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PHYTOCHEMICAL SCREENING, ANALGESIC, ANTI-INFLAMMATORY AND ANTI-DIARRHEAL EFFECT OF *VERNONIA PATULA* WHOLE PLANT

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Abstract

Vernonia patula has been used in the traditional medicine for the treatment of a wide range of medical problems. The present study screened phytochemicals and also investigated the anti-nociceptive, anti-inflammatory and anti-diarrheal activities of *Vernonia patula*. Swiss albino mice of either sex weighing 25-30 gm were divided into control, standard and test extract groups with 6 animals in each group. The extract was used at 250 and 500mg/kg dose per orally. Analgesic activity was evaluated by acetic acid-induced writhing and formalin induced paw licking test. Anti-inflammatory effect was investigated by ear edema test with xylene and croton oil. Antidiarrheal effect was evaluated by castor oil and $MgSO_4$ induced diarrheal test in mice. Phytochemical screening of the alcoholic extract exhibited the presence of carbohydrate, glycosides and diterpenes. In the acetic acid-induced writhing model, the extract had a significant ($p<0.01$) analgesic effect characterized by a reduction in the number of writhes at 500mg/kg when compared to the control. The results showed that the extract had significant analgesia in formalin test ($p<0.001$). The test extract reduced the ear edema in xylene test ($p<0.05$) and croton oil test ($p<0.01$). The extract had a slight antidiarrheal property in castor oil as well as in $MgSO_4$ induced diarrhea model. *Vernonia patula* whole plant extract showed antinociceptive and anti-inflammatory effect against acute and chronic inflammation. It also possessed mild antidiarrheal properties and further details investigations are required to evaluate these effects with exact mechanism.

Keywords: *Vernonia patula*, Phytochemicals analgesic, anti-inflammatory, anti-diarrheal effect.

Introduction

Inflammation refers to a complex biological response of vascular tissues against harmful stimuli like pathogens, damaged cells or irritants. It is also the protective attempt by the organism to the injurious stimuli as well as initiate healing process for the tissue and considered to be the major cause of rheumatoid arthritis [1,2]. The inflammatory response can lead different diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis. Pain is a sensorial modality, primarily protective in nature, but often causes discomfort. It is the most important symptom that brings the patient to physician. Analgesics relieve symptoms of pain, but hardly affect its underlying cause [3].

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical complications, though relatively little knowledge about their mode of action is available. From the very broad flora of Bangladesh, several native Bangladeshi medicinal plants have a long tradition of use with great phytotherapeutic potential [4]. Thus, a scientific evaluation of herbs can incorporate an alternative medical option with easy access to underprivileged population.

Vernonia patula (family: Asteracea), known as "kukshim" in Bengali, also called little iron weed is a perennial grass with erect stem seen in the mainland of China, Fujian, Guangdong, Guangxi, Jiangxi, Hunan, Sichuan, Yunnan and Vietnam, Myanmar, India, Bangladesh, Srilanka, Malay island, the Philippines, Australia, Africa, New Zealand, Asia and other places [5,6]. The whole plant is edible and can be used as a medicine [7]. The plant is used in smoking cessation, cough, fever, malaria, urinary calculi, arthritis and leprosy. It possesses antimicrobial, antioxidant, antihelmentic, antiinflammatory, analgesic, antipyretic, antiflautulent, antispasmodic and antidiuretic properties [8-16]. Therefore, in the present study we attempted to evaluate the phytochemical screening, antinociceptive, anti-inflammatory and antidiarrheal activities of *Vernonia patula* whole plant.

Methods

Plant materials collection and preparation of sample

The plant, *Vernonia patula* (abbreviated as VP) whole plant was collected from Savar, Dhaka. The plant was taxonomically authenticated and identified from the Bangladesh National Herbarium, Mirpur, Dhaka (Accession number: DACB 45771). The collected materials were thoroughly washed in water and shed dried at 35°–40°C for a week and pulverized in electric grinder to get extractable powder. Then powder was extracted in soxhlet apparatus with ethanol, dried with a rotary evaporator and finally the extract was preserved in the refrigerator for further experimental use.

Experimental Animals

Swiss albino mice of either sex, 6-7 weeks of age, weighing 25 to 30g and Wister rats weighing 150-180gm of either sex were collected from the animal research lab in the Department of Pharmacy Jahangirnagar University, Savar, Dhaka. Animals were maintained under standard environmental conditions and had free access to food and water *ad libitum*. The animals were acclimatized to laboratory condition for one week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Toxicity studies

Toxicity studies of the extracts were carried out in Swiss Albino mice of either sex weighing 25 to 30g. The extract was found to be safe till 5000 mg/kg p.o [17].

