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Antidiarrhoeal activity of *Aristolochia argentina* Gris. (Aristolochiaceae) in rodents

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ABSTRACT

The roots of *Aristolochia argentina* are used in folk medicine for the treatment of colitis, diarrhoea and hemorrhoids. In this study, based on ethnobotanical lead, we evaluated the antidiarrhoeal activity of *Aristolochia argentina* lyophilized aqueous extract (AALE) in rats and mice using various models. The castor oil and magnesium sulphate induced diarrhoea, the small intestinal transit in mice and the intestinal fluid accumulation were used in this study. At the doses of 62.5, 125 and 250 mg/kg *p.o*., the AALE showed significant antidiarrhoeal activity in both models. The AALE significantly reduced the intestinal fluid accumulation in the castor oil induced enteropooling. AALE delays small intestinal transit possibly, at least in part, involving opioid and α_2-adrenergic receptors. The phytochemical analysis revealed the presence of carbohydrates, flavonoids, tannins, saponins and anthraquinones. The results suggest that AALE showed antidiarrhoeal activity by inhibiting intestinal motility and enteropooling property, justify its use in traditional medicine.

INTRODUCTION

Aristolochia is a genus native to South America, in Argentine is represented by 21 species (Jaime *et al*., 2006), among which *Aristolochia argentina* grows in the following Argentine provinces: Catamarca, Chaco, Córdoba, Jujuy, La Rioja, Misiones, Salta, San Juan, San Luis, Tucumán (de la Peña and Pensiero, 2004; Dimitri, 2004; Barboza *et al*., 2009). *Aristolochia argentina* Gris. (Aristolochiaceae) is popularly known as "Charrúa", "Charruga", "Patito", "Buche de pavo" (de la Peña and Pensiero, 2004). The roots of this plant are used in folk medicine and are commercially available. The infusions and tinctures are used mainly for the treatment of colitis, diarrhoea and hemorrhoids (Priestap *et al*., 2003). Traditionally, has been used as antirheumatic, emmenagogue, diuretic, diaphoretic, antidiarrhoeal and antihemorrhoids (Hieronymus, 1882; Saggesse *et al*., 1959; Ratera and Ratera, 1980; Martinez *et al*., 2005; Arias Toledo *et al*., 2007, 2009; Arias Toledo, 2009; Barboza *et al*., 2006, 2009; Trillo *et al*., 2010; Ceballos *et al*., 2014).

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Externally can be used for dermatological treatments, as antiseptic, and for treating poisoning and pruritus (Barboza *et al*., 2009; Hieronymus, 1882). Extracts of *Aristolochia argentina* also showed antibacterial and antifungal activities (Gutkin *et al*., 1981). Diarrhoea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual. Though most episodes of childhood diarrhoea are mild, acute cases can lead to significant fluid loss and dehydration, which may result in death or other severe consequences if fluids are not replaced at the first sign of diarrhoea. Diarrhoea can be caused by an increased osmotic load within the intestine resulting in retention of water within the lumen; excessive secretion of electrolytes and water into the intestinal lumen; exudation of protein and fluid from the mucosa; and altered intestinal motility resulting in rapid transit and decreased fluid absorption (Shakey and Wallace, 2012). Diarrhoea is considered as one of the leading causes of growth retardation and death in infants (Petri *et al*., 2008). Diarrhoea remains the second leading cause of death among children under five globally. Nearly one in five child deaths – about 1.5 million each year – is due to diarrhoea. It kills more young children than AIDS, malaria and measles combined. Each year, an estimated 2.5 billion cases of diarrhoea occur among children under five years of age, and estimates suggest that overall incidence has remained relatively stable over the past two decades (UNICEF/WHO, 2009).

In recent years there has been great interest in herbal medicines for treating all kinds of diseases, including diarrhoea. Medicinal herbs constitute an indispensable component of the traditional medicine practiced worldwide due to the economic viability, accessibility and ancestral experience. The plant remedies or naturally products are known to contain synergistic and/or side effects neutralizing potentials, and usually offer their pharmacological actions mediated through multiple patways (Gilani and Rahman, 2005). There are numerous studies that report the antidiarrhoeal activity of plant extracts, prepared with varied parts of them, in different parts of the world (e.g., Mujumdar *et al*., 2005; Jia *et al*., 2008; Mazzolin *et al*., 2010: Awe *et al*., 2011; Nansunga *et al*., 2014). There is no scientific proof justifying the traditional use of *Aristolochia argentina* root in the treatment of diarrhoea. This study was conducted to investigate the possible usefulness of the roots of *Aristolochia argentina* in the treatment of diarrhoea.

