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Phytochemical analysis and reappraisal of diuretic activity of *Delphinium brunonianum* Royle and its mode of action in experimental rats

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Abstract: The aim of this study was the evaluation of diuretic potential of *Delphinium brunonianum*. Acute diuretic effect in rats was evaluated 8 h after administration of various doses of crude extract, fractions and hydrochlorthiazide. While, prolonged effect of butanolic fraction was assessed after 7days of oral administration in rats. Thereafter, involvement of different pathways in diuretic activity was also appraised. Furthermore, polyphenolic contents in butanolic fraction were assessed using HPLC/UV-VIS technique. All doses of extract and fractions induced a prominent increase in urine and Na+ excretion with no effect on excretion of K+. Prior administration of indomethacin and atropine considerably avoided the diuretic effect of butanolic fraction. Regarding the quantitative chemical analysis the polyphenolic contents were recorded as 28.78 µg/mg. Thus results of present investigation suggested that *Delphinium brunonianum* possess remarkable diuretic potential.

Keywords: *Delphinium brunonianum*, diuretic activity, prostaglandins, muscarinic pathway, HPLC/UV-VIS analysis.

INTRODUCTION

Diuretic agents increase urine output by interfering with blood circulation and reabsorption of water and ions in renal tubules (Gallagher *et al*., 2006). Currently many synthetic diuretics are available in market, in spite of wide spread use of these agents, several synthetic diuretics have been associated with many adverse effects (Ellison and Loffing, 2009), thus there is a need to develop new diuretic agents with lesser side effects. Since centuries, in developing countries medicinal plants have been extensively used for the treatment of various diseases. In the recent years medicinal plants have been a highly revered and exemplary source of chemical substances with prospective therapeutic potential and low toxicity.

Several medicinal plants are purportedly used as diuretic agent in folklore medicine to treat cardiovascular diseases without sufficient scientific basis. Thus there is an urgent need for development of scientific basis for these aboriginal drugs through modern pharmacological methods, emphasizing the mechanism underlying their therapeutic effects before use in human. *Delphinium burnonianum* royle (Local Nmae: Mareal/Mukhoti (Shina), Gul- e- Mamoon (Urdu) belonging to family Rananculaceae is found in Gilgit Baltistan where it is being used by locals for treatment of various disorders (Hussain *et al*., 2011, Khan *et al*., 2009). Since no pharmacological study has been reported on diuretic activity of *Delphinium burnonianum* till date, hence present study was designed to evaluate diuretic activity of aqueous ethanolic extract obtained from aerial parts of *Delphinium brunonianum* and its butanolic and aqueous fractions after oral administration in Sprague dawley rats.

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MATERIALS AND METHODS

Plant material

Aerial parts of *Delphinium burnonianum* were collected from Gilgit -Baltistan in August 2016. After identification from Dr Sher Wali Khan, Assistant Professor in Department of Botany, Karakoram International University, Gilgit Baltistan a voucher was deposited in herbarium of College of Pharmacy, University of Sargodha and catalogued as DB-16-09.

Extract and fractions preparation

Aerial parts of *D. brunonianum* were cleaned, air dried and pulverized into coarse powder. Crude extract was prepared by maceration (three times of 72 h each) with aqueous ethanol mixture (30:70) at room temperature. Afterwards, extract was filtered and solvent was eliminated by rotary evaporator. Subsequently obtained extract was dried in open air and stored in air tight containers. A part of crude extract of *D. burnonianum* (DB-Cr) was subjected to fractionation using butanol as

solvent to yield butanolic (DB-B) and aqueous fraction (DB-Aq) (Alamgeer *et al*., 2016).

Animals

Sprague dawley rats (200-250g) of either sex obtained and housed in Animal Resource Center of University of Sargodha, were used in this study, according to the guidelines of institutional animal ethical committee and comply with instructions of National Research Council (Alamgeer *et al*., 2017). All study protocols were approved from Institutional Animal Ethics Committee, College of Pharmacy, University of Sargodha (Approval No. 51A26 IEC UOS).