Phytochemical screening

Following phytochemical tests were performed [18].

Chemical Constituents	Test	Method
Test for Carbohydrates	Molish Test	2ml of filtrate +2 drop of molish reagent few drops of concentrated H_2SO_4.
	Fehling's Test	2 ml of filtrate was taken and 5-8 drops of Fehling's solution was added and heated on water bathe for half an hour.
Test for Tannins	Ferric Chloride Test	2ml of filtrate+10ml distilled water +a drops of $FeCl_3$
Test for Flavonoids	Hydrochloric Acid Test	2ml of filtrate + few drops of concentrated HCL and Mg turnings were added.

Test for Saponins	Foam Test	2ml of filtrate + 4ml of distilled water were mixed well and shaken vigorously.
Test for Alkaloids	Mayer's Test	Filtrate was treated with Potassium Mercuric Iodide.
	Wagner's Test	Filtrates were treated with (iodine in potassium iodide).
	Dragendroff's Test	Filtrates were treated with solution of Potassium Bismuth Iodide.
Test for Glycosides	Modified Borntrager's test	Filtrates were with FeCl3 solution boiling for 5 mins & mixture was cooled & extracted with equal volume of Benzene. The benzene layer was separated & treated with Ammonia solution.
Test for Diterpenes	Copper acetate test	Filtrates were dissolved in water treated with 3-4 drods of copper acetate solution

Experimental Design

Animals were divided into four groups of six animals each.

Group	**Administered Substance and Dose**
Group I	Control (distilled water, 10ml/kg b.w.)
Group II	Standard (Specific to the model)
Group III	*Vernonia patula* ethanolic extract (250 mg/kg b.w.)
Group IV	*Vernonia patula* ethanolic extract (500 mg/kg b.w.)
All doses were administered per orally	

Analgesic activity evaluation

Acetic acid induced writhing test

The method according to Koster *et al.* (1959) was employed for this test. Four groups of six mice each were pre-treated with normal saline (10 mL/kg), diclofenac-Na (100 mg/kg) and the *Vernonia patula* alcoholic extract (250 mg and 500 mg/kg) respectively. Forty five minutes later each mouse was injected with 0.7% acetic acid at a dose of 10 mL/kg body weight. The number of writhing responses was recorded for each animal during a subsequent 5 min period after 15 min of the I.P. administration of acetic acid and the mean abdominal writhes for each group was obtained [19].The percentage inhibition was calculated using the formula:

$$Inhibition(\%) = \frac{\left(\begin{matrix}Mean\ number \\ of\ writhes\ (control)\end{matrix}\right) - \left(\begin{matrix}(Mean\ number \\ of\ writhes\ (drugs)\end{matrix}\right)}{Mean\ number\ of\ writhes\ (control)}$$

Formalin-induced Paw licking test

The method of Hunskaar and Hole (1987) was used for the study. Mice were divided into 4 groups of 6 animals each. Group 1, the control group received normal saline, p.o., group 2, the standard group received Diclofenac-Na (100 mg/kg). Groups 3, 4, received Ethanolic extract (250 mg/kg and 500 mg/kg). After 1 hour of drug administration, 2.7% formalin was injected into the dorsal surface of the left hind paw. The time spent licking the injected paw was recorded. Animals were observed for the 5 min post formalin (acute phase) and for 5 min starting at 15th min post formalin (delayed phase) [20].

Anti-inflammatory activity evaluation

Xylene-Induced Ear Edema test

Mice were divided into 4 groups of 6 animals each. Group 1, the control group received normal saline, p.o., group 2, the standard group received diclofenac-Na (100 mg/kg). Groups 3 and 4 received alcoholic extract (250 and 500 mg/kg). One hour later, each animal received 20µl of xylene on the anterior and posterior surfaces of the right ear lobe. The left ear was considered as control. Mice were sacrificed one hour after xylene application and circular sections were taken, using a cork borer with a diameter of 3 mm, and weighed. The percentage of ear edema was calculated as inflammation based on the weight of left ear without xylene [21].