MATERIALS AND METHODS

Plant material

Roots of *Aristolochia argentina* were collected in the San Luis province, Ayacucho Department, Luján locality. The botanical identification of specie was made through the application of classical taxonomic methods and certified by Dr. Luis Del Vitto. For future reference, the voucher specimen was deposited in the Herbarium of the Universidad Nacional de San Luis, San Luis, Argentina, under the registry No. 9258. The collection of plant material was approved by the chief of "Biodiversity" program, Ministry Environment of the government of San Luis province (Resolution Nº 588-PBD-2014).

Extraction procedure

The roots collected were cleaned, selected and desiccated, and then the dry material was mechanically milled to powder. Infusion of roots of the plant at 10% was prepared according to VI Ed Argentine National Pharmacopoeia. The plant material was separated by filtration and the aqueous extract was concentrated and lyophilized to preserve it.

Preliminary phytochemical screening

Chemical tests were carried out on aqueous extract of the roots of *Aristolochia argentina* using standard procedures, to identify their major groups of chemical constituents.

Animals

Adult albino Wistar rats (150–180 g) and mice (20–25 g) were used. They were housed in standard environmental conditions and fed with rodent diet and water ad libitum. The animals were housed at a room temperature of 24 ± 1°C with 12 h light/dark cycle. The animals were randomly assigned to different groups and a period of 4 days was allowed to adapt to each experiment. All experiments were in compliance with the ANMAT No. 6344/96 for animal care guidelines. Experimental protocols were approved by Animal Care and Use Institutional Committe (CICUA) of Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis (approval numbers, F-101/12, Res. N° 1498/13; F-145/13, F-146/13, F-167/13, Res. N° 10/14).

Acute toxicity test

The *Aristolochia argentina* lyophilized extract (AALE) was studied for acute oral toxicity as per revised OECD guidelines No. 423. Thirty albino mice (20 - 25 g) of both sexes were randomly divided into five groups of six animals each. The mice were fed on mice pellets and water *ad libitum*. The animals were starved for 4 h prior to testing. The AALE was re-dissolved in distilled water and administered intragastrically (5, 50, 300 and 2000 mg/kg). The fifth group, served as control, was treated only the vehicle (distilled water). The volume of the AALE were administered to each animal in the test group was calculated based on the body weight (0.2 ml/mice). Animals were observed daily, for 14 days. The parameters studied were body weight and macroscopic analysis of the vital organs: heart, lungs, liver, spleen and kidneys.

Normal defaecation

The test performed in mice according to Izzo et al. (1992). Four groups of 6 mice each, starved for 18 h, were placed individually in polythene cages with filter paper at the bottom. AALE (125 and 250 mg/kg) was administered to two groups, loperamide (10 mg/kg) to another, while the fourth group, served as control, was treated only the vehicle (distilled water). The total number of faeces in each group was counted every h for the next 3 h. The percent reduction in the number of faeces in the treated group was obtained by comparison with the control animals.

Castor oil-induced diarrhoea in mice

Diarrhoea was induced by oral administration of castor oil to mice (0.2 ml/animal) (Izzo *et al*., 1992). Food but not water was withdrawn 12 h before the experiments. AALE (62.5, 125 and 250 mg/kg) and loperamide (10 mg/kg), a standard antidiarrhoeal agent, were administered orally 30 min before castor oil administration. Control mice received the same volume of distilled water. The animals were placed in individual cages over clean filter paper. Mice were scored (double blind) for copious (++), mild (+) or lack (0) of diarrhoea, 2 h after castor oil challenge. The activity score was calculated by taking the sum of the number of "+" mice and twice the number of "++" mice. A score of 0 indicated a complete absence of diarrhea (Di Carlo *et al*., 1993).

Magnesium sulphate induced diarrhoea

A similar protocol as for castor oil-induced diarrhoea was followed. The mice were divided into different groups for the treatment either with various doses of AALE, vehicle or loperamide. After 30 min each of these animals was given 2 g/kg magnesium sulphate by oral route (De Melo *et al*., 1988). Mice were scored (double blind) for copious (++), mild (+) or lack (0) of diarrhoea, 4 h after magnesium sulphate challenge.

