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THE ANTI-DIARRHEAL EFFECTS OF WHOLE PLANT EXTRACTS OF VIOLA BETONICIFOLIA AGAINST MAGNESIUM SULPHATE INDUCED DIARRHEA IN PIGEONS

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ABSTRACT

Background: Diarrhea is still a major health problem worldwide, especially in developing countries which experience high morbidity and mortality rates from it. Banafsha (Viola betonicifolia) has been used in traditional medicine of the Indo-Pak sub-continent for treating gastrointestinal disorders such as diarrhoea, however this is not scientifically supported. In this study, the anti-diarrheal activity of the aqueous whole plant extract of Viola betonicifolia was investigated in the magnesium sulphate induced diarrhoea model in pigeons.

Methods: The method used was maceration of dried powdered plant material in distilled water, filtration and evaporation to yield crude extract (3.1kg of dried powdered plant material gave 52g of crude extract in 14 days). A total of 60 healthy pigeons (250-350g) of both sexes were divided into six groups (n=8): Distilled water (negative control), loperamide (0.2 mg/kg, as positive control) and metronidazole (7 mg/kg, as positive control), and VBAE (Groups IV-VI) 50, 100, and 200 mg/kg, respectively. The drugs were injected into the muscle and diarrhea was produced 30 minutes after magnesium sulphate (2 mg/kg, orally) was given. Anti-diarrheal activity was evaluated during 6 hours using four parameters, as the time to first stool, latency to first stool, the number of stool and stool weight, the number of watery stool was scored.

Results: The result demonstrated that the dose dependent anti-diarrheal activity of the VBAE is significant. At 200 mg/kg, the extract significantly prolonged latency to first stool (27±2.30 minutes vs. 7.45±0.69 minutes in control, $p<0.001$), reduced the number of watery stools (5.43±1.44 vs. 12.27±0.15, $p<0.001$), and decreased total stool weight (3348.39±43.30 mg vs. 16613.31±17.32 mg, $p<0.001$). The 100 mg/kg dose also showed significant effects on latency (23±2.02 minutes, $p<0.001$), stool count (10±1.44, $p<0.05$), and stool weight (5023.83±37.53 mg, $p<0.001$), while the 50 mg/kg dose was less effective. The anti-diarrheal effects were comparable to standard drugs loperamide and metronidazole, particularly at the highest dose.

Conclusion: Aqueous extract of Viola betonicifolia exhibits a significant dose dependent anti-diarrheal activity against magnesium sulphate induced diarrhea in pigeon which may be due to anti-motility and anti-secretory activity. The results of this study validate the traditional application of this plant in diarrheal diseases and indicate the potential of this plant as a source of natural anti-diarrheal agents.

KEYWORDS: Anti-diarrheal, Viola betonicifolia, magnesium sulphate, pigeons, aqueous extract, loperamide, gastrointestinal motility

INTRODUCTION

Diarrhea is a common health problem in the world, which induces 1.3 million deaths every year especially for infants and young children. It is defined by changes in bowel habits and stool consistency associated with disturbances in intestinal fluid and electrolyte absorption or secretion, and is associated with increased bowel movementsn.(1) It is defined by changes in bowel habits and stool consistency associated with disturbances in intestinal fluid and electrolyte absorption or secretion, and is associated with increased bowel movements.(2, 3) There are some synthetic anti-diarrheal drugs (e.g., loperamide, metronidazole) available, but these may have side effects, develop resistance, and be less readily available in resource-limited settings. This has led to the search for alternative medicines such as medicinal plants used traditionally to control diarrheal diseases.(4, 5)

Viola betonicifolia is a member of Violaceae family and is widely found all over Pakistan, India, Nepal, Sri Lanka, China, Malaysia and Australia and is locally called as "Banafsha" in Indo-Pak subcontinent.(6, 7) Viola betonicifolia is a member of Violaceae family and is widely found all over Pakistan, India, Nepal, Sri Lanka, China, Malaysia and