Croton oil induced ear edema test

The croton oil induced ear edema test was performed as described Zitterl-Eglseer (1997). Mice were divided into 4 groups of 6 animals each. Group 1, the control group received normal saline, p.o., group 2, the standard group received diclofenac-Na (100 mg/kg). Groups 3 and 4 received alcoholic extract at dose of 250 and 500 mg/kg p.o. respectively. One hour later, each animal received 15µl of croton oil on the posterior surfaces of the right ear lobe and 15µl acetone on the inner surface of left ear lobe. Ears of mice were cut one hour after croton oil application and circular sections were taken, using a cork borer with a diameter of 3 mm, and weighed [22].

Antidiarrheal potential evaluation

Castor oil induced diarrhea in mice

The method as described by Jebunnessa *et. al.,* (2009) was used with slight modification. Twenty four mice of either sex fasted for 12 h were allocated to four groups of six animals each. Group I (received DW at a dose of 10 ml/kg p.o.) served as control group, Group II (received loperamide 10 mg/kg, p.o.) served as standard, Group III and IV received extract of *V. patula* at the dose of 250 & 500 mg/kg b.w. p.o., respectively. One hour after administration, mice were fed castor oil orally at a dose of 0.5ml per mouse to induce diarrhoea. Each animal was placed in an individual cage, the floor of which was lined with white blotting paper which was changed every hour. The total number of both dry and wet faeces excreted by the animals was counted every hour for a period of 4 hours [23]. The total number of diarrheal faeces of the control group was considered 100%. The activity of each group was expressed as percent inhibition (%) of defecation and percent inhibition (%) of diarrhoea.

Magnesium sulphate-induced diarrhea

Twenty four mice of either sex fasted for 12 hours were divided into four groups of 6 mice each. Group I (received DW at a dose of 10 ml/kg p.o.) served as control group, Group II (received loperamide 10 mg/kg, p.o.) served as standard, Group III and IV received extract of *V. patula* at the dose of 250 & 500 mg/kg b.w. respectively. All treatments were given orally. After 1 hour, each mouse received magnesium sulphate by oral route. The animals were placed individually in cages over white filter paper. The number of wet feces was recorded for a period of 4 h. [24]. The activity of each group was expressed as percent inhibition (%) of defecation and percent inhibition (%) of diarrhea.

Statistical Analysis

Microsoft Office Excel (2007) was used as a statistical tool for % inhibition assay data. Statistical analysis for animal experiments was carried out using One way ANOVA following Dunnett's post hoc comparison test by SPSS 16.0 for windows. Data were presented as Mean±SEM. The results obtained were compared with the vehicle control group. $p<0.05$, $p<0.01$ and $p<0.001$ were considered to be statistically significant, highly significant and very highly significant respectively.

Result and Discussion

Phytochemical screening of the plant extract confirmed the presence of several bioactive compounds **carbohydrates, glycosides** and **diterpenes** which may be responsible for the versatile medicinal properties of this plant and for *in-vivo* findings.

The result of acetic acid induced writhing presented in table 1 and figure 1 which showed that the alcoholic extract of *V. patula* at 250mg/kg reduced the number of writhing insignificantly whereas at 500 mg/kg it reduced the number of writhing response highly significantly ($P<0.01$) when compared to the control group. The antinociceptive power was 17.52% and 58.07% respectively indicating that the extract has potent analgesic effect at 500mg/kg which was slightly lower but comparable to the reference drug (diclofenac-Na, 100 mg/kg).

Formalin induced paw licking test indicated that, the extract of *V. patula* caused a dose-dependent decrease in licking time. At both first five as well as second five minutes, the effect was significant ($p<0.001$ & $p<0.05$) at dose 500 mg/kg as compared to the control group treated with vehicle only. At early phase, the extract at 250 mg and 500 mg/kg dose, inhibited algesia by 11.65% and 48.35% respectively. This inhibitory effect at late phase was 31.55% and 78.86% by 250mg and 500mg/kg doses respectively as compared to the control group (table 2 and figure 2).

The result of xylene induced ear edema test showed that the effect was not significant at 250mg/kg but at 500 mg/kg, significantly ($p<0.05$), suppressed the ear swelling in mice. The rate of inhibition was 27.23% and 55.19% at 250 mg and 500 mg/kg

respectively. Diclofenac (100 mg/kg) showed significant ($p<0.05$) anti-inflammatory activity with a 59.60% reduction of inflammation as compared to the control (table 3 and figure 3).