Effect of small intestinal transit in mice

The effect of AALE on small intestinal transit in mice was tested using the charcoal method (Mascolo *et al*., 1992). Mice were fasted for 18 h and pretreated orally with AALE at dose of 62.5, 125 and 250 mg/kg. The charcoal meal (a suspension containing 10% charcoal in 5% arabic gum; 0.1 ml/10 g) was administered 30 min after the administration of AALE. A control group was established, which was administered the same volume of vehicle (distilled water).

Morphine sulphate (10 mg/kg, *p.o*.) was used to reduce gastrointestinal motility (standard drug). Mice were euthanized by cervical dislocation after 20 min and the small intestine was rapidly removed and laid out on white filter paper for inspection and measurement of distances traversed by the charcoal. The length traversed by the charcoal marker was calculated as a percentage of the intestine length.

To obtain information about the mechanism of action of AALE (250 mg/kg), we experienced with different drugs acting by a well know mechanism such as atropine 0.25 mg/kg, phentolamine 1 mg/kg, yohimbine 1 mg/kg, propranolol 2.5 mg/kg, cyproheptadine 2.5 mg/kg, verapamil 5 mg/kg, and naloxone 10 mg/kg. These drugs were dissolved in 0.9 % NaCl (saline) and given subcutaneously 10 min before extract administration, with the exception of verapamil, yohimbine, and naloxone given intraperitoneally (Di Carlo *et al*., 1993, Capasso *et al*., 2004).

Intestinal fluid accumulation

The method is based on that of Robert *et al*. (1976), that evaluate the net accumulation of fluid in the lumen of the small intestine. The rats were fasted for 12 h before the experiment. AALE (62.5, 125 and 250 mg/kg) was administered (*p.o*.) to rats, followed 1 h later by castor oil (2 ml/rat). Control rats received only vehicle. The standard chlorpromazine (30 mg/kg, i.p.) was administered 30 min before the oral administration of castor oil. The animals were euthanized by inhalation of carbon dioxide, 30 min later.

The small intestine was clamped at the pyloric valve and the ilio-caecal juntion and carefully removed from the abdomen. The small intestine was weighed (W_1), emptied of fluid, reweighed (W_2) and the length (L) measured. The difference in weight divided by the length shows the "enteropooling" in milligrams of fluid per centimeter of intestine (Valle *et al*., 2000).

$$\text{Enteropooling} = (W_1 - W_2) / L$$

Statistical analysis

Diarrhoea was expressed as total score and the chi-square test was used to determine the significance between groups. Intestinal fluid accumulation and small intestinal transit were expressed as means ± S.E.M. and Student´s t-test was used to determine the significance of difference between means. A P value less than 0.05 was taken as statistically significant.

RESULTS

The present study was designed to investigate the antidiarrhoeal properties of the *A. argentina*. Thus, four experimental models (the castor oil and magnesium sulphate induced diarrhoea, the small intestinal transit in mice and the intestinal fluid accumulation) were used, which are applied because they are very simple and reproducible.

Phytochemical screening

The phytochemical analysis of the aqueous root extracts of *A. argentina* revealed the presence of, carbohydrates, flavonoids, tannins, saponins and anthraquinones.

Acute toxicity test

Acute toxicological studies have showed that an oral administration of 2000 mg/Kg of AALE did not produce any sign of acute toxicity in the animals (male and female). Over the 14 days following the oral administration of AALE, none of the animals died and no significant changes in daily body weight or organ weight were observed through the end of this period (data not shown).

Normal defaecation

The results of the AALE effect on normal defaecation show that doses of 125 and 250 mg/kg in an average time of 3 h, normal defaecation was inhibited by 27.78 and 44.45%, respectively, and for loperamide by 72.23%. After 3 h, this inhibitory effect could not be detected as there was no defaecation in the control group after this time, and thus, no comparison could be made.

Castor oil-induced diarrhoea in mice

All mice in the control group produced copious diarrhoea (total score 12). In the castor oil-induced diarrhoeal experiment in mice, AALE, at the doses of 62.5, 125 and 250 mg/kg reduced its cathartic effect (Table 1). These results were shown to be statistically significant.

Table 1: Effect of AALE on castor oil-induced diarrhoea in mice.