Australia and is locally called as "Banafsha" in Indo-Pak subcontinent.(8) Phytochemical studies have identified various bioactive compounds such as saponins, flavonoids, tannins, proteins, phenolic compounds, alkaloids and vitamin C, and this has been responsible for its diverse pharmacological properties such as antioxidant, antibacterial, antifungal, anti-inflammatory, analgesic and neuroprotective effects. Previous studies showed that the methanolic extract of Viola betonicifolia had significant prokinetic and laxative properties in rodents, at least partially through cholinergic mechanisms mediated by the activation of muscarinic receptors.(9, 10)

The extract showed some spasmogenic activity in the isolated preparation of rabbit jejunum and guinea-pig ileum which was partially atropine-sensitive, suggesting the presence of acetylcholine-like components, as well as other stimulant components in the gut(11) Magnesium sulphate, an osmotically active saline purgative, induces diarrhea by reducing water absorption from the intestine and promoting fluid accumulation, which distends the bowel and stimulates peristalsis.(12) nterestingly, the same plant is also known to have anti-diarrheal activity, likely due to its ability to reduce water absorption from the intestine, cause fluid accumulation, expand the bowel and stimulate peristalsis, through the action of magnesium sulphate (an osmotically active saline purgative) which stimulates the release of cholecystokinin from the duodenal mucosa, thereby increasing intestinal secretion and motility.(13, 14) Thus, the present study aimed at the scientific evaluation of the aqueous whole plant extract of Viola betonicifolia for its anti-diarrheal activity by using its established experimental model which mimics secretory diarrhoea in pigeon and assessing its anti-diarrheal activity quantitatively using various parameters such as stool frequency, consistency and weight of stools in order to provide scientific support for its traditional medicinal use and investigate its possible role as a source of novel anti-diarrheal agents.

METHODOLOGY

2.1. Plant material: Whole plant of Viola betonicifolia were collected from Bodigram (Swat), Khyber Pakhtoonkhwa, Pakistan in the month of April and May 2015. The identification and confirmation of the plant species was done by Prof. Dr. Muhammad Ibrar (Taxonomist and Chairman, Department of Botany, University of Peshawar, Pakistan). All collected plant materials were washed well with distilled water to get rid of contaminants and debris and bulk collection was prepared for further processing.

2.2. Drugs and chemicals: In the study, various drugs and chemicals were used such as Sodium Chloride (Unisia Pharmaceutical Pakistan), Dimenhydrinate (Searle Pakistan Pvt. Ltd.), Magnesium Sulphate (Unisia Pharmaceutical Pakistan), Loperamide (Jansosen-Cliag Pharmaceutical Pakistan Pvt. Ltd), and Metronidazole (Unisia Pharmaceutical Pakistan). Chemicals used were all analytical grade and purchased from well-known pharmaceutical companies, assuring quality and uniformity in experimental practices.

2.3. Experimental models: Pigeons (250-350g) of both sexes were used in this experiment. Before and during the experimental procedures all pigeons had free access to a mixture of millet and wheat, locally available foodstuffs, and drinking water, which were provided in the 12-hour light (12 L) phase of the 12-hour light/dark (12:12 L/D) cycle, under room temperature conditions. Pigeons were divided into six groups (8 birds per group): Group-I as negative control received distilled water (10 ml/kg, IM) and Group-II received Loperamide (0.2 mg/kg, IM) as a positive control group while Group-III to VI received the test extract at 25, 50, 100, and 200 mg/kg, IM respectively.

2.4. Preparation of juice and extract: The whole plant with 6 Kg was dried at room temperature under the shade for 45 days. Next, the dried material (3.1 kg) was ground into a fine powder and macerated with distilled water (5 L) for 14 days at room temperature, with special agitation and stirring to guarantee the extraction process. The residue was filtered through muslin cloth and then by filter paper (Whatman No. 01) under water. This was repeated 3 times, now using a total of 3 L of distilled water. All the filtrates were collected and kept at room temperature for 40 days for complete evaporation of water. Distilled water was completely evaporated and crude aqueous extract of about 52 g was obtained which was kept safely for future use in pharmacological studies.