Antiinflammatory effect of VP in croton oil induced ear edema was shown in table 4 & figure 4. The VP extract reduced the inflammation insignificantly at 250mg/kg. But at 500mg/kg reduced inflammation highly significantly ($p<0.01$) as compared to the control group. The rate of inhibition of ear swelling at 250mg and 500mg/kg was 16.06% and 53.35% respectively. The standard reference drug (diclofenac-Na, 100 mg/kg) inhibited ear swelling very highly significantly ($p<0.001$) and it was 78%.

The castor oil induced diarrhea test was carried out to assess the effect of ethanolic extract of VP in experimentally induced diarrhea in rodent. The results showed that, there has been a statistically insignificant reduction in the incident and severity of diarrhea with higher dose of the crude extract of VP in experimental animals (table 5 and figure 5). Ethanolic extract of VP at 250 and 500mg/kg showed no significant reduction of defecation. The plant crude extract of VP at 250 mg/kg and 500 mg/kg reduced the number of fecal episodes by 21.15% & 30.77% respectively and reduced the diarrheal episodes by 24.24% & 35.33% respectively. Loperamide (10mg/kg, p.o.) profoundly ($p<0.01$), inhibited the fecal output (53.19%) and the diarrheal episodes (60.61%) produced by castor oil.

The results of the effect of VP on $MgSO_4$ induced diarrhea have been showed in table 6 and figure 6. Ethanolic extract of VP at both the doses (250mg/kg and 500 mg/kg) showed no significant effect on defecation and diarrhea. The crude extract at 250mg/kg and 500mg/kg reduced the diarrheal episodes by 20.14% and 35.97% respectively when compared to the control group. The standard drug loperamide (10mg/kg, p.o.) profoundly ($p<0.01$), inhibited both the fecal output (48.23%) and the diarrheal episodes (71.94%) produced by magnesium sulfate.

In traditional system of medicine, certain plants are claimed to provide relief of pain and inflammation. Different parts of *vernonia patula* are used by traditional practitioner for their anti-inflammatory, anticonvulsant, antiulcer, antitumor, antimicrobial, antifungal, antipyretic, blood purifier and anti-asthmatic. The analgesic, anti-inflammatory and anti-diarrheal effects of the extract was investigated in the present study. The analgesic activities were evaluated by chemically induced tissue damage animal models, which could provide response to two different grades of noxious stimuli [25]. Acetic acid causes an increase in peritoneal fluids of PGE2 and PGF2α, serotonin and histamine involved in part, which is a model commonly used for screening peripheral analgesics [26, 27].

Formalin test is believed to be a more valid analgesic model which is better correlated with clinical pain [28,29]. Formalin test is biphasic and measures pain of both neurogenic (first phase) and of inflammatory origin (second phase). The first phase (0–5min) being a result of direct stimulation of nociceptors measures centrally mediated effects and is insensitive to anti-inflammatory agents while the second phase (15 – 30 min) which is qualitatively different from the first phase is dependent on peripheral inflammation and changes in central procession due to chemical mediators release from damaged cells that stimulate nociception and thus induce pain [20]. This test measures the response to a long lasting nociceptive stimulus similar to clinical pain [28] and is recommended as a tool in basic pain research for studying the mechanisms of analgesic agents because of its connection to tissue injury. The ability of VP extract to inhibit first phase of the formalin test more prominently indicates its involvement in peripherally mediated neurogenic pain activity.

The application of mouse models of ear edema induced by different irritant agents (Croton oil, xylene, caVPaicin, AA, phenol, histamine) have been widely used to identify the probable topical anti-inflammatory effect of the substance in study and to propose its possible mechanism of action [30]. Xylene induced neurogenous swelling, a common inflammatory model, was selected for evaluating vascular permeability which was partially associated with substance P [31]. Xylene causes instant irritation of the mouse ear, which leads to fluid accumulation and edema characteristic of the acute inflammatory response [32]. The results showed that VP extract at the dose of 500 mg/kg, significantly suppressed the ear swelling caused by xylene in mice.

Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main

irritant agents. TPA is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen activated protein kinases (MAPK), and phospholipase A2 (PLA2), leading to release of platelet activation factor (PAF) and AA. This cascade of events stimulates vascular permeability, vasodilation, polymorphonuclear leukocytes migration, release of histamine and serotonin and moderate synthesis of inflammatory eicosanoids by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes [33,34]. COX and 5-LOX inhibitors, leukotriene B4 (LTB_4) antagonists and corticosteroids show topical anti-inflammatory action in animal models of Croton oil or TPA-induced skin inflammation [35]. The crude extract of VP significantly reduced the inflammation which may due to the inhibition of histamin release as well as eicosanoids synthesis.