Treatment	Dose (mg/kg)	Diarrhoea score			Total score (max=12)
		++	+	0	
Castor oil (control)	-	6	0	0	12
Loperamide	10	0	3	3	3[b]
AALE	62.5	2	2	2	6[a]
AALE	125	2	2	2	6[a]
AALE	250	0	4	2	4[b]

AALE (*Aristolochia argentina* lyophilized extract) and loperamide were administered (*p.o*.) 30 min before castor oil administration. Diarrhoea score was analyzed by Chi square test; [a]$P<0.05$ vs control; [b]$P<0.01$ vs control. Six animals were used for each group.

Magnesium sulphate induced diarrhoea

In the magnesium sulphate-induced diarrhoeal model in mice, AALE at the above dose levels significantly reduced the extent of diarrhoea in test animals (Table 2).

Table 2: Effect of AALE on magnesium sulphate-induced diarrhoea in mice.

Treatment	Dose (mg/kg)	Diarrhoea score			Total score (max=12)
		++	+	0	
Magnesium sulphate (control)	2000	6	0	0	12
Loperamide	10	0	3	3	3[b]
AALE	62.5	1	2	3	4[a]
AALE	125	2	2	2	6[a]
AALE	250	0	3	3	3[b]

AALE (*Aristolochia argentina* lyophilized extract) and loperamide were administered (*p.o.*) 30 min before magnesium sulphate administration. Diarrhoea score was analyzed by Chi square test; [a] $P<0.05$ vs control; [b] $P<0.01$ vs control. Six animals were used for each group.

Effect of small intestinal transit in mice

In control animals, the charcoal meal traversed 54.91 ± 1.79% of the total length of the small intestine. Both AALE and morphine inhibited the intestinal propulsion of charcoal in mice (Fig. 1). The inhibitory effect of AALE on gastrointestinal transit was found to be significant at 250 mg/kg but not at 62.5 and 125 mg/kg.

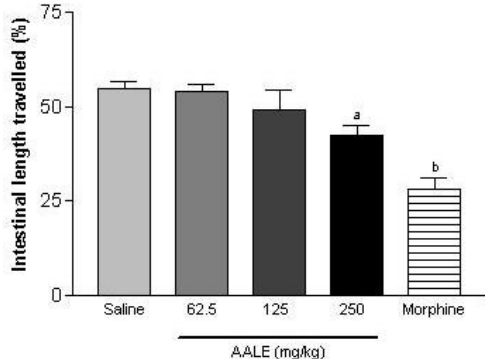

Fig. 1: Effect of AALE (*Aristolochia argentina* lyophilized extract) on gastrointestinal transit in mice. AALE and morphine 10 mg/kg were administered (30 min and 15 min, *p.o.*, respectively) before the charcoal suspension. Results are mean ± S.E.M. of 6-8 animals for each experiments group. [a] $P<0.001$, [b] $P<0.0001$ vs control (Student´s *t*-test).

Pretreatment (Fig. 2) with yohimbine, phentolamine and naloxone, but not with propanolol, cyproheptadine or atropine, antagonized the effects the AALE.

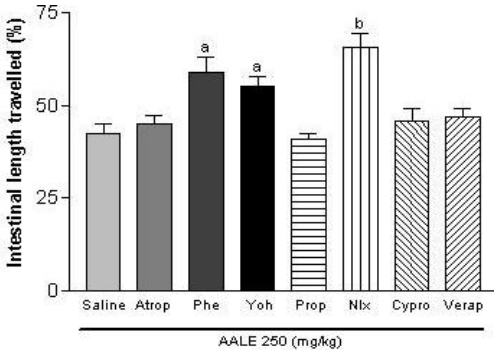

Fig. 2: Effect of AALE (*Aristolochia argentina* lyophilized extract) (250 mg/kg, *p. o.*) on upper gastrointestinal transit alone (saline) or in mice treated (s.c.) with atropine (Atrop, 0.25 mg/kg), phentolamine (Phe, 1 mg/kg), propanolol (Prop, 2.5 mg/kg), and cyprohetadine (Cypro, 2.5 mg/kg). Other group was treated (i.p.) with yohimbine (Yoh, 1 mg/kg), naloxone (Nlx, 2 mg/kg) and verapamil (Verap, 5 mg/kg). Results are mean ± S.E.M. of 6-8 animals for each experiments group. [a] $P<0.01$, [b] $P<0.001$ vs AALE, 250 mg/kg (Student´s *t*-test).

Intestinal fluid accumulation

Oral administration of castor oil produced accumulation of water and electrolytes in intestinal loop. AALE (62.5, 125 and 250 mg/kg) significantly inhibited castor oil-induced intestinal fluid accumulation in the rats (Fig. 3).

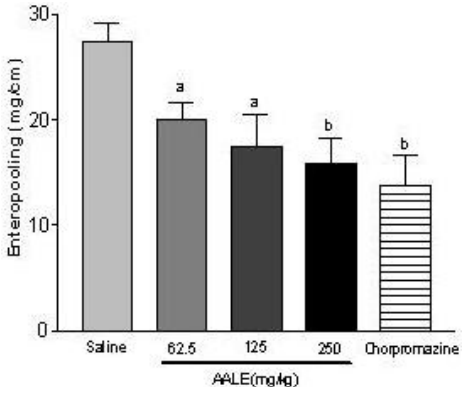

Fig 3: Effect of AALE (*Aristolochia argentina* lyophilized extract) on intestinal fluid accumulation in rats. AALE was administered (*p.o.*) to rats, followed 1 h later by castor oil (2 ml/rat). The standard chlorpromazine (30 mg/kg, i.p.) was administered 30 min before the oral administration of castor oil. Results are mean ± S.E.M. of 6-8 animals for each experiments group. [a] $P<0.01$, [b] $P<0.01$ vs control. (Student´s *t*-test).

DISCUSSION

Diarrhoea is the frequent passage of liquid faeces and it involves both an increase in the motility of the gastrointestinal tract, along with increased secretion and decreased absorption of fluid, and thus a loss of electrolytes (particularly sodium) and water (Rang and Dale, 2008). Hence to restore personal comfort and convenience, many patients require antidiarrhoeal therapy and treatment is carried out to achieve, among other objectives, increased resistance to flow (segmental contraction, decreased propulsion and peristalsis) and increased mucosal absorption or decreased secretion. The use of native plants for the treatment of diarrhea is a common practice in many folk medicines. In this context, the investigation of the antidiarrhoeal effect of AALE in this study comprised the evaluation of its effect on intestinal transit and the fluid accumulation.

Orally administration of AALE up to 2g/kg produced no mortality and visible signs of delayed toxicity 14 days post-treatment. These results ensured the continuance of pharmacological studies on this species using the oral route and motivated us to proceed with the biological assays.

The effect of AALE on experimentally induced diarrhoea was evaluated using castor oil-induced diarrhoea. This is in recognition of the fact that in some diarrhoeas, the secretory component predominates while other diarrhoeas are characterized by hypermotility of the gastrointestinal tract.

Castor oil increase volume of intestinal content by prevention of the re-absorption of water and the liberation of ricinoleic acid, from action of lipases. Ricinoleic acid markedly increased the prostaglandins content in the gut lumen and also caused an increase of the net secretion of the water and electrolytes

into the small intestine. Also, ricinoleic acid stimulates the production of several mediator substances that include nitric oxide, platelet activating factor, cAMP and tachykinins (Beuoler *et al*., 1979; Izzo *et al*., 1990, Mascolo *et al*., 1992). It has been well known that castor oil causes motility and secretory diarrhoea (Rouf *et al*., 2003), therefore is a useful model for screening for antimotility and antisecretory compounds. The effect of AALE showed significant antidiarrhoeal activity reducing the castor oil-induced diarrhoea in mice. The highest effect was observed at the dose of 250 mg/kg, which suggest that the antidiarrhoeic activity revolves around this dose. AALE may have brought about this activity by stimulation of the re-absorption of water from the intestinal lumen or by anti-prostaglandin activities that contribute to the pathophysiological functions in the gastrointestinal tract. AALE inhibited the castor oil- induced diarrhoea, it can be assumed that the antidiarrhoeal effect was excerted by antisecretory mechanism. This was also evident from the reduction in enteropooling.

Intraluminal fluid accumulation was determined by castor oil-induced enteropooling. The prevention of intraluminal fluid secretion by AALE in this study may be due to inhibition of prostaglandin biosynthesis with resultant decrease in secretion of fluid into the lumen or may be due to promotion of absorption of water and electrolytes in the gut.

