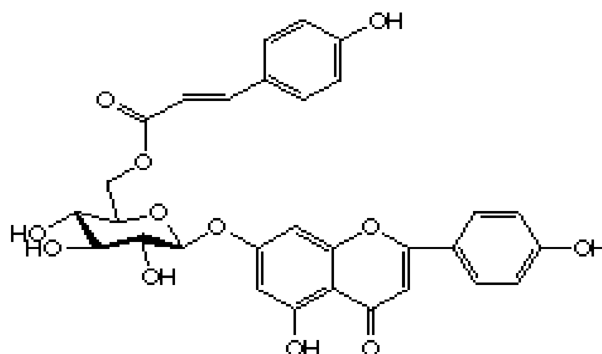

Figure 1. Structure 0f TFGN

β-glucopyranoside (TFGN) exhibited significant *in vivo* anti-inflammatory activities. However, until recently, there have been few studies about the *in vivo* antioxidative effect of TFGN. It is necessary to study its *in vivo* antioxidative effect, since inflammation is closely related to oxidative stress. Therefore, focus was on the *in vivo* antioxidative potential of TFGN.

Oxidative stress has been connected with many chronic diseases such as atherosclerosis, diabetes and cancer. Oxidative stress in diabetes mellitus (DM) is produced through multiple mitochondrial, enzymatic and non-enzymatic pathways (Bebrevska et al., 2010). Many flavonoids from foods and herbal medicines have been tested already *in vitro* to determine their antioxidative effect. As these *in vitro* tests do not pay attention to problems of malabsorption, distribution, excretion and metabolism, it is indispensable to study the activity of a promising antioxidant *in vivo*. In this study, the effects of the TFGN on the plasma concentration of MDA and some fat-soluble antioxidants (co-enzyme Q_9, α-and γ-tocopherol) were determined (Figure 1).

MATERIALS AND METHODS

Chemicals

α-Tocopherol, α-tocopherol acetate, γ-tocopherol, co-enzymes Q_9 and Q_{10}, 1,1,3,3-tetramethoxypropane, butylated hydroxyanisole, butylated hydroxy toluene (BHT), retinol, streptozotocin, thiobarbituric acid and pentobarbital (20% solution) were purchased from Sigma. All other used reagents were of analytical grade and were purchased from Shanghai Biochemical Co. (Shanghai, China). K3E Vacutainer tubes and diabetes test kits were purchased from Becton, Dickinson and Company (BD) and Abbott, respectively.

Plant material

P. alaschanica (aerial parts) were collected in Eerduosi, Inner Mongolia, China, in July, 2015. The plant material was identified by Prof. Wuxiangjie (Inner Mongolia University for Nationalities) and a voucher specimen (NO. 20150802) was stored in the Mongolian Medicine Research Center, Inner Mongolia University for Nationalities.

Extraction and isolation

The air-dried and powdered aerial parts of *P. alaschanica* (6.0 kg) were extracted twice under reflux with EtOAc (25 L) after extracting with $CHCl_3$ (10 L). The combined extracts were concentrated to a residue (510 g, yield 8.5 %) under reduced pressure. The EtOAc extract was isolated by column chromatography on silica gel and eluted by a gradient of $CHCl_3$-CH_3OH (40:1 to 5:1) to give seven fractions (Fractions 1-7). Fraction 5 (4.0 g) was further eluted on a Sephadex LH-20 column with MeOH yielding TFGN (405 mg) (Wang et al., 2015b). The purity of TFGN was determined to be above 98.0% by normalization of the peak areas detected by HPLC.

TFGN: Yellow needles; UV (MeOH) λ_{max} nm (log ε): 269 (4.35), 327 (4.23). IR (neat) v (cm^{-1}): 3447, 3365, 1688, 1654, 1639, 1602, 1588, 1510, 1487, 1361, 1278, 1167, 1074 cm^{-1}. ^1H NMR (500 MHz, in DMSO- d_6) δ_H: 6.89 (1H, s, H-3), 6.47 (1H, d, J = 1.5 Hz, H-6), 6.81 (1H, d, J = 1.5 Hz, H-8), 7.93 (2H, d, J = 8.0 Hz, H-2′, 6′), 6.93 (2H, d, J = 8.0 Hz, H-3′, 5′), 5.17 (1H, d, J = 7.0 Hz, H-1″), 7.36 (2H, d, J = 8.0 Hz, H-2‴, 6‴), 6.66 (2H, d, J = 8.0 Hz, H-3‴, 5‴), 7.51 (1H, d, J = 16.0 Hz, H-7‴), 6.34 (1H, d, J = 16.0 Hz, H-8‴). ^{13}C-NMR (125MHz, in DMSO- d_6) δ_C: 164.6 (C-2), 103.3 (C-3), 182.5 (C-4), 161.7 (C-5), 99.8 (C-6), 163.0 (C-7), 95.5 (C-8), 157.3 (C-9), 105.6 (C-10), 121.3 (C-1′), 129.1 (C-2′), 116.6 (C-3′), 161.3 (C-4′), 116.6 (C-5′), 129.1 (C-6′), 100.3 (C-1″), 73.4 (C-2″), 76.7 (C-3″), 70.1 (C-4″), 74.1 (C-5″), 63.9 (C-6″), 125.3 (C-1‴), 130.6 (C-2‴), 116.0 (C-3‴), 160.2 (C-4‴), 116.0 (C-5‴), 130.6 (C-6‴), 145.5 (C-7‴), 114.1 (C-8‴), 166.9 (C-9‴); HR-ESI-MS: *m/z* 723.1705 $[M-H]^-$ (calcd for 723.1708).

Animals and experimental design

Male Wistar rats at the age of 3 months were provided by Changchun Yisheng Laboratory Animal Technology Co., Ltd. (Changchun, China). The 50 animals were housed in an air-conditioned room with 12/12h-light/dark cycles and provided with standard laboratory food (Rat sterile granulated feed, product executive standard: GB14924–2001, license: SCXK–(JI) 2010–0001) and water *ad libitum.* All animals received humane care in compliance with local regulations of laboratory animal care and

institutional guidelines. At day 7, after an acclimatisation period of 1 week, rats were randomly assigned to five experimental groups (n = 10) as described later. Streptozotocin was administered intraperitoneally (i.p.) at a single dose of 60 mg/kg, prepared with citrate buffer (10.01 mg/ml) to animals in groups 3 to 5.

Group 1 (G1): Healthy control, received 2.0 ml/kg i.p. of citrate buffer.
Group 2 (G2): Toxicity group, received 2.0 ml/kg i.p. of citrate buffer.
Group 3 (G3): Negative control, received 60 mg/kg i.p. of streptozotocin solution.
Group 4 (G4): Treatment group, for 7 weeks (streptozotocin solution, 60 mg/kg, i.p.).
Group 5 (G5): Positive group, for 7 weeks (streptozotocin solution, 60 mg/kg, i.p.).

The animals were treated with the TFGN after an induction period of 7 weeks. G2 received a high dose of 500 mg/kg TFGN solution (10.0 mg/mL); G4 received the TFGN solution (50 mg/kg); G5 received a dose of 50 mg/kg α-tocopherol acetate solution (10.0 mg/mL). G1 and G3 received water during the treatment period. Treatment was given once at approximately the same time interval of 24 h every day. The weights of animals were determined every 7 days. The presence of diabetes was confirmed by measuring blood glucose levels using a portable blood sugar monitor. During the induction period, the blood glucose level (BGL) was tested at equal intervals of 14 days. Rats with BGL less than 10 mM were excluded at the end of the induction period of 7 weeks. The BGL was measured at equal intervals of 4 days during the treatment period.

Sample preparation

At the end of the treatment period, plasma samples were collected from the arteria carotis in 1 ml tubes (Eppendorf) containing potassium EDTA (30 µl 7.5% EDTA/ml blood sample) and immediately centrifuged at 1000 × g for 12.5 min. The plasma samples were stored at -70°C.

Lipid peroxidation assay

A HPLC-fluorimetric detection method was used to determine the oxidative damage due to lipid peroxidation (Hermans et al., 2005). Briefly, this method quantifies MDA after reaction with thiobarbituric (TBA) in acid and heat conditions, and the resulting pink fluorescent complex is analysed by HPLC fluorescence detection.

Determination of fat-soluble antioxidants

For the determination of α- and γ-tocopherol and coenzyme Q plasma levels, an optimized and validated HPLC-coulometric detection method was used as described by the published method (Hermans et al., 2005).

Statistical analysis

SPSS 15.0 and GraphPad Prism 5 software packages was used to evaluate the data. The difference between the means was estimated using an appropriate test. All data are shown as mean ± standard error of mean (SEM). The Levene's test was used to test the homogeneity of variances. If variances were unequal data were mathematically transformed (logarithmic or power transformation). A value of $P < 0.05$ was considered significant.

RESULTS

The BGL and body weight of all animals were determined before the induction of diabetes mellitus. As shown in Figure 2a, there were no statistically significant differences between the groups. Oxidative stress was permitted to develop during 7 weeks after streptozotocin injection. From Figure 2b, it is found that the BGL in G3 significantly increased at the end of this induction period. Treatment was begun after development of DM. The results of the MDA-TBA and the determination of body weight of the animals after 3 weeks of treatments are shown in Figures 3 and 4, respectively. Moreover, the results of the effect on the plasma concentration of some fat-soluble antioxidants (co-enzyme Q_9, α- and γ-tocopherol) were shown in Figures 5 to 7.

DISCUSSION

The BGL in G3 significantly increased at the end of this induction period, confirming the successful development of DM (Figure 2b). Moreover, the characteristic symptoms of DM such as the increased appetite, polydipsy, polyuria, and loss of weight were observed. From Figure 3, all animals which received oxidative stress displayed a significant decrease in body weight compared to G1, even after treatment with G4 or G5. There were no significant differences between the body weight of G4 or G5, with G3. As shown in Figure 4, the level of MDA-TBA complex in G3 was significantly promoted as compared to the G1 ($P<0.001$), which indicated the suitability of the chosen system for *in vivo* lipid peroxidation inhibitory activity evaluation. Comparing to G3, there was a significant reduction in the oxidative stress damage in G4 and G5. However, the damage of lipid peroxidation was not decreased to G1. The level of lipid damage in G4 and G5 was similar. Thus, the G4 and G5 had similar effect on the *in vivo* oxidative stress status of the diabetic animals. The potent lipid peroxidation chain breaking activity in G3 compound with G2 or G4 produced a similar effect.

From Figure 5, it is found that the level of plasma α-tocopherol in G3 significantly reduced compared to G1. However, there was no significant difference between G4 and G1 or G3. In addition, no significant difference was observed between G5 and G3. The G1 and G2 showed similar α-tocopherol levels. Concerning γ-tocopherol, there was no significant difference between G1 and G3 (Figure 6). It is surprising that the levels of γ-tocopherol in G4 and G5 were significantly reduced with the G1, and the observed levels were not different from G3. It should be noted that the levels of γ-tocopherol were very low. The discoveries showed that in G3, G4 and G5 where oxidative stress was induced almost complete depletion of γ-tocopherol had happened. Any positive influence of the treatment with TFGN on the level of γ-tocopherol

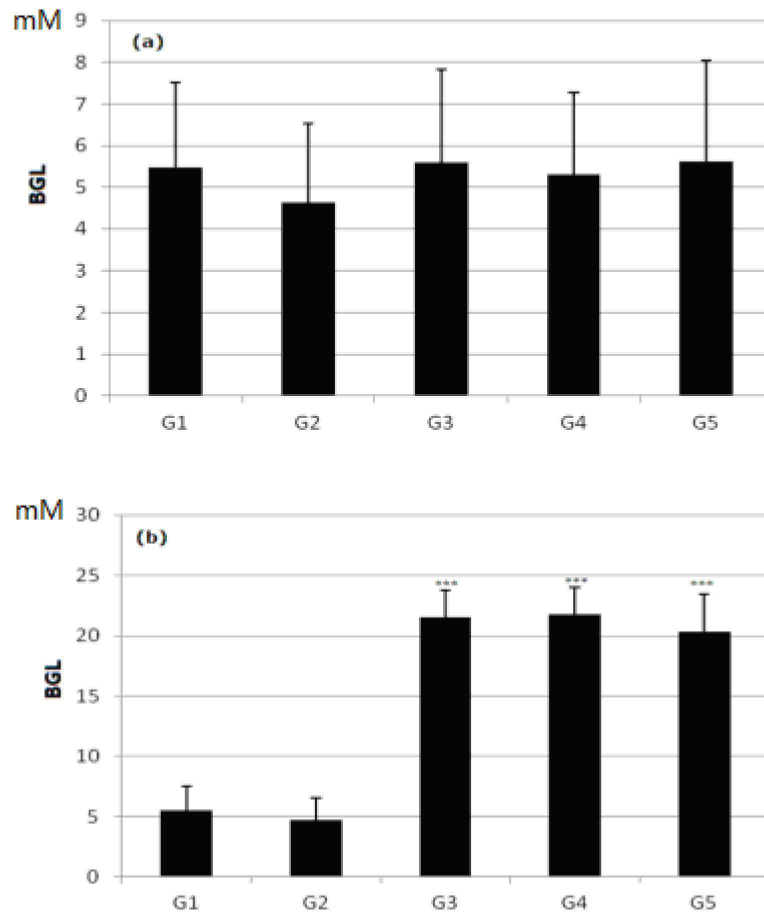

Figure 2. (a) BGL in G1-G5 at the start of the study. (b) BGL in G1-G5 at the end of the induction period. ***P<0.001, compared to G1. There was no statistically significant difference between G4 or G5, compared to G3.

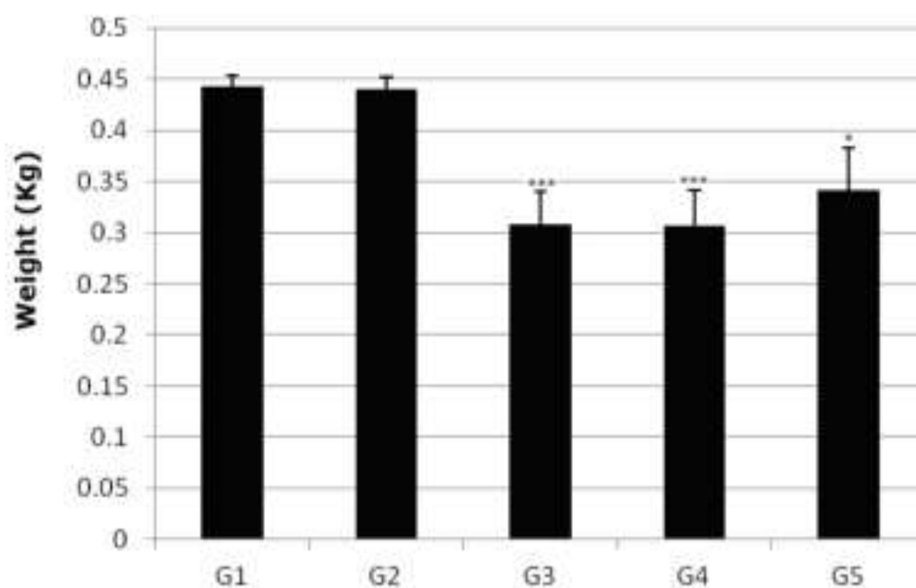

Figure 3. Body weight in G1-G5. ***P<0.001; *P<0.05; compared to G1. There were no significant differences between G4 or G5, compared to G3.

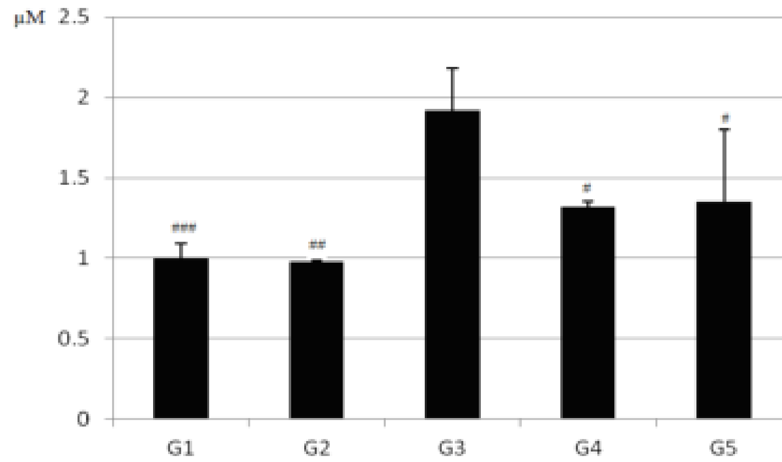

Figure 4. The levels of MDA–TBA complex in G1-G5. ### P<0.001, ## P<0.01, # P<0.05, compared to G3.

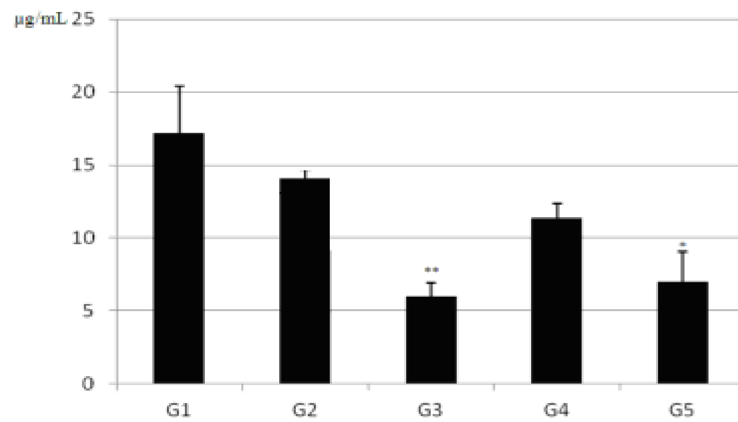

Figure 5. The levels of α -tocopherol plasma in G1-G5. **P<0.01, *P<0.05, compared to G1. There was no significant difference between G4 or G5, compared to G3.

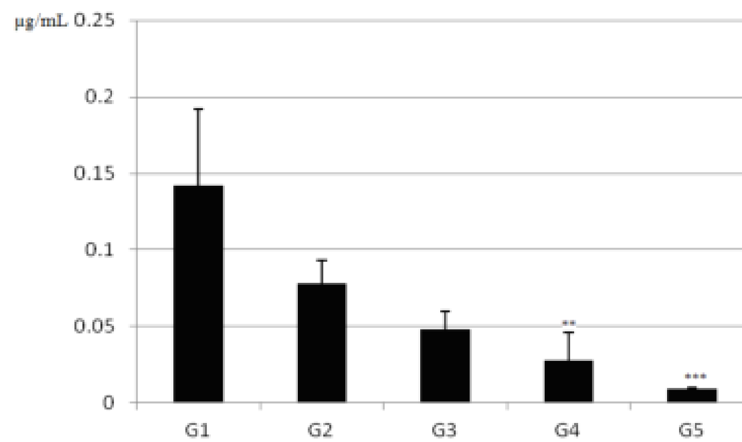

Figure 6. The levels of γ -tocopherol plasma in G1-G5; ***P<0.001, **P<0.01, compared to G1. There was no significant difference between G1 compared to G3.

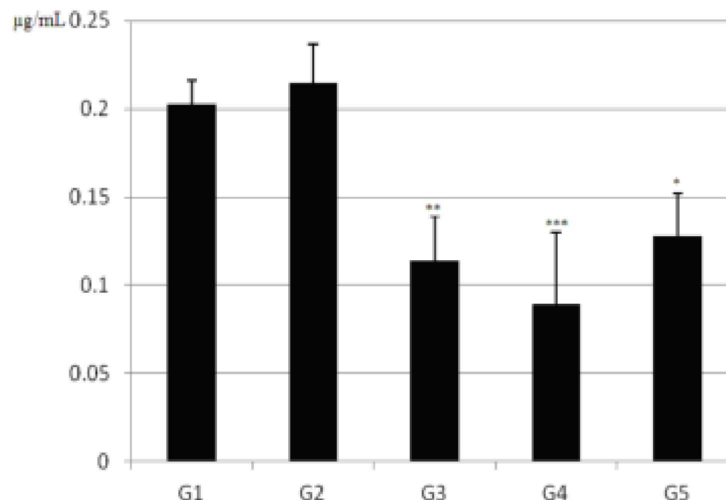

Figure 7. The levels of Q_9 plasma in G1-G5; ***P<0.001, **P<0.01, *P<0.05, compared to G1. There was no significant difference between G4 or G5, compared to G3.

under the condition of oxidative stress could not be observed. From Figure 7, the level of Q_9 was higher in the G1 as compared to the G3. The plasma level of Q_9 in the G4 or G5 was different from the G1 but not from the G3. The diabetic animals in G3 and G4 displayed a significant decrease at this phase of the experiment. This might be an after effect of its depletion as a factor in the antioxidant defence against the induced oxidative stress, which was not equalized by upregulation of its synthesis in this case. There was no significant difference between G4 or G5 on the Q_9 level compared to G3. Thus, the dynamics of the up-regulation and depletion of this molecule are needed to further investigate. For detecting potential toxic effects of TFGN, the animals in G2 received 10× the treatment dose (500 mg/kg). The animals in G2 were not injected with streptozotocin. With regard to the level of BGL, MDA-TBA complex, the fat-soluble antioxidants and body weight measured, no significant differences were observed between G1 and G2.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Short Communication

Diuretic, insecticidal and leishmanicidal profile of the whole plant of *Viola betonicifolia*

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In the present study, the crude methanolic and its succeeding solvent fractions of the whole plant of *Viola betonicifolia* were investigated for its diuretic, insecticidal and leishmanicidal profile. The methanolic and *n*-hexane fractions were screened (at the dose of 200, 300 and 400 mg/kg intraperitoneally [i.p.]) for diuretic effects on BALB/c mice. The methanolic extract demonstrated a weak, but not statistically significant diuretic activity compared to the standard drug, while the diuretic effect of *n*-hexane was similar to negative control. The insecticidal effect shown was in low to moderate effect range. The crude methanolic extract and aqueous fraction exhibited 40% mortality against *Callosobruchus analis*, while chloroform and *n*-hexane exhibited 40% activity against *Tribolium castaneum* and *Rhyzopertha dominica.* No leishmanicidal effect was observed against any of the tested samples.

Key words: *Viola betonicifolia,* diuretic, insecticidal and leishmanicidal.

INTRODUCTION

Viola betonicifolia belongs to family Violaceace locally which is known as banafsha. *V. betonicifolia* is grown in various countries of the world like Pakistan, India, Nepal, Srilanka, China, Malaysia and Australia. In Pakistan, it is available in Swat, Hazara and Dir districts. The folk use of this plant is as antipyretic, astringent, diaphoretic, anticancer, purgative and diuretic (Husain et al., 2008). It has been used in the treatment of various neurological disorders including epilepsy and insomnia (Hamayun, 2005). Additionally, it has been used in the treatment of sinusitis, skin and blood disorders and pharyngitis (Bhatt and Negi, 2006). The roots are used for kidney diseases, pneumonia and bronchitis. The flowers are recommended for the treatment of asthma, cough and colds while the leaves are useful for the treatment of boils (Husain et al., 2008). Recently, we have tested the crude methanolic as well as the subsequent solvent fraction of *V. betonicifolia* for various pharmacological activities (Muhammad and Saeed, 2011; Muhammad et al., 2012). In the current study, the diuretic, insecticidal and leishmanicidal of *V. betonicifolia* whole plant were carried out.

MATERIALS AND METHODS

Animals

BALB/c mice of either sex were used in all experiments. Animals were purchased from the Pharmacology Section of the Department of Pharmacy, University of Peshawar, Peshawar, Pakistan. The

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animals were maintained in standard laboratory conditions (25°C and light/dark cycles 12/12 h) and were fed with standard food and water *ad libitum*. The experimental protocols were approved by the ethical committee of the Department Pharmacy, University of Peshawar, Peshawar, Pakistan.

Plant

Whole plant of *V. betonicifolia* was collected from Swat, Khyber Pakhtunkhawa, Pakistan, in April 2010. Plant specimen was identified by Taxonomist, Department of Botany, University of Peshawar and a specimen was deposited there in the herbarium with voucher number 6410/Bot. The collected whole plant (12 kg) was air dried and powdered. The powder was extracted by maceration with methanol at room temperature for 14 days with occasional shaking. The methanolic extract was filtered and concentrated under vacuum using rotary evaporator at low temperature (45°C). The methanolic extract was dissolved in distilled water and further fractionated with chloroform, *n*-hexane, ethyl acetate, *n*-butanol and water.

Pharmacological study

Diuretic activity

The diuretic activity of *V. betonicifolia* crude methanolic and its n-hexane fraction was determined using male BALB/c mice. The animals were divided into eight groups (n = 6). The animals were fasted for 24 h and were fed laboratory diet and water *ad libitum*. On the day of experiment, the animals of group I was treated with distilled water (15 ml/kg intraperitoneally [i.p]) and this group served as control. Similarly, the animals of group II were treated with frusemide (10 mg/kg i.p.). Groups III, IV and V were treated with 200, 300 and 400 mg/kg (i.p) methanolic extract, respectively. The remaining groups VI, VII and VIII were injected 200, 300 and 400 mg/kg (i.p) *n*-hexane fraction, respectively. All the treated animals were placed in metabolic cages (1 animal in each metabolic cage). Urine was collected in graduated cylinders and its volume was recorded at 2, 3 and 6 h. Cumulative urine excretion was calculated in relation to body weight and expressed as ml/100 g body weight.

Insecticidal activity

In vitro insecticidal assay was carried out against *Tribolium castaneum, Rhyzopertha dominica* and *Callosobruchus analis* following method available in literature (Saeed et al., 2010). The test sample was prepared (200 mg of crude extract was dissolved in 3 ml of methanol and served as stock solution). The sample (1019.10 µg/cm^2) was loaded over the filter paper of appropriate size (9 cm or 90 mm) on Petri plate using micropipette. The plate was left overnight (24 h) to evaporate the solvent. Next morning, 10 healthy and active insects of each species of same size and age were added to each plate including control (methanol) and standard drug (Permethrin, 239.50 µg/cm^2). Thereafter, the plates were incubated in growth chamber at 27°C for 24 h with 50% relative humidity. For calculation, the number of survived insects was counted and the mortality.

$$\text{Inhibition (\%)} = 100 - \frac{\text{Number of insects alive in test}}{\text{Number of insects alive in control}} \times 100$$

Leishmanicidal assay

Leishmanicidal assay was carried out using previously described method (Saeed et al., 2010). *Leishmania major* (MHOM/SU/73/5-ASKH) promastigotes were cultured at 22 to 25°C in RPMI-1640 (Sigma). The medium was supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS). Promastigote culture in the logarithmic phase of growth was centrifuged at 2000 rpm for 10 min, and washed with saline three times in the same condition. Parasites were diluted with fresh culture medium to a final density of 106 cells/ml. In a 96-well microtiter plate, 180 ml of medium was added in first row and 100 ml of medium was added in others wells. Test extracts (20 ml) was added in medium and serially diluted. 100 ml of parasite culture was added in all wells. One row was used for control (DMSO) which received medium, while one for standard drugs (Amphotericin B, Pantamidine). The plate was incubated at 21 to 22°C for 72 h and the numbers of surviving parasites were counted microscopically in Neubauer chamber. Results are the replicates of three different experiments. The 50% inhibitory concentrations (IC_{50}) were calculated by a Windows based EZ-Fit 5.03 Perrella Scientific Software.

RESULTS AND DISCUSSION

Diuretic effect

The crude methanolic of the whole plant of *V. betonicifolia* was 200, 300 and 400 mg/kg and its *n*-hexane solvent fraction was 200, 300 and 400 mg/kg. It is clear from our results that both tested extracts were devoid of diuretic effect as shown in Figure 1. The diuretic effect of methanolic extract was slightly greater than the negative control group, but was statistically non significant. In the traditional medicines, *V. betonicifolia* is used as diuretic (Husain et al., 2008), but our results were able to provide a scientific background to the folklore of this plant. The reason of the failure of our test extract as diuretic might be due to the use of *V. betonicifolia* as polypharmacy (in combination with other plants) for diuretic effect. It means that the methanolic extracts have diuretic molecules, but their action is mild. So our tested compound is mild diuretic.

Insecticidal effect

The crude methanolic extract and its succeeding solvent fractions were screened for insecticidal effect against three insects, that is, *T. castaneum, R. dominica* and *C. analis.* The tested samples showed low to moderate insecticidal activity as shown in Table 1. The crude methanolic extract showed 20 and 40% activity against *T. castaneum* and *C. analis,* while chloroform exhibit 40% mortality against *T. castaneum* as presented in Table 1. The percent mortality of *n*-hexane was 40 and 20% against *R. dominica* and *C. analis* ethyl acetate was inactive against any of the tested insect. The butanolic extract showed low mortality (10%) against *T. castaneum,* while a moderate mortality (40%) was shown by aqueous against *C. analis.*

Leishmanicidal effect

The leishmanicidal effect of the crude methanolic and its

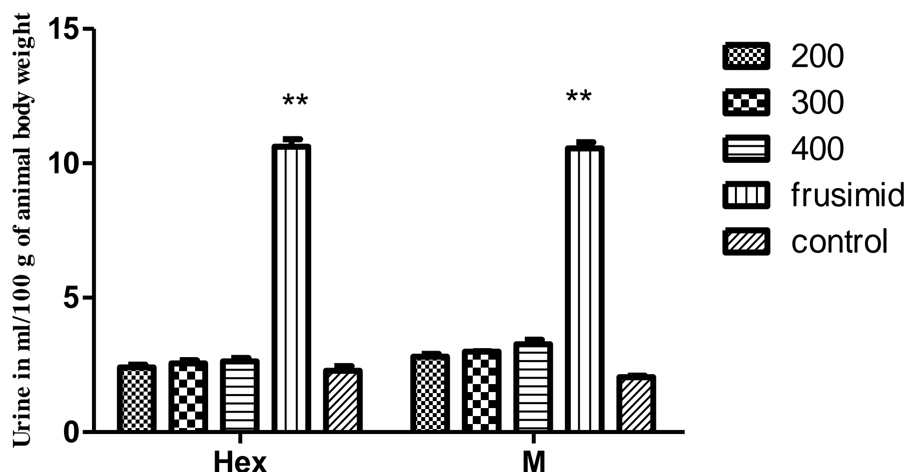

Figure 1. Diuretic effect of M (methanolic) and Hex (*n*-hexane) fraction of *Viola betonicifolia* in mice. Bar presents the volume of urine after 5 h of the treatment with frusemide (10 mg/kg) and extracts (200, 300 and 400 mg/kg).

Table 1. Insecticidal activity of *Viola betonicifolia*.

Name of insect	Standard	Control	Mortality (%)					
			M	Ch	Hex	Et	Bu	Aq
Tribolium castaneum	100	-	20	40	-	-	10	-
Rhyzopertha dominica	100	-	-	-	40	-	-	-
Callosobruchus analis	100	-	40	-	20	-	-	40

M: Methanolic, Ch: chloroform, Hex: n-hexane, Bu: butanol, Aq: aqueous.

Table 2. Leishmanicidal effect of *Viola betonicifolia*.

Test organism	Sub-fraction of oil	IC_{50} (µg/ml) ± SD
Leishmania major (MHOM/SU/73/5-ASKH)	M	> 100
	Ch	> 100
	Hex	> 100
	Et	> 100
	Bu	> 100
	Aq	> 100
	Amphotercin-B	0.29 ± 0.05
	Pentamidine	5.09 ± 0.04

M: Methanolic, Ch: chloroform, Hex: n-hexane, Bu: butanol, Aq: aqueous. IC_{50}: values indicate the effective concentration of a compound in µg/ml necessary to achieve 50% inhibition. Incubation period 72 h and incubation temperature was 22°C.

succeeding solvent fractions is depicted as shown in Table 2. None of the tested sample showed any leishmanicidal activity with IC_{50} more than 100. The diuretic effect of methanolic extract and its subsequent solvent fractions at the test dose of 200 and 400 mg/kg of *Viola odorata* (related species of *V. betonicifolia*) have been reported (Vishal et al., 2009).

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