Diarrhoea is one of the most prominent reasons (7.1 million incidents per year) of malnutrition and death among the children in the world, especially in the developing countries [36,37]. It is manifested by increased gastrointestinal movement, watery or wet stool, and abdominal pain [38].

Castor oil stimulates the peristaltic activity as well as generation of giant contractions of the intestine and transverse and distal colon, leading to the changes in the electrolyte (Na^+ and Cl^-) permeability of the intestinal mucosa [39,40]. The extract *Vernonia patula* showed insignificant effect in castor-oil induced diarrhea.

Magnesium sulfate acts as laxative. $MgSO_4$ may stimulate the release of cholecystokinin, which leads to intraluminal fluid & electrolyte accumulation & to increased intestinal motility [41]. The ethanolic extract of *Vernonia patula* showed insignificant effect in $MgSO_4$ induced diarrhea.

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Table 1: Effect of VP 250mg/kg and VP 500mg/kg in the acetic acid induced writhing test.

Group	Number of Writhing (Mean ±SEM)	% inhibition of algesia
Control	12.33±2.54	00
STD (Diclofenac-Na, 100 mg/kg)	4.83±1.58***	60.83
VP 250mg/kg	10.17±1.35	17.52
VP 500mg/kg	5.17±0.60**	58.07

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

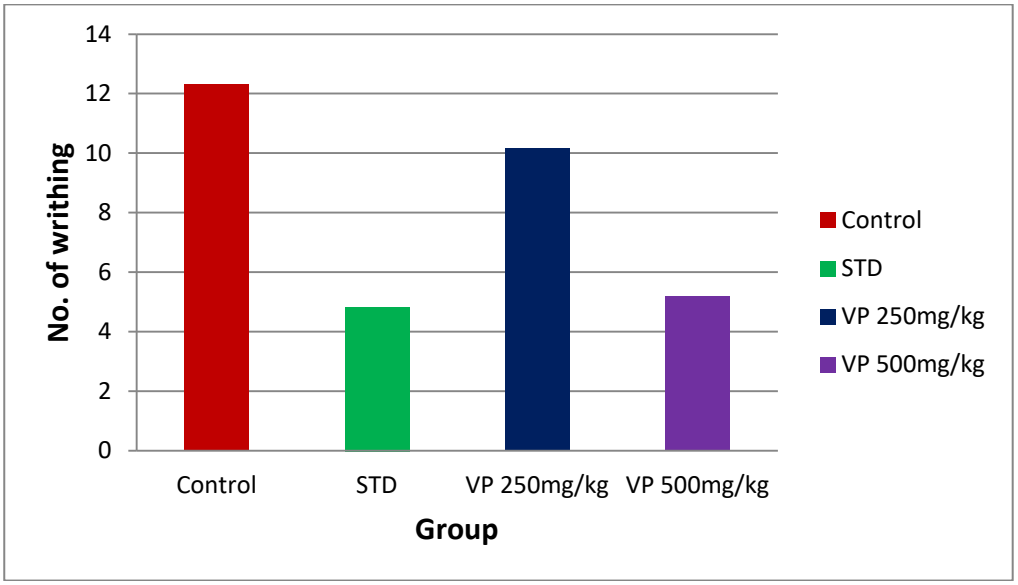

Figure 1: Effect of VP 250mg/kg and VP 500mg/kg in the acetic acid induced writhing test.

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

Table 2: Effect of VP 250mg/kg and VP 500mg/kg in the formalin induced paw licking test (1st and 2nd 5 minutes).

Group	1st 5 minutes		2nd 5 minutes	
	Licking Time (Mean±SEM)	% inhibition of algesia	Licking Time (Mean±SEM)	% inhibition of algesia
Control	20.0±1.59	00	3.17±0.87	00
Control	7.33±0.56***	63.35	1.00±0.45*	68.45
VP 250mg/kg	17.67±1.02	11.65	2.17±0.54	31.55
VP 500mg/kg	10.33±1.26***	48.35	0.67±0.33*	78.86