2.5. Anti-diarrheal activity: All healthy pigeons (both males and females) used in the experiments were starved for 6 hours prior to the experiment to reduce the gastrointestinal conditions. They were subsequently moved to individual cages and the bases of cages were lined with white milky plastic to facilitate easy observation and stool weighing and accurate counts for watery stools, dry stools, stool frequency and stool weight were obtained. Excess food and water were given ad libitum to them during the experimental period. The six groups of pigeons were divided into group 01 received distilled water (10ml/kg, IM) as negative control, group 02 received Loperamide (0.2 mg/kg, IM) as positive control, group 03 received Metronidazole (7 mg/kg, PO) as positive control, while group 04, 05 and 06 received Viola betonicifolia aqueous extract in doses of 50, 100 and 200 mg/kg IM respectively. Each pigeon in the characterized groups was administered a diarrheal agent after 30 minutes of a tested administration of the sample. All pigeons were carefully watched for 6 hours for first stool, latency to first stool, number of watery stool, number of defecation and stool weight.

2.6. Magnesium Sulphate Model: The tested samples, namely distilled water (negative control), loperamide (positive control), and VBAE (test groups) at 50, 100 and 200 mg/kg were administered to the previously described pigeon groups. Half an hour after, they were made to have diarrhea using Magnesium Sulphate at the dose of 2

mg/kg orally (PO). The anti-diarrheal activity was assessed using this model, using osmotic and secretory diarrhoea induced by magnesium sulphate.

Table 1: List of drugs and chemicals used, their routes of administration, concentration, and purpose of use:

Drugs	Dose mg/kg	Route of administration	Purpose
Distilled water	10 ml/kg	IM	Negative control
Magnesium Sulphate	2	PO	To induce diarrhea
Loperamide HCl	0.2	IM	Antidiarrheal
Metronidazole	7	PO	Antidiarrheal
Charcoal	8	PO	Charcoal meal GI transit test

2.7. Statistical analysis: Data are reported as mean ± SEM (Standard Error of Mean). One way analysis of variance (ANOVA) and Dunnett's test were used for the evaluation of anti-diarrheal activity, performed using GraphPad Prism for Windows (GraphPad Software, San Diego, California, USA). Differences were deemed statistically significant at *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ when compared to the negative control group.

RESULTS:

3.3.4. Effect of VBAE against Magnesium Sulphate induced diarrhea

Magnesium Sulphate shows successful induction of diarrhea in different experimental animals. The mechanism by which Magnesium Sulphate induces diarrhea is associated with anticipation of the reabsorption of electrolytes and water from the small intestine and increasing the contents of experimental animal's intestine which can induce soft and watery stool. Report shows that cholecytokinin is liberated from the mucosa of duodenum by the action of administration of Magnesium Sulphate which increase the motility and the secretion of small intestine (15).