Assessment of diuretic effect of Delphinium brunonianum

Diuretic effect was determined according to the method adopted by De Souza (de Souza *et al*., 2013) with slight alteration. Rats were divided in different groups (n=5) and12 h before starting of experiments rats were kept on fasting but free access to water. All animals were treated with 5 mL/100 g bw of normal saline as oral salt load to inflict a controlled water and salt balance.

Acute diuretic activity

To assess acute diuretic effect rats in control group received vehicle orally (0.5 mL/100 g BW) while, other groups of rats received crude extract (250, 500, 1000 mg/kg), butanolic fraction (25, 50 and 100 mg/kg), aqueous fraction (100, 200 and 400 mg/kg) and 10 mg/kg of hydrochlorthiazide (HCTZ) respectively. Immediately after treatments rats were placed in separate cages. Urine sample was collected at 1, 2, 4, 6 and 8 h. After that cumulative urine volume was quantified and presented as mL/100 g. Electrolyte concentration, pH and conductivity was estimated from sample.

Prolonged diuretic activity

To evaluate the effect of DB-B on repeated oral administration rats were divided in 3 groups and received vehicle, DB-B (50 mg/kg) and hydrochlorthiazide (10 mg/kg) separately for a period of 7 days. For collection of urine sample all animals were placed in metabolic cages and urine was collected in graduated cylinder at 1, 2, 4, 6 and 8 h. Volume, pH, density and electrolyte concentration was measured in each urine sample on daily basis. Moreover, at the end of experiment (7th day) plasma concentration of sodium, potassium, urea and creatinine were also assessed by enzymatic method using automated chemistry analyzer (Prando *et al*., 2016).

Appreciation of the mechanisms assimilated in diuretic activity

Valuation of role of the cholinergic pathway, nitric oxide and prostaglandin/cAMP pathway in the diuretic effect

This experiment was conducted with an intent to evaluate the possible mechanism involved in diuresis induced by butanolic fraction. For this purpose previously described procedure was adopted with slight changes (Gasparotto *et al*., 2009, Gasparotto Junior *et al*., 2012). Different groups of rats given normal saline as salt load and then treated with L-Name (40mg/kg), atropine (1 mg/kg) and indomethacin (5 mg/kg) 1 h prior to administration of DB-B (50 mg/kg). All rats were placed in separate cages to collect urine for next 8 h. At the end urine volume, pH, conductivity and electrolyte contents were estimated for each rat.

Evalauation of phenolics in butanolic fraction of Delphinium brunonianum using HPLC

HPLC analysis was performed on a Waters liquid chromatograph (Waters Spa, Milford, MA, USA) was used for attainment of data. The column was stabilized at 30 ± 1°C and 20 uL injection volume was used. The acquisition wavelength was adjusted at 200–500 nm. Gradient elution was performed by mobile phase water-acetonit-rile (93:7, v/v, 3% acetic acid) as reported in literature (Di Sotto *et al*., 2018, Locatelli *et al*., 2017, Zengin *et al*., 2018). Sample solution was centrifuged to obtain the supernatant for injection into HPLC.

Preparation of standard solutions and samples

The stock of phenolics (1 mg/mL) was prepared in a final volume of 10 mL of methanol. Whereas, mixed standard solution was obtained at the concentrations of 10, 25, 50, 75, 100, 150 and 200 µg/mL by dilution with the mobile phase and injected in HPLC. Working solutions of standards at various concentrations (0.25, 0.5, 1, 2.5, 5, 10, and 20 µg/mL) were obtained by dilution with the mobile phase and instilled into +9/ system. Each sample was weighted and mixed in mobile phase in 1:1 (*w:v*). In this case, the obtained concentrations (µg/mL) correspond to the total amount (µg/mg). After solubilization, the sample was centrifuged at 12000 x *g* before All chromatograms obtained by each sample will be sent in case (based on the paper structures). The reported values are mean ± standard deviation of three independent measures.

STATISTICAL ANALYSIS

The results of present investigation are presented as mean ± Standard error of mean (SEM) of 5 animals in each group. Values were analyzed by two and one way ANOVA followed by Dunnet or Bonferreni post test using GraphPad Prism version 6.

RESULTS

Acute diuretic activity of Delphinium brunonianum

Acute oral administration of HCTZ (10 mg/kg), DB-Cr, DB-B and DB-Aq produced significant ($p<0.001$) diuresis at 8 h as compared to control group. However DB-B (50 mg/kg) produced most pronounced effect as compared to

Table 1: Acute diuretic effect of *Delphinium brunonianum*

Group	Urine vol (mL/100g/8 h)	DI	Na+ mMol/L	K+ mMol/L	Saluretic index		Na+/K+
Control (NS)	1.3 ±.20	-	89.43±8.86	43.36±4.97	-	-	2.062
HCTZ 10 mg/kg	6.7±.15[a]	4.89	237.86±6.605 [a]	79.23±5.21[a]	2.65	1.827	3.002
DB-Cr 250 mg/kg	5.077±.81 [a]	3.705	242.56±6.16 [a]	45.76±1.97	2.712	1.055	5.3006
DB-Cr 500 mg/kg	5.80±.24 [a]	4.233	249±3.31 [a]	47.53 ± 0.34	2.78	1.096	5.238
DB-Cr 1000 mg/kg	3.53±.06 [a]	2.576	86.32±6.45	43.67±2.11	.965	1.007	1.976
DB-B 25 mg/kg	5.003±.135 [a]	3.84	268.63 ±16.54 [a]	42.833±3.96	3.00	.987	6.271
DB-B 50 mg/kg	6.173±.514 [a]	4.748	280.96±12.65 [a]	45.93±12.46	3.141	1.059	6.11
DB-B 100 mg/kg	2.95±.138 [a]	2.26	177.93±3.87 [a]	37.833±1.14	1.989	.872	4.703
DB-Aq 100 mg/kg	3.02±.092 [a]	2.323	131.3±1.04 [a]	38.86±2.578	1.468	.896	3.37
DB-Aq 200 mg/kg	4.360 ±.287[a]	3.35	149.43±3.01 [a]	43.13±2.614	1.670	.994	3.464
DB-Aq 400 mg/kg	2.55±.336 [a]	1.96	138.13±9.77 [a]	43.703±1.26	1.544	1.007	3.160

Values are expressed as mean ±SEM. Statistical analysis is performed by applying Two way ANOVA followed by Bonferrani's test. Where [a] = p<0.001 in comparison to control group. Saluretic index= mmol/L of test group/ mmol/L of control group. DI= Diuretic index= volume of test group/ volume in control group. HCTZ= hydrochlorthiazide. DB-Cr= crude extract of *D.brunonianum*, DB-B= butanolic fraction. DB-Aq= aqueous fraction.