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

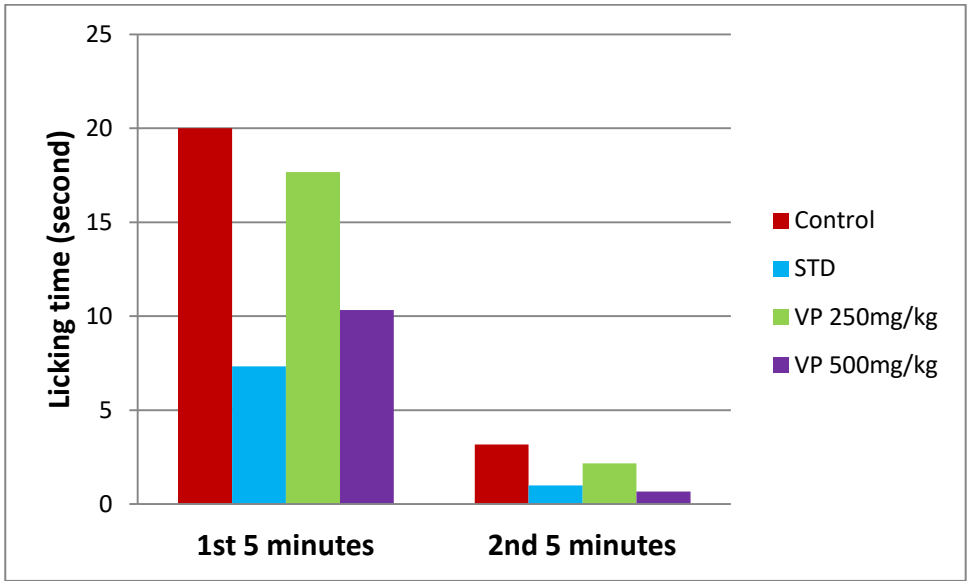

Figure 2: Effect of VP 250mg/kg and VP 500mg/kg in the formalin induced paw licking test (1st 5 minutes and 2nd)

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

Table 3: Effect of VP 250mg/kg and VP 500mg/kg in xylene induced ear edema test.

Group	% inflammation (Mean±SEM)	% inhibition of inflammation
Control	89.36±21.44	00
STD(Diclofenac-Na, 100 mg/kg)	36.10±4.55*	59.60
VP 250mg/kg	65.03±12.73	27.23
VP 500mg/kg	40.04±8.99*	55.19

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

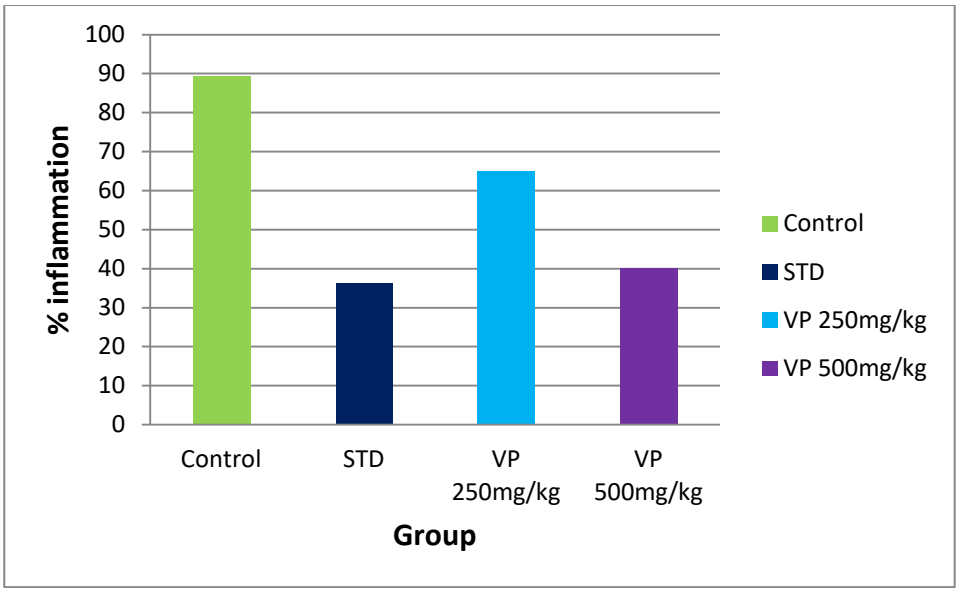

Figure 3: Effect of VP 250mg/kg and VP 500mg/kg in xylene induced ear edema test.

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

Table 4: Effect of VP 250mg/kg and VP 500mg/kg in croton oil induced ear edema test.

Group	% inflammation (Mean±SEM)	% inhibition of inflammation
Control	82.24±13.77	00
STD (Diclofenac-Na, 100 mg/kg)	18.09±3.42***	78.00
VP 250mg/kg	69.03±5.78	16.06
VP 500mg/kg	38.20±4.09**	53.55

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

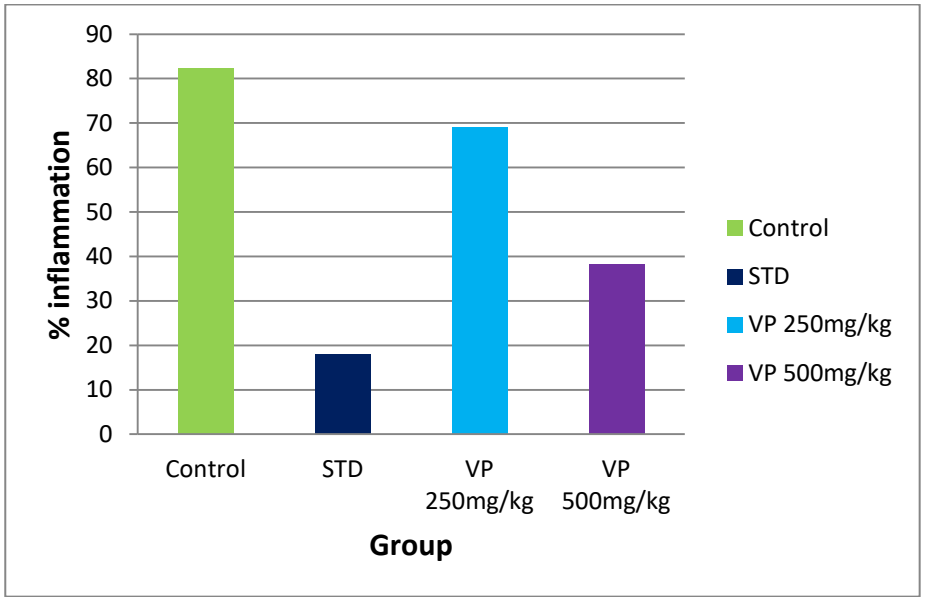

Figure 4: Effect of VP 250mg/kg and VP 500mg/kg in croton oil induced ear edema test.

NB: Data were analyzed by Independent-Sample T test and presented as Mean±SEM, (n=6). * (p<0.05) = significance, ** (p<0.01) = highly significance, and *** (p<0.001) = very highly significance as compared to control group.

Table 5: Effect of VP 250mg/kg and VP 500mg/kg on the total number of feces and diarrhea in castor oil induced diarrhea test in mice.

Group	Total number of feces (Mean±SEM)	% inhibition of defecation	Total number of Diarrhoeal feces (Mean±SEM)	% inhibition of diarrhea
Control	26.00±2.77	00	16.50±2.59	00
STD (Loperamide 10 mg/kg)	12.17±3.82**	53.19	6.50±2.46**	60.61
VP 250mg/kg	20.50±1.50	21.15	12.50±0.96	24.24
VP 500mg/kg	18.00±1.65	30.77	10.67±0.67	35.33

N.B: *(p< 0.05) =Significant, ** (p< 0.01) = Highly Significant, *** (p< 0.001) = Very Highly Significant as compared to control group. (n=6).

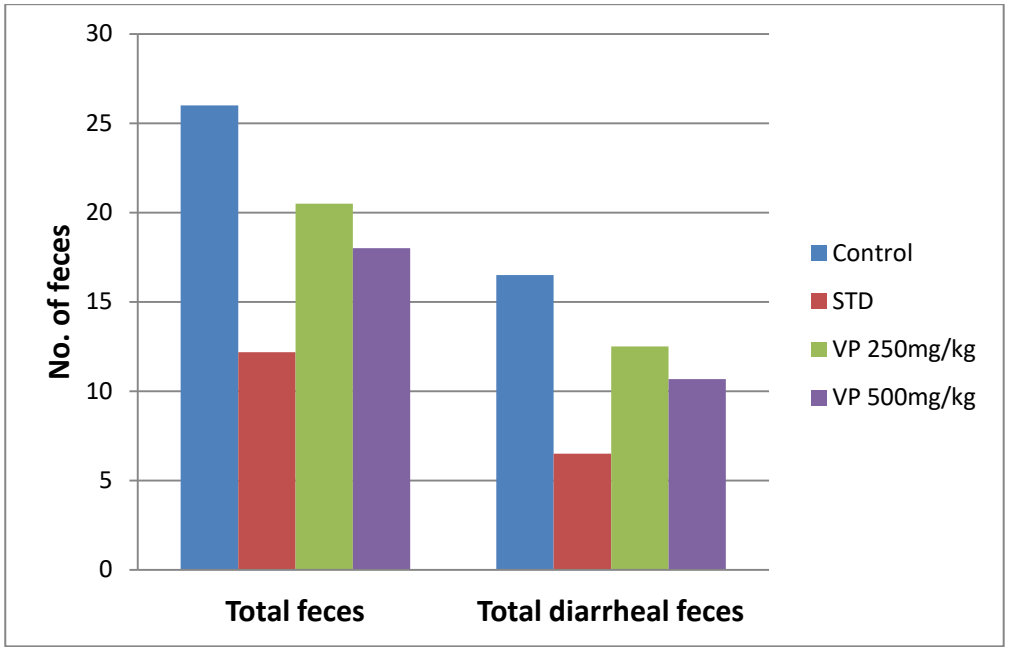

Figure 5: Effect of VP 250mg/kg and VP 500mg/kg on the total number of feces and diarrhea in castor oil induced diarrhea test in mice.

Table 6: Effect of VP 250mg/kg and VP 500mg/kg on the total number of feces and diarrhea in $MgSO_4$ induced diarrhea test in mice.

Group	Total number of feces (Mean±SEM)	% inhibition of defecation	Total number of Diarrhoeal feces (Mean±SEM)	% inhibition of diarrhea
Control	9.33±0.92	00	4.17±0.60	00
STD (loperamide 10 mg/kg)	4.83±0.79**	48.23	1.17±0.40**	71.94
VP 250mg/kg	6.17±1.76	33.87	3.33±1.61	20.14
VP 500mg/kg	5.33±1.56	42.87	2.67±0.76	35.97

N.B: *(p< 0.05) =Significant, ** (p< 0.01) = Highly Significant, *** (p< 0.001) = Very Highly Significant as compared to control group.. (n=6).

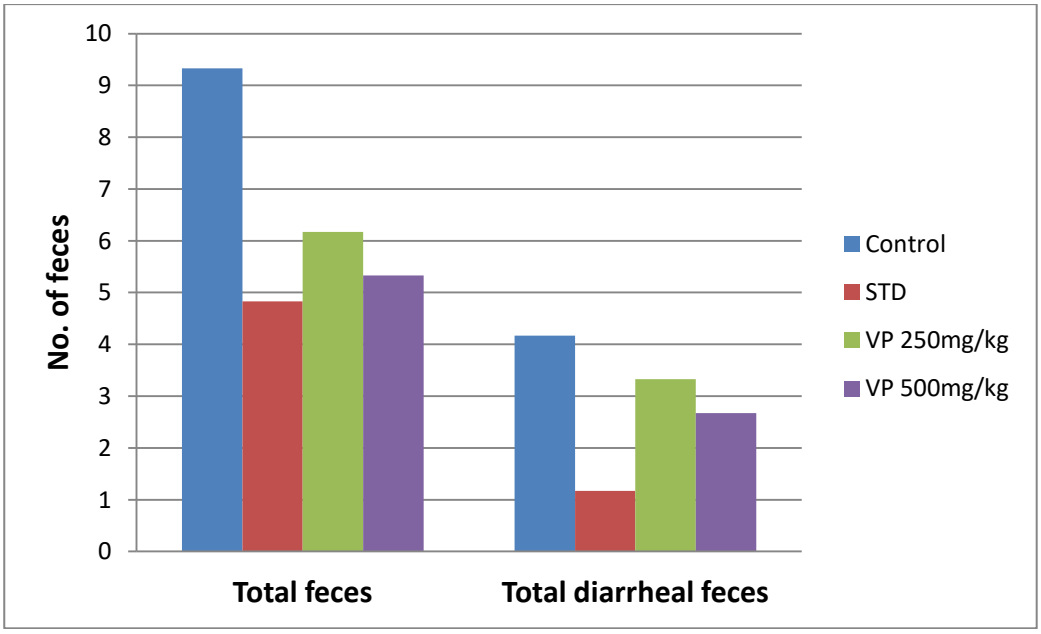

Figure 6: Effect of VP 250mg/kg and VP 500mg/kg on the total number of feces and diarrhea in $MgSO_4$ induced diarrhea test in mice.