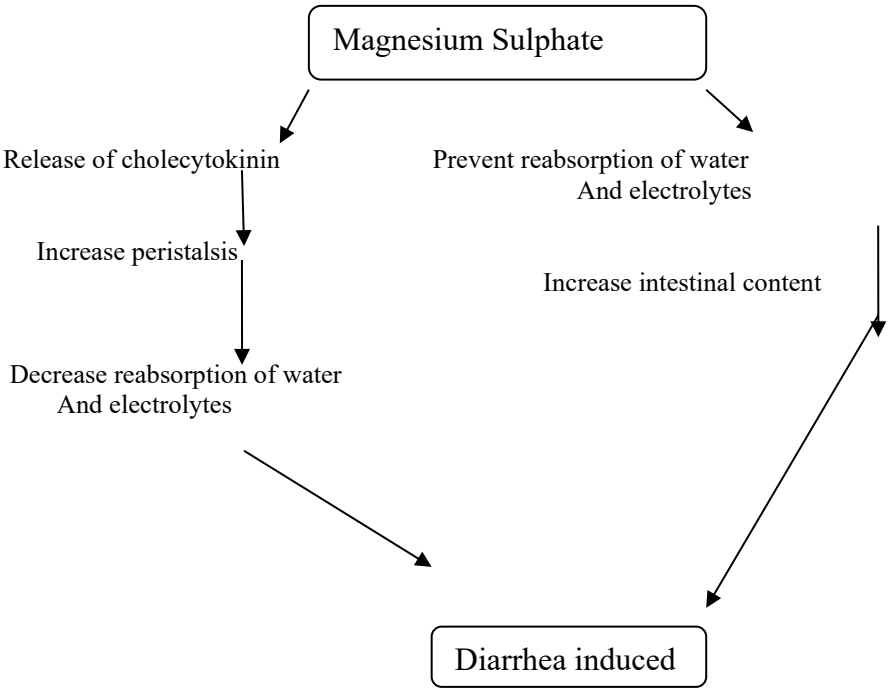


Figure 1: Mechanism of Magnesium Sulphate induced diarrhea

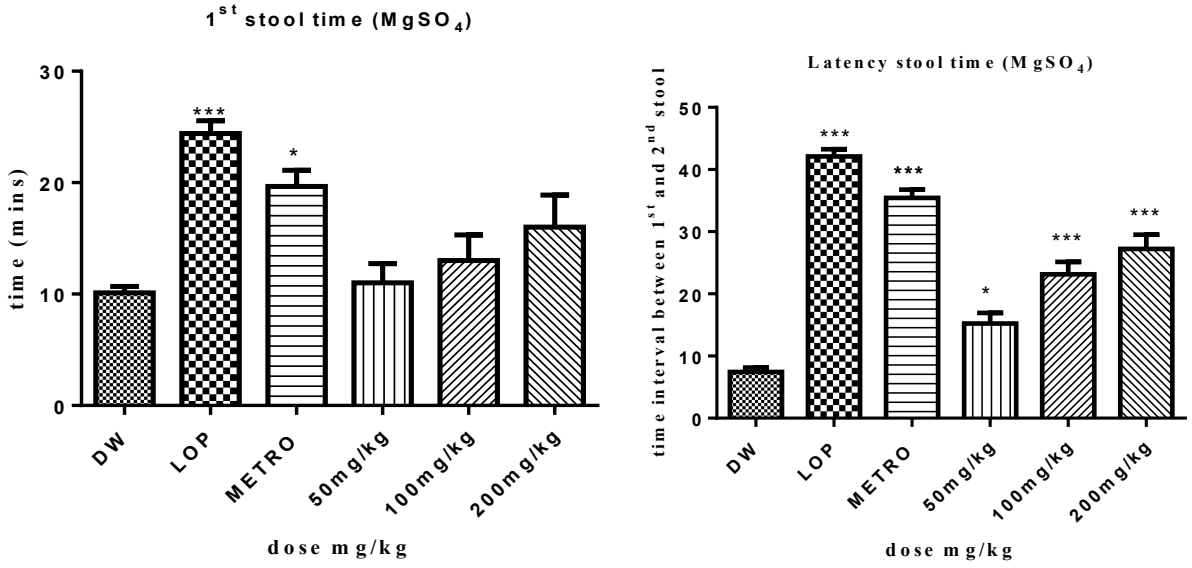
VBAE at the dose of 50mg/kg were not significant to 1st stool, number of stool and number watery stool, whereas showing significant ($p<0.05$) to latency to 1st stool time, but express more significant ($p<0.001$) to weight of stool. Tested sample at 100mg/kg was not significant for start of stool time, but was largely ($p<0.001$) and significantly ($p<0.05$) for number of stool and number of watery stool and was also significantly ($p<0.001$) for weight of stool.
At 200mg/kg our extract was not significant to 1st stool time, at the same time confirm significant ($p<0.01$) to number of stool, even more significant ($p<0.001$) in case of latency to 1st stool time, no watery stool moreover to weight of stool in compared to negative control group. VBAE at high dose is not significant in 1st stool time