On the other hand, magnesium sulphate has been reported to induce diarrhoea by increasing the volume of intestinal content through prevention of reabsorption of water. It has also been demonstrated that it promotes the liberation of cholecystokinin from the duodenal mucosa, which increases the secretion and motility of small intestine and thereby prevents the reabsorption of sodium chloride and water (Galvez *et al*., 1993). AALE was found to alleviate the diarrhoeic condition in this model. AALE may have increased the absorption of water and electrolyte from the gastrointestinal tract, since it delayed the gastrointestinal transit in mice as compared to the control. The delay in the gastrointestinal transit prompted by AALE might have contributed, at least to some extent, to their antidiarrhoeal activity by allowing a greater time for absorption. This activity is probably due to the ability of the AALE to inhibit intestinal motility.

The reduction in percentage distance travelled can be used to establish the intestinal smooth muscle relaxation. The property of reducing intestinal contractions (and consequently, intestinal transit) is demonstrated by most antidiarrhoeal drugs and this property was shown by AALE further demonstrating its antidiarrhoeal activity. These conditions tend to suggest that AALE reduced diarrhoea by increasing reabsorption of electrolytes and water or by inhibiting induced intestinal accumulation of fluid just as loperamide. Loperamide acts by decreasing the transit velocity and increasing the capacity of the intestine to retain their fluids, furthermore it inhibits calmodulin-sensitive phosphodiesterases (Awouters *et al*., 1983; Daly and Harper, 2000).

In the evaluation of intestinal transit, morphine was used as standard drug. Morphine and other opiates have been demonstrated to inhibit intestinal transit *in vivo* and to be spasmolytic *in vitro*, possibly by an action on neurotransmitter release. Evidence exists for both centrally and peripherally mediated inhibition of intestinal motility by opiates. Thus, opioids may act at more than one site to counteract the pathophysiological basis of diarrhoeal disease. Putative roles for endogenous opiates in control of gastrointestinal motility have been well reviewed by Kromer (1988).

However, the exact mechanism involved is difficult to clarify as control mechanism of intestinal functions are various and imply intrinsic and extrinsic nerves, autacoids and hormones (Di Carlo *et al*., 1994).

Catcholamines stimulate sodium and chloride absorption from the intestine by interacting with α_2-adrenergic receptors on enterocytes (Brun *et al*., 1998), clonidine delays normal small intestinal transit in rat (Ruwart *et al*., 1980) and inhibits defaecation in basal conditions (Doherty and Hancock, 1985). Also, adrenergic agonists with actions at α_2-adrenergic receptors promote fluid and electrolyte absorption hence yohimbine, a specific α_2-receptor antagonist will do the opposite thus promoting diarrhoea (Adeyemi *et al*., 2008). Our results show that the α_2-adrenoreceptor antagonists yohimbine and phentolamine counteracted the effects of AALE on intestinal transit while propranolol, a β-adrenoceptor antagonist, failed to do so. This indicates a role for the α_2-adrenergic system in the action of AALE examined.

Atropine, a muscarinic receptor antagonist, inhibits gastrointestinal motility (propulsion), reduces intestinal fluid secretion, and delays gastric emptying thus ameliorating diarrhoea (Adeyemi *et al*., 2007). In the present study the effect of AALE on intestinal motility was affected by naloxone but unaffected by atropine. Further, 5-hydroxytryptaminergic (cyproheptadine) antagonist did not modify the action of AALE on intestinal transit, indicating that this action did not involve cholinergic and 5-hydroxytryptaminergic mediation, but influenced by opioid. This study has demonstrated that AALE delays small intestinal transit possibly, at least in part, involving opioid receptors, which are found in the gastrointestinal wall.

Phytochemical screening of the aqueous extract of *Aristolochia argentina* revealed the presence of tannins, flavonoids, and saponins. Tannins, flavonoids and other plant metabolites possess antidiarrhoeal activity in different experimental animal models and flavonoids also inhibit diarrhoea induced by castor oil. Tannins are known to reduce secretion and make the intestinal mucus resistant through the formation of protein tannate (Adzu *et al*., 2003). Di Carlo *et al*. (1993) demonstrated that flavonoids inhibit intestinal motility and secretion in rodents mediated by α_2-adrenergic receptors and calcium. These properties may explain the reason for the effective use of the plant as antidiarrhoeal agent in traditional medicine. It is therefore possible that tannins and flavonoids content of *Aristolochia argentina* among others may be responsible for the antidiarrhoeal activity of AALE. In conclusion, the antidiarrhoeal effect of the AALE may, thus, be attributable to its inhibitory

action against gastrointestinal motility and the inhibition of enteropooling property. This justifies the use of *Aristolochia argentina* for the treatment of diarrhoea in traditional medicine.

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