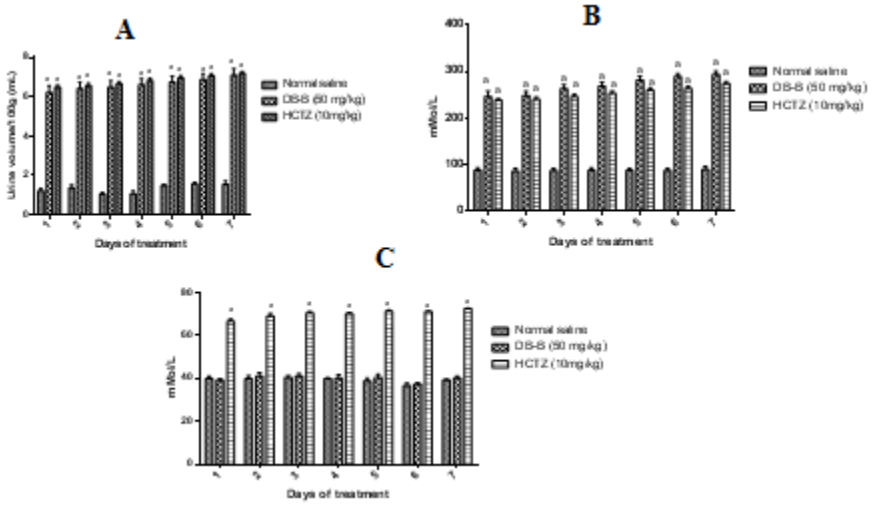


Fig. 1: Diuretic activity induced by butanolic fraction of *D.brunonianum* (DB-B) in rats. A) Alteration in urine output after daily oral treatment of DB-B. B) Effect of butanolic fraction of *D.brunonianum* (DB-B) on excretion of Na+. C) Effect of butanolic fraction of *D.brunonianum* (DB-B) on excretion of K+. Results are presented as mean ± SEM of 5 rats in each group as compared to the control group using two way ANOVA where, a = p<0.001.

all other treatment groups (table 1). Further pH and conductivity of urine sample obtained from treatment groups remains unaltered (data not shown).

Prolonged administration of DB-B showed persistent diuretic effect in rats

Daily oral administration of DB-B (50 mg/kg) and HCTZ (10 mg/kg) produced marked increase in urine excretion starting from day 1 and remains persistent till day 7 of treatment (fig. 1 A). Similarly DB-B enhanced excretion of sodium throughout the study period (fig. 1 B,C). Further, hydrochlorthiazide also increased urine excretion of sodium and potassium from day 1 to 7. The concentration of plasma electrolyte, urea and creatinine, estimated on last day of study were not significantly altered in treatment groups as compared to control group (data not shown).

Involvement of the cholinergic pathway and prostaglandin/cAMP pathway in the DB-B mediated diuretic effect

Prior administration of indomethacin to rats significantly (p<0.001) reduced DB-B induced diuresis and natriuretic effect. Similarly increase in urine and sodium excretion by treatment of DB-B was completely blocked by prior administration of atropine. However pretreatment with L-NAME did not affect diuresis and natriuresis in treatment and control group (fig. 2). All other parameters (pH and conductivity) remains unaffected in comparison to control group therefore, data was not presented.

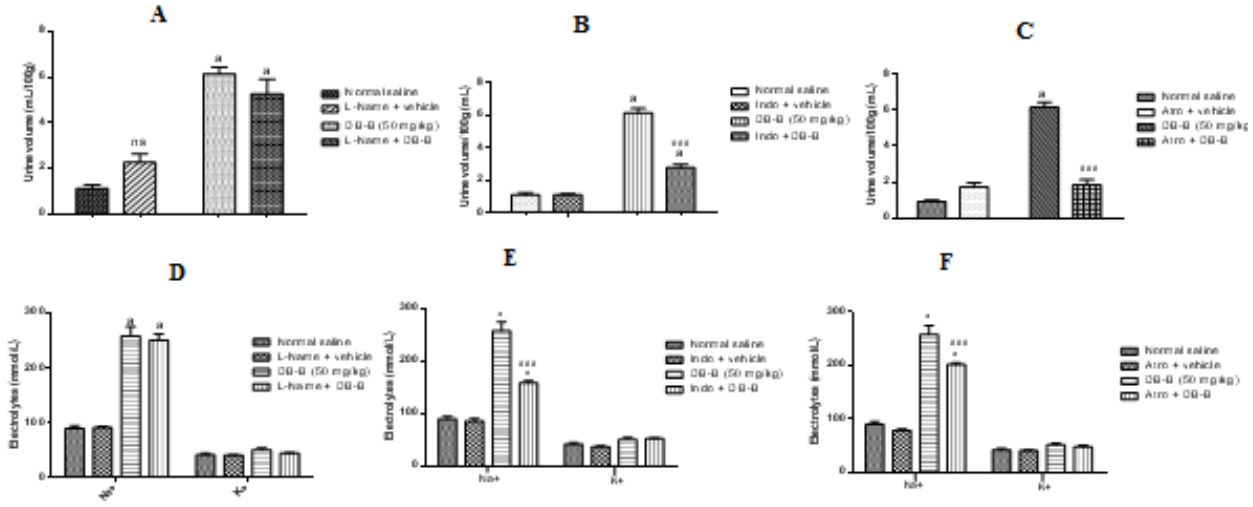
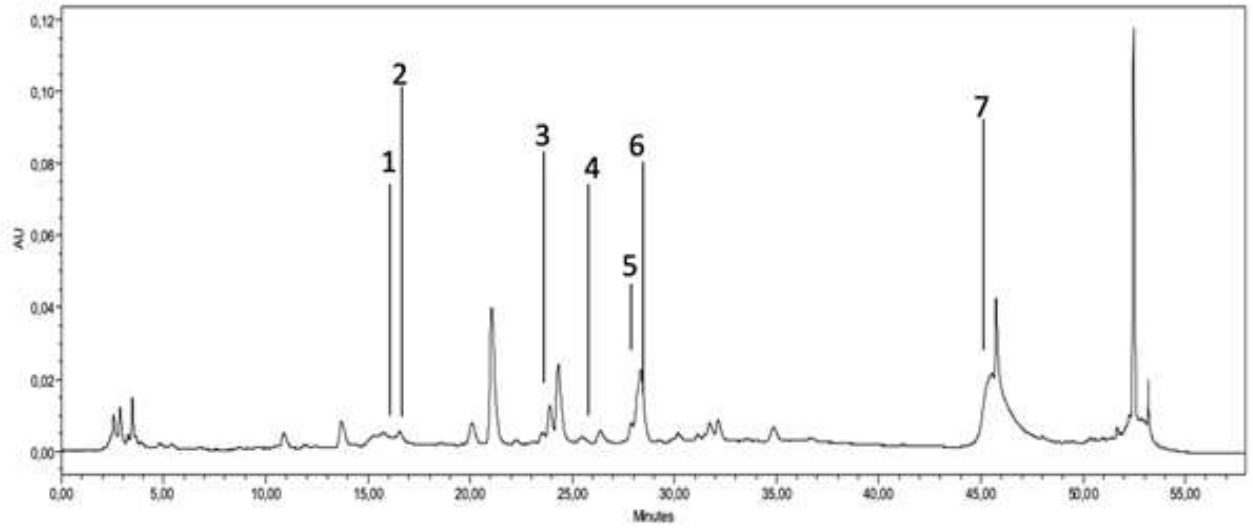


Fig. 2: Effect of muscarinic receptor blockade, nitric oxide synthase and cyclooxygenase inhibition in diuresis induced by DB-B. Effect of DB-B (50 mg/kg) on urine volume 1 h after administration of (A) L-Name (60 mg/kg) (B) indomethacin (5 mg/kg) and (C) atropine (1 mg/kg) . Alteration in urinary electrolyte excretion induced by DB-B by co-administration of various antagonists (D, E, F). Statistical analysis is performed by applying Two ANOVA using GraphPad Prism and values are expressed as mean ± SEM. a= $p<0.001$ in comparison to control group, ###= $p<0.001$ as compared to DB-B group



The peaks identified the quantified phenolics as, 1: epicatechin; 2: 3-OH benzoic acid; 3: rutin; 4: *t*-ferulic acid; 5: naringin; 6: 2,3-diMeO benzoic acid; 7: naringenin

Fig. 3: HPLC/UV-Vis Fingerprints showing presence of phenolics in DB-B

Quantification of polyphenolic contents present in DB-B

Qualitative analysis showed the presence of polphenolics 28.78 µg/mg including, individual phenolics (µg/mg) epicatechin 2.84±0.36, 3-OH benzoic acid 0.86±0.09, rutin 18.83±2.54, t-ferulic acid 1.21±0.13, Naringin 0.76±0.08, 2,3-diMeO benzoic acid 1.54±0.16, Naringenin 2.74±0.29. Fingerprint of HPLC analysis is given in fig. 3 while the retention times of standard compounds have already been published (Zenigen *et al*., 2016)

DISCUSSION

In contemporary world, medicinal plants are being extensively used for treatment of various disorders including cardiovascular diseases, hence it is the need of hour to validate the pharmacological effects of these plants. Taken into account, crude extract, butanolic and aqueous fractions of *Delphinium brunonianum* were studied for their diuretic potential and results showed that significant diuretic and natriuretic effect that was

comparable to hydrochorthiazide. Similar effects were also obtained by prolonged treatment of DB-B (50 mg/kg). Hypokalemia is major side effect observed with clinically used diuretics (Sarafidis *et al*., 2010), however, the findings of present investigation revealed that *Delphinium brunonianum* did not interfere with excretion of potassium, thus *Delphinium brunonianum* could be a promising herbal diuretic drug with minor side effects. A possibility for diuretic action of DB-B may be due to involvement of prostaglandins that interfere with renal blood flow. Prostaglandins not only play role in homeostasis of salt and water but also act as mediators of vascular tone. In addition prostaglandins are also involved in hormonal regulation of renal system and by their vasodilatory effect they affect the renal perfusion thus altering glomerular filtration rate (Gasparotto *et al*., 2009). Cyclooxygenase 1 and 2 are important enzymes that are involved in prostaglandin synthesis and are abundantly present in kidney (Green *et al*., 2012), in present study when sprague dawley rats were pretreated with indomethacin (Cox inhibitor) diuretic and natriuretic effect induced by DB-B was significantly reduced proposing that might possible DB-B is provoking diuretic effect by mobilizing the renal prostaglandins. Nonetheless, there is a need of further studies to elucidate this mechanism of diuresis and involvement of prostaglandins in diuretic effect of *Delphinium brunonianum*.

Diuretics are classified depending upon their mechanism and site of action. In this context the results of this study proposed that DB-B acts through a different mechanism than HCTZ which affects Na+/Cl- cotransporter(Wile, 2012). Numerous studies suggested that the active phytoconstituents and foods containing epicatechin and naringenin showed a promising increases in diuresis (Ansari *et al*., 2018, Mariano *et al*., 2018) as HPLC analysis revealed the presence of epicatechin and naringenin in DB-B (fig. 3) therefore we can assume that these phytoconstituent may partly be responsible for diuretic activity of this plant.

Furthermore, diuretic response elicited by DB-B was completely abolished by pretreatment with atropine a muscarinic antagonist, depicting activation of muscarinic receptors is necessary for triggering diuretic activity of *Delphinium brunonianum*. It is well known that activation of muscarinic receptors by action of acetylcholine causes vasodilation and increase urinary blood flow results in increased diuresis (Wierema *et al*., 1997).

On the other hand, in order to evaluate role of nitric oxide in diuretic response of *Delphinium brunonianum*, L-Name was given to rats 1 h prior to administration of DB-B, but the diuretic and natriuretic effect induced by DB-B remains entirely unaffected. These findings corroborate with previous study which stated diuretic effect of *Maytenus ilicifolia* in normotensive rats (Leme *et al*., 2013) and described similar results when rats were pretreated with L-Name.

CONCLUSION

In conclusion results disclosed in current study divulged that hydroalcoholic extract and fractions (butanolic and aqueous) obtained from aerial parts of *Delphinium brunonianum* presented significant diuretic and natriuretic effect when given to rats through oral route. In addition current findings revealed that diuretic effect of DB-B is reliant on generation of prostaglandins and activation of muscarinic receptors. As described herein, *Delphinium brunonianum* does not affect excretion of potassium thus it could serve as a promising alternative for development of safer diuretic formulations. In addition HPLC analysis revealed the presence of polyphenols which may responsible for diuretic potential of BD-B.

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