induced by Magnesium Sulphate while valuable ($p<0.001$) which is (27 min) decrease in latency to 1st stool time in contrast to negative control group. Our test sample express noteworthy ($p<0.01$) result in case of number of stool but showing most significant ($p<0.001$) in reduction of watery stool as well as weight of stool.

Table 2: Effect of VBAE on Magnesium Sulphat induced diarrhea

Sample	Dose(mg/kg)	1st stool time (min)	Latency to 1st stool time (min)	Number of stool	Number of watery stool	Weight of stool (mg)
Administration of Magnesium Sulphate after 30 minutes						
DW+ $MgSO_4$	5ml/IM,1gm/kg, PO	10.12±0.57	7.45±0.6928	16.56±0.40	12.27± 0.15	16613.31±17.32
LOP	0.2	$24.42\pm1.15^{***}$	$42.12\pm1.15^{***}$	$7.23\pm0.57^{***}$	$2.87\pm0.50^{***}$	$2969.76\pm20.21^{***}$
METRO	7	$19.67\pm1.44^{*}$	$35.45\pm1.38^{***}$	$9.51\pm1.32^{***}$	$4.77\pm0.57^{***}$	$3212.83\pm25.00^{***}$
VB	50	11 ± 1.73^{ns}	$15\pm 1.73^{*}$	12 ± 1.15^{ns}	9.65 ± 0.86^{ns}	$8998.42\pm34.64^{***}$
VB	100	13 ± 2.30^{ns}	$23\pm 2.02^{***}$	$10\pm 1.44^{*}$	$7.51 \pm 1.15^{*}$	$5023.83\pm37.53^{***}$
VB	200	15 ± 2.88^{ns}	$27\pm 2.30^{***}$	$8\pm 1.73^{**}$	$5.43\pm1.44^{***}$	$3348.39\pm43.30^{***}$

The data is shown as mean ± SEM (n = 8). For Data analysis One way ANOVA followed by Dunnett's were applied. The asterisks indicate significant value compared to the negative control group, P< 0.05, **p< 0.01 and ***p< 0.001.



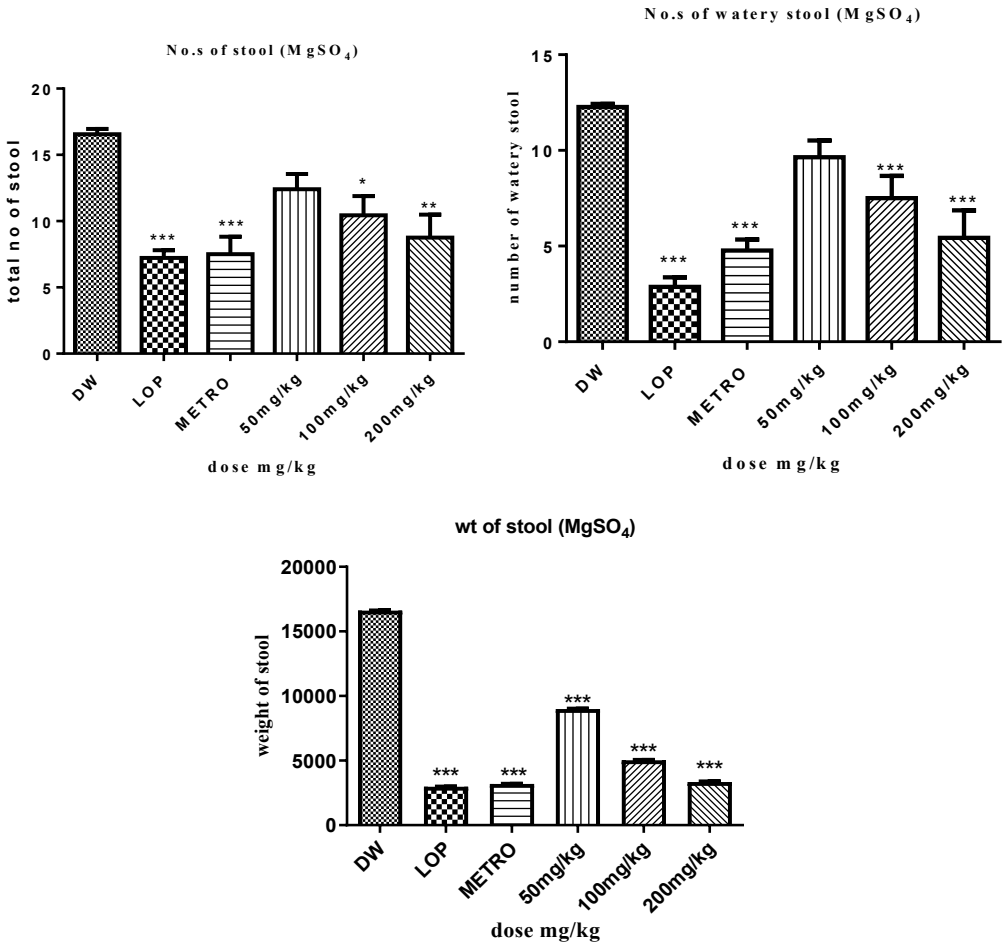


Figure 2:Bar diagram indicating dose dependent effect of VBAE against Copper Sulphte induced emesis in pigeons.

Bar represent as mean ± SEM for group (n=8). One way ANOVA followed by Dunnett's test was applied for data analysis. *$p<0.05$,**$p<0.01$, ***$p<0.001$ versus negative group(Asterisks representing statistically significant value from control)

DISCUSSION

The results of the present study indicate that the aqueous extract of Viola betonicifolia whole plant showed significant dose dependent anti-diarrheal activity in pigeon induced with magnesium sulphate which supports the use of Viola betonicifolia in gastrointestinal problem. Magnesium sulphate-induced diarrhea is an established experimental model which mimics secretory diarrhea by causing intestinal fluid accumulation in the lumen through osmotic effect and stimulation of intestinal motility, and is appropriate to test anti-motility and anti-secretory drugs.(16) Diarrhea is induced by a decrease in water and electrolyte absorption from the small intestine resulting in increased intestinal content and soft, watery stools, and release of cholecystokinin from the mucosa of the duodenum, which potentiates peristalsis and secretion.(17)

The administration of different doses of VBAE (50, 100 and 200 mg/kg) gave a dose dependent protection from magnesium sulphate diarrhea as reflected by the increase in the time taken to expel the first stool, reduction in the numbers of stools and watery stools and reduction in the weight of the stool, compared to the negative control group. Specifically, the latency period to first stool was significantly increased with the use of VBAE at 200 mg/kg (27 minutes, $p<0.001$), the number of watery stools was reduced (5.43±1.44, $p<0.001$), and the stool weight was decreased (3348.39±43.30 mg, $p<0.001$) when compared with the standard anti-diarrheal agents, loperamide and metronidazole. Loperamide is a synthetic opioid receptor agonist that is used to reduce the motility of the intestines and to increase the absorption of fluid, and metronidazole is an antimicrobial agent that has anti-diarrheal activity due to its anti-bacterial and anti-inflammatory activity. (18)

The antidiarrheal effect of VBAE may be due to several mechanisms that might include both anti-motility and anti-secretory activities, which are in accordance with the pharmacologic properties of Viola betonicifolia. The previous

investigations were conducted using mice and rats, and revealed that crude methanolic extract of V. berthingiana (VBME) stimulated the gut in a dose-dependent manner that was partially blocked by atropine, suggesting the involvement of cholinergic pathways via activation of muscarinic receptors. VBME induced dose-dependent contractions in isolated rabbit jejunum and guinea-pig ileum preparations, which was partially inhibited by atropine but not by pyrilamine (a histamine receptor blocker), hexamethonium (ganglion blocker) or SB203186 (serotonergic receptor antagonist), indicating the presence of acetylcholine-like components as well as other stimulatory mechanisms to the gut besides histamine, nicotine, or 5-HT receptor pathway(19) Viola betonicifolia is rich in phytochemicals such as alkaloids, saponins, flavonoids, and phenolic compounds, which underlie its various pharmacological properties. Alkaloids and saponins are known to have gut stimulatory activity, and the presence of these compounds in the plant extract may be responsible for the anti-diarrheal activity by interacting with the gut receptors and the modulation of the smooth muscle contractility.(20)

n addition, the antioxidant activity of the plant (high scavenging activity on DPPH assays with IC50 values of 80-500 μg/mL, depending on the solvent used) may play a role in their protective activity against oxidative stress generated by diarrhea.(21) The aqueous extract of Viola betonicifolia has been found to possess antimicrobial activity against a wide range of pathogens such as Escherichia coli, Bacillus subtilis, Shigella flexneri, Staphylococcus aureus, Pseudomonas aeruginosa and Salmonella typhi with zone of inhibition up to 20mm for chloroform fraction of Viola betonicifolia against E. coli and 17mm against S. typhi. The anti-diarrheal activity of this plant may be due to both symptomatic relief and etiological treatment, as the plant has been known to have antibacterial activity which may be more relevant in the treatment of infectious diarrhea, in which bacterial pathogens are prominent.(22)

The use of pigeons as experimental animals in this study is well justified because they have been used extensively in previous pharmacological studies of anti-diarrheal drugs such as that of Cuscuta reflexa that showed strong anti-diarrheal properties in a dose dependent manner.(23) The method of intramuscular injection of the extract is not conventional for gastrointestinal studies, but guarantees the correct dosage and eliminates any problems with oral administration that may result from gastrointestinal absorption and first-pass metabolism, while making it possible to assess the pharmacological activity accurately.(24) The dose-dependent nature of the anti-diarrheal effects observed in this study, with 200 mg/kg showing maximum efficacy, suggests the presence of active phytoconstituents that act in a concentration-dependent manner.(25)

The positive anti-diarrheal activity of the extract shown in this study is dose dependent, which indicates that it has some active phytoconstituents with concentration-dependent activity. (25)

Diarrhea may be alleviated if CCK release is inhibited or CCK receptors are blocked, which will decrease intestinal secretion and motility. The increased latency period to first stool with VBAE treatment is further evidence of anti-motility mechanism as longer gastrointestinal transit will provide more time for fluid and electrolyte absorption, leading to formed stool. This is consistent with the action of loperamide, which directly targets the opioid receptors to cause a reduction in peristalsis and increase in sphincter tone, thus minimizing fluid loss (26, 27)

In this study, the standard positive controls, loperamide and metronidazole, were used as a reference to evaluate the efficacy of VBAE. The extract had effects similar to loperamide (0.2 mg/kg) on weight and frequency of stool; loperamide was more effective in the prolongation of the latency for the first stool and reduction of the number of watery stools. This indicates that VBAE has a high anti-diarrheal activity effect that might need optimisation of the dose or formulation to obtain maximum therapeutic efficacy. The moderate efficacy of the extract when compared to the standard drugs is not surprising since crude plant extracts are usually composed of both active and inactive compounds and the isolation of the active principles could possibly lead to the development of more effective anti-diarrheal drugs from the extract. This marked decrease in stool weight is of note, specifically, because it was a significant decrease in stool output and loss of fluids; which is the main treatment objective in a case of diarrhea. In negative control group, the stool weight after 200 mg/kg of VBAE was 16613.31±17.32 mg, whereas the 200 mg/kg of administration group stool weight was 3348.39±43.30 mg, which is 80% reduction in stool weight. This remarkable effect indicates that the extract completely reverses the osmotic and secretory effect of magnesium sulphate, perhaps by increasing the reabsorption or decreasing the secretion in the intestinal epithelium. (28)

The safety profile of viola betonicifolia is supported by its long tradition of use and the fact that no toxicity has been reported at the dose used in the present study, or in previous experimental studies showing its safety in animal models. The neuropharmacological investigations of hexane extract of the plant have revealed anxiolytic, muscle relaxant, sleep inducing and sedative activities at the doses of 0.3-0.5 g/kg of mice with a large safety margin. Considering this safety profile and its proven anti-diarrheal effectiveness, Viola betonicifolia is a useful plant for formulation of new anti-diarrheal drugs, especially in areas where conventional drugs are prohibited due to lack of resources. (29)

The results of this study also demonstrate the value of the magnesium sulphate modèle for the assessment of anti-diarrheal drugs, since the methodology is reproducible and quantifiable and represents an experimental model that closely mimics the clinical picture of diarrhoea. Multiple parameters were used such as latency to first stool, stool frequency, watery stool count and stool weight, to evaluate the anti-diarrheal effects in order to account for the anti-

motility and anti-secretory activity of the extract's activity. The results are statistically significant as p-values for the various parameters range between <0.05 and <0.001, which further strengthens the findings and validity of the conclusions drawn. In conclusion, the present study supports the traditional medicinal applications of Viola betonicifolia aqueous extract in gastrointestinal ailments and further research is warranted to elucidate the mechanism of action and clinical uses of this extract in diarrhea.

CONCLUSION

In pigeons, the aqueous extract of Viola betonicifolia showed dose dependent anti-diarrheal activity as manifested by increase in latency to first stool, decrease in watery stool passage and decrease in stool weight (200mg/kg) as compared to the standards. The results confirm the traditional application of this plant in diarrheal disorders, which are likely to be mediated by anti-motility and anti-secretory properties. The study has found that medicinal plants have the potential to be used in the treatment of gastrointestinal diseases and has highlighted the importance of maintaining traditional knowledge by critically examining it. Viola betonicifolia is recommended as a potential source for future studies towards safe and effective anti-diarrheal formulation. This is especially important for those areas of the country where access to traditional medicines is restricted.

RECOMMENDATIONS

HPLC and GC-MS analysis for phytochemical characterization should be used to identify bioactive compound which can be used for standardization and quality control of extracts. Mechanistic studies with receptor antagonists are needed to elucidate molecular pathways involving cholinergic, opioid, and serotonergic systems. The compounds isolated should be subjected to screening for finding lead compounds to develop new anti-diarrheal agents. Standardized preparations must be validated in humans through clinical trials to ensure safety, efficacy, dosing and drug interaction. To ensure that stable formulations are developed and thorough toxicity testing is conducted, as well as comparison cost-effectiveness assessments with standard drugs in resource-limited environments.

LIMITATIONS

Since there are physiological differences in the gastrointestinal tract and drug metabolism between species, the extrapolation of results from pigeon models to humans is limited. Traditional oral use is not reflected in the intramuscular administration, which needs to be taken into account for the assessment of bioavailability. The complex aqueous extract has undergone no phytochemical standardization, which makes it difficult to repeat and find the constituents. Only efficacy in magnesium sulphate-induced diarrhea was considered and no mechanistic study of ion transport or neurotransmitter effects were performed. There is limited generalizability and evaluation of toxicity for clinical use due to the small sample size (n=8), short observation time (6 hours) and lack of toxicity studies.

Ethics approval

The protocols and procedures used for animals and their care followed the ethical guidelines approved by the Department of Pharmacy,

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