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Pharmacological studies on toxicological, antidiarrhoeal and vasodilatory activities of *Sclerocarya birrea* (A. Rich) Hochst (Anacardiaceae) aqueous leaf extract

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Abstract
This study indicated the pharmacological basis for the use of *Sclerocarya birrea* in some diseases. In mice, the aqueous leaf extract of *S. birrea* showed antidiarrhoeal, antisecretory effects against castor oil and magnesium sulphate induced diarrhoea and castor oil induced fluid accumulation at 300 and 500 mg/ kg. The extract was found safe up to tested dose of 3g/ kg. In rat aortic rings, *S. birrea* demonstrated concentration dependent relaxation of NE and 5-HT induced contractions in endothelium denuded rings. In rings treated with CPA, 2-APB and ryanodine, relaxation was not blocked by CPA, indicating that the extract vasodilating properties are not mediated by this route but by IP_3 and ryanodine receptors located in sarcoplasmic reticulum. Our results suggest that *S. birrea* possesses antidiarrhoeal, antisecretory and vasorelaxation properties mediated through calcium antagonist pathway and probably explains its medicinal use in diarrhoea and hypertension.

Keywords: *sclerocarya birrea*, antidiarrhoeal, antisecretory CPA, 2-APB, sarcoplasmic reticulum, IP_3 receptor

1. Introduction
Scler*ocarya birrea* (*S. birrea*) is a tropical plant widely used to treat many human ailments such as hypertension and diarrhoea.

S. birrea is a tree distributed from Subsaharian regions to South Africa. In South Africa it is called ″cider tree″ and the ripe fruits contain vitamin C and edible nuts. The fruit pulp is used to product a jelly and to flavour liqueur [1] and alcoholic drink. The leaves are divided into ten or more pairs of leaflets, each about 60 cm long. Male and female flowers occur separately but not on separate trees. The different parts of the plant are used to treat human ailments including: hypertension, dysentery, diarrhoea and gastro-intestinal disorders. The leaves and bark decoctions are used to treat diabetic mellitus patients [2].

Previous chemical screening showed mainly tannins and flavonoids in leaf extract [3]. A new flavonol glycoside, quercetin 3-O-alpha-1-(5″-galloyl)-arabinofuranoside and phenolic components are also isolated from leaves [4]. In a recent review, phytochemistry, pharmacology and toxicology studies of the different parts of the plant are reported [5].

Pharmacological studies showed antidiarrhoeal activity [6], angiotensin converting enzyme inhibition [7], intracellular calcium decrease in rat skeletal muscle cells [3], anti-inflammatory properties [8], vasorelaxant and hypotensive effects [9] and contractile effects on isolated vascular smooth muscle cells [10].

In developing countries, hypertension is now considered as the main cause of mortality and in Burkina Faso its prevalence is estimated to 40% of cardiovascular diseases [11]. Many people utilize plant extracts to treat this disease among which *S. birrea*.

In this study, we investigated whether *S. birrea* leaf extract which is used to treat hypertension would exert relaxation of isolated arteries *in vitro*. If so, we would also examine the nature of the vasorelaxation and its possible mechanism of action.

2 Materials and Methods
2.1 Plant collection
Fresh leaves of *S. birrea* were collected from Gampéla (Burkna Faso, West Africa) in July 2015. This plant is identified by Pr Millogo-Rasolodimby, Department of Botany, University Ouaga I Pr Joseph KI-ZERBO. The herbarium specimens have been deposited in this Department.

2.2 Preparation of plant extracts
Crude decoction is prepared from the shade dried leaves. Forty grams of leaves powder of the plant were macerated in one liter (1 L) of deionized water for 24 h at room temperature and then boiled for 10 min to mimic the traditional preparation methods. After cooling, the resulting extract was filtered through whatman n°2 and freezed for 24 h and then lyophilized to give brown powder which was utilized for experiments (yield: 8.5% on dried weight).

2.3 Animals
Male Naval Medical Research Institute (NMRI) mice (28 – 30 g) and male Wistar rats (250 – 300 g) were used in this study. The animals were fed with standard diet and were kept at 24 ± 2°C, 60 ± 10% humidity and submitted to a 12 h light/dark cycle with free access to food and water. All animals procedures were strictly within national laws and guidelines (the animals used in accordance with the local ethic committee of Ouaga I Pr Joseph KI-ZERBO for the use and care of animals).

In vivo experiments
Male mice were used in these studies.

Acute toxicity test
The test was performed as described by Bayala (2005) [12]. Fifty six male mice were used and fourteen groups were made, each group contained four animals. The animals were fasted for twenty four hours with free access to water but water was withdraw ten hours before the experiment.

Different groups were treated with crude extract applied in increasing doses (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500 and 3000 mg/ kg). One hour after administration of the extract, the animals were allowed to feed. The animals were observed regularly for time period of 1, 24, 48 and 72 hours for lethality or toxic effects as gastrointestinal cramps diarrhoea and behavioral changes.

Castor oil induced diarrhoea

This study was assessed with slight modification according to the method described by Shah et *al*. (2011A) [13]. Twenty mice were used and divided into four groups and each group contained five mice. The animals were housed in individual cage where the floor was lined with transparent paper and they were fasted for twenty four hours with free access to water prior the start of the experiment. The group I received saline solution (NaCl, 0.9%) 10 mL/ kg orally and served as negative control. The groups II and III received the extract of *S. birrea* at doses of 300 and 500 mg/ kg respectively by oral route. The group IV served as positive control and received 10 mg/ kg of loperamide orally. One hour after the treatment, each mouse received 10 mL/ kg of castor oil orally. Four hours of the treatment of castor oil, each cage was inspected for the presence or absence of diarrhoeal feces and recorded.

Magnesium sulphate induced diarrhoea

The method used was that used for castor oil induced diarrhoea. Briefly twenty male mice were divided into four groups containing five mice each. Group I was administrated with saline solution orally (10 mL/ kg). Groups II and III received the extract of *S. birrea* at doses of 300 and 500 mg/ kg orally respectively and group IV was administrated with loperamide 10 mg/ kg orally and served as positive control. One hour of this treatment, all the animals received orally 2g/ kg of magnesium sulphate. Four hours after the treatment of magnesium sulphate, each individual cage was inspected to detect the presence or absence of diarrhoeal feces and noted. The absence indicated protection from diarrhoea.

Castor oil induced fluid accumulation

Effect of leaf extract of *S. birrea* on castor oil induced fluid accumulation was described according to the method of Mehmood and Gilani (2010) [14] with slight modification. Thirty male NMRI mice (28 – 30g) were fasted for 24h before the beginning of the experiment and were divided into five groups of six animals each. Groups I and II were treated with 10 mL/ kg of normal solution orally and served as negative controls. Group III received verapamil 100 mg/ kg intraperitoneally using insulin syringe needle and served as positive control. Groups IV and V received the extract at doses 300 and 500 mg/ kg intraperitoneally respectively. One hour after the treatment, each mouse received 10 mL/ kg of castor oil orally except the mice of group I. Thirty minutes of the treatment of castor oil, the animals were killed by cervical dislocation and the small intestine was removed carefully after tying the ends with thread and weighed. The results were expressed as (*Pi*/*Pm*) x 1000. *Pi* is the weight of small intestine and *Pm* is the weight of animal (in g).

***In vitro* experiment**

The wistar rats of both sexes were used in this study.

The animals were killed by cervical dislocation. The thoracic aorta was immediately isolated and put in Krebs solution and cleaned of adherent connective tissue and cut into rings (3 – 4 mm length). Each ring was suspended in organ bath between two parallel stainless hooks. One hook was fixed while the other was connected to a force transducer for the isometric tension. The organ bath contained 5 ml Krebs' solution at 37°C, bubbled with carbogen (95% O_2 and 5% CO_2). The Krebs' solution contained the following composition (mM): NaCl, 120; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 15; KH_2PO_4, 1.2; D-glucose, 11; Hepes, 10; PH = 7.4.

The rings were stretched progressively to a basal tension of 2 g and allowed to equilibrate for at least 90 min, during which time the bath solution was replaced with prewarmed solution every 15 min to protect against interfering metabolites [15]. The presence or lack of functional endothelium was examined by demonstrating the presence or absence of relaxation induced by Ach 100 µM on aortic rings precontracted with 1 µM Norepinephrine (NE). Aortic rings showing an Ach induced relaxation ≤ 10%, indicative of an effective removal of the endothelium were utilized for experiments. After a washout and an equilibration period of 60 min, the rings returned to the basal level and the experiments can begin.

2.4 Evaluation of the relaxant effects of the plant extracts.

2.4.1 Protocol 1

The aortic rings were precontracted by a single concentration of NE (1 µM) or

5-hydroxytryptamine (5-HT) (100 µM) and then different increasing concentrations of

S. birrea were added cumulatively to the organ bath.

When required by the experimental protocol, cyclopiazonic acid (CPA) (20 µM), ryanodine (20 µM) and 2-amino-ethoxy-diphenylborate (2-APB) (50 µM) were performed 30 min before administration of NE, or 5-HT.

2.4.2 Protocol 2

The aortic rings were contracted with increasing cumulative concentrations of NE or 5-HT. Subsequently pre-treatment with the plants extracts were performed 30 min before the administration of NE or 5-HT. In control conditions, the first concentration response to NE or 5-HT was almost identical to the second one.

2.5 Drugs

Norepinephrine bitartrate, 5-hydroxytryptamine, cyclopiazonic acid, ryanodine, 2-amino-ethoxy-diphylborate (2-APB), verapamil, magnesium sulphate and loperamide were purchased from Sigma Chemicals Co. St Louis, MO, USA.

Castor oil was purchased from Ouagadougou Pharmaceutical Office (Burkina Faso).

The crude extract of *S.birrea* is dissolved in deionized water. Cyclopiazonic acid was dissolved in dimethylsulfoxide (DMSO) and further diluted in deionized water, then NE and 5-HT were dissolved in deionized water.

All solutions were freshly prepared on the day of the experiment.

2.6 Data analysis

One way ANOVA followed by Dunnett's test was used for fluid accumulation experiments.

Relaxant effects were expressed as percentage relaxation from NE, or 5-HT precontraction levels. Data were shown as mean ± SEM. Statistical significance was estimated by student's t-test. A p value of less than 0.05 was considered to be significant. All statistical analysis was done using Graph Pad Prism (Graph Pad Software, San Diego, CA, USA). Concentration – response curves were analysed by non-linear regression (Graph Pad Prism).

3 Results

3.1 Acute toxicity test

The acute toxicity test conducted in different doses of *S. birrea* showed no mortality or behavioural changes up to the dose of 3g/ kg suggesting that the extract is relatively safe to the maximum tested dose.

3.2 Castor oil induced diarrhoea

The group administrated orally saline and castor oil showed no protection against diarrhoea but the positive control loperamide caused 100% protection at the dose of 10 mg/ kg. The groups treated with different doses of the extract of *S. birrea* (300 and 500 mg/ kg) exhibited dose dependent inhibition effect against castor oil induced diarrhoea by producing 40 and 60% respectively as shown in table 1.

Table 1: Antidiarrhoeal effect of the extract of *S. birrea* against castor oil induced diarrhoea in mice.

Treatment	Number of mice with diarrhoea	Percentage of protection
Group 1 Saline (10 mL/kg) plus castor oil (10 mL/kg)	5/5	0
Group 2 Extract (300 mg/kg) plus castor oil (10 mL/kg)	3/5	40
Group 3 Extract (500 mg/kg) plus castor oil (10 mL/kg)	2*/5	60
Group 4 Loperamide (10 mg/kg) plus castor oil (10 mL/kg)	0**/5	100

*p < 0.05 and **p < 0.01 versus saline + castor oil treated group (χ^2 test).

3.3 Magnesium sulphate induced diarrhoea

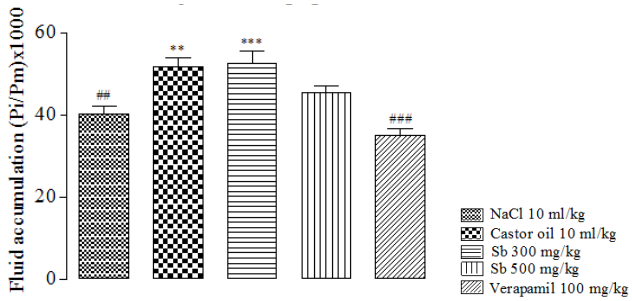
Administration of magnesium sulphate to mice enhanced diarrhoea and caused only 20% protection of saline treated group. Treatment of *S. birrea* at the doses 300 and 500 mg/ kg inhibited $MgSO_4$ induced diarrhoea by offering 40 and 60% protection versus saline group respectively as shown in table 2. The positive control loperamide offered 80% protection at the dose of 10 mg/ kg.

Table 2: Antidiarrhoeal effect of the extract of *S. birrea* against magnesium sulphate induced diarrhoea in mice.

Trematent	Number of mice with diarrhoea	Percentage of protection
Group 1 Saline (10 mL/kg) plus $MgSO_4$ (2g/kg)	4/5	20
Group 2 Extract (300 mg/kg) plus $MgSO_4$ (2g/kg)	3/5	40
Group 3 Extract (500 mg/kg) plus $MgSO_4$ (2g/kg)	2/5	60
Group 4 Loperamide (10 mg/kg) plus $MgSO_4$ (2g/kg)	1/5	80

3.4 Castor oil induced fluid accumulation

In fluid accumulation study, castor oil induced a significant increase fluid accumulation in mice with a value of 51.74 ± 2.25 while saline solution which is a negative control exhibited 40.18 ± 2.03. The extract of *S. birrea* exhibited 52.64 ± 2.94 and 45.33 ± 1.75 at the doses of 300 and 500 mg/ kg respectively. Verapamil produced significant reduction in castor oil induced fluid accumulation with a value of 34.96 ± 1.64 at the dose 100 mg/ kg (*i.p*). All values are expressed as mean ± S.E.M, n = 6 (Fig.1).



$^{**}p$<0.001 and $^{***}p$<0.001 compared saline treated group with other groups

$^{\#\#}p$<0.001 and $^{\#\#\#}p$<0.001 compared castor oil treated group with other groups (One way ANOVA followed Dunnett's test).

Fig 1: Dose dependent inhibitory effect of *S. birrea* on castor oil induced fliud accumulation in the small intestine of mice. The values are mean ± S.E.M.

3.5 Effects of *S. birrea* on NE induced contraction on rat aorta

The aqueous extract of *S. birrea* has no activity on rat aorta basal tone when applied to the organ bath. Then we utilized NE, a spasmogen on rat aorta to research some antagonistic effects.

NE (1 µM) induced contraction on rat aorta with or without endothelium (fig.3A). As there was no significative difference of contraction on these two kinds of aortic rings, then we choiced to work on aortic rings without endothelium. When the contraction induced by NE (1 µM) reached a plateau, cumulative doses of *S. birrea* were added (fig.2). Under this condition *S. birrea* extract produced a dose dependent manner relaxation of the contraction developed by NE (fig.3A). The extract relaxed the contraction by 100%.

In another sets of experiments, the different doses of the extract of *S. birrea* were added to the bath containing aortic rings and 20 min after the cumulative concentration of NE were applied. In this case, the extract of *S. birrea* shifted the contraction curves of NE to the right in concentration dependent and competitive manner (fig.3B).

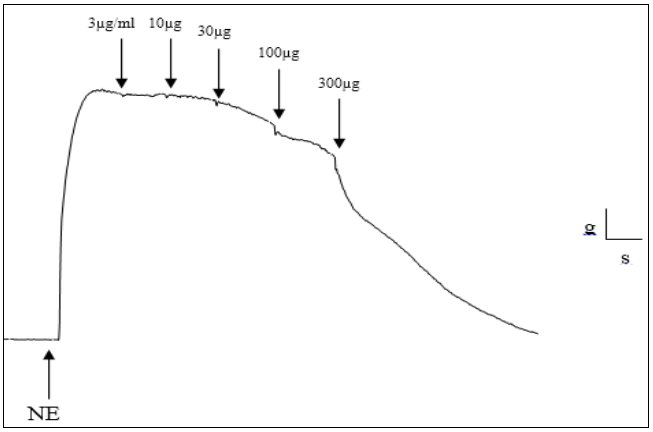
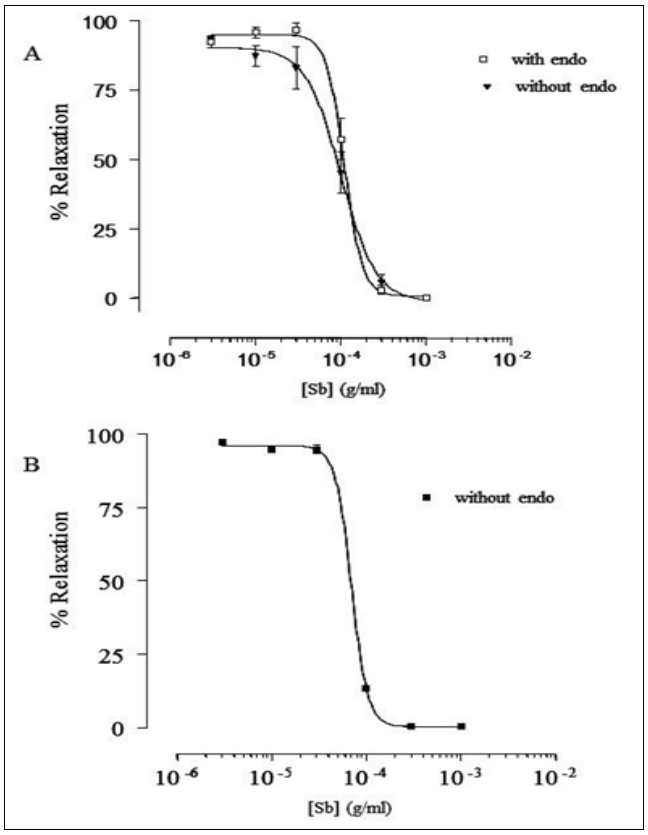


Fig 2: Example of typical tracing showing the relaxant effect of *S. birrea* on contraction induced by NE (1µM) in isolated rat aorta.



With endo = with endothelium
Without endo = without endothelium

Fig 3: Effects of aqueous extract of S. *birrea* on aortic ring with endothelium or without endothelium precontracted with NE (1µM) (A) or precontracted with 5-HT (100µM) (B).

3.6 Effects of *S. birrea* on 5-HT induced contraction on rat aorta

5-HT (100 µM) induced a contraction on rat aorta. When this contraction reached a plateau, cumulative doses of the extract of *S. birrea* applied to the bath induced a vasorelaxation by 100% (fig.4A). When the aortic rings were incubated with different doses of the extract of *S. birrea* for 20 min, the extract shifted the contraction curves of 5-HT to the right in concentration and non-competitive manner (fig.4B). It is interesting to observe that 100 µg/ml inhibited the contraction by 15% and 300 µg/ml induced an inhibition by 70% (fig.4B).

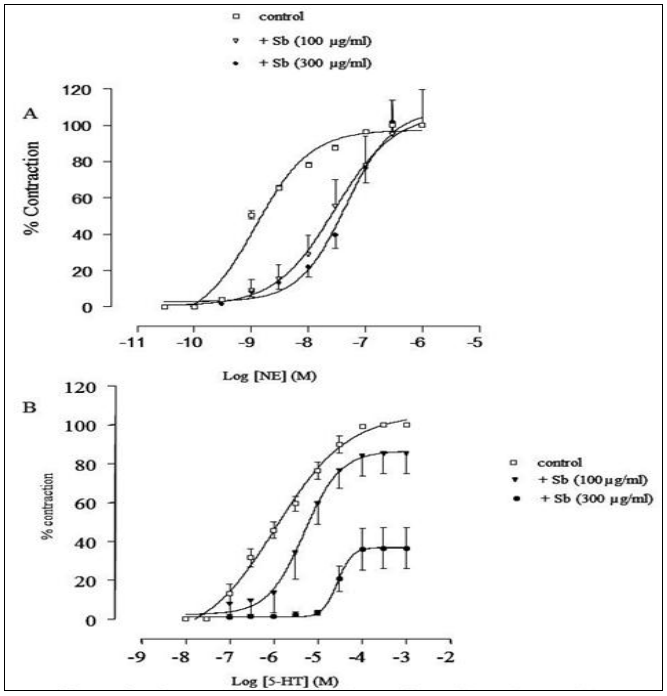


Fig 4: Inhibitory effects of *S. birrea* on the contractions of NE (A) and SHT (B) in endothelium-denuded aortic rings. Results are presented as mean ± S.E.M.

3.7 Effects of *S. birrea* on NE induced contraction on rat aorta treated with CPA

When the aortic rings were treated with CPA (20 µM) to block Ca^{2+}-ATPases in reticulum sarcoplasmic for 20 min, NE (1 µM) induced a contraction which was relaxed by cumulative doses of the extract of *S. birrea*. This vasorelaxation had an IC_{50} = 113 µg/ml versus 110 µg/ml for the control (fig.5A). It is interesting to observe that this vasorelaxation was 90%.

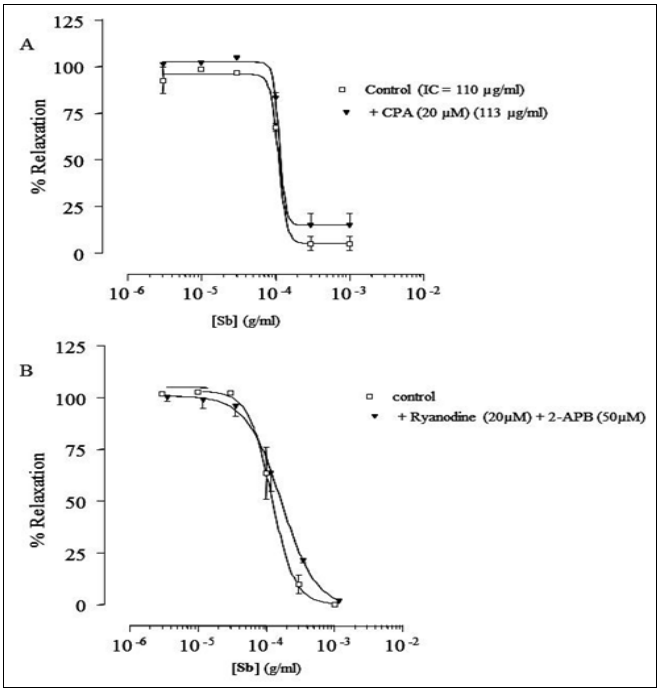


Fig 5: Effect of aqueous extract of *S. birrea* in aortic ring without endothelium precontracted with NE (1µM) in presence of CPA, inhibitor of Ca^{2+} -ATPase (A), Ryanodine, inhibitor of ryanodine receptor and 2-APB, inhibitor of IP_3 receptor (B).

3.8 Effects of *S. birrea* on NE induced contraction on rat aorta treated with ryanodine and 2-APB.

Ryanodine (20 μM) and 2-APB (50 μM) were used to block ryanodine receptors and inositol trisphosphate receptors respectively in vascular smooth muscle cells. When the aortic rings were treated with these two substances for 20 min, application of NE induced contraction. When the contraction reached a plateau, cumulative doses of the extract of *S. birrea* induced a vasorelaxation by 100% with an IC_{50} = 141 μg/ml versus 120 μg/ml for the control (fig.5B).

4. Discussion

The acute toxicity of the extract showed no death with higher dose of 3g/kg, suggesting that LD_{50} was above 3g/ kg compared to that obtained by *i.p* route [16]. This result shows that the extract of *S. birrea* is safe for oral route at tested doses.

The extract of *S. birrea* administrated at the doses of 300 and 500 mg/ kg showed 40% and 60% inhibition of diarrhoea induced by castor oil after four hours, but the standard drug, loperamide showed 100% of protection. Indeed, castor oil in lumen of the small intestine is metabolized into ricinoleic acid which stimulates release of prostaglandins, which in turn results in stimulating of secretion [17]. In the other hand, this ricinoleic acid induces release of some mediators of intestinal tract such as histamine, nitric oxide and prostaglandins which in turn stimulates intestinal secretion, motility, permeability and prevents reabsorption of sodium, potassium and water [18, 19].

Aqueous extract of *S. birrea* (300-500 mg/ kg) significantly reduced diarrhoea induced by castor oil. At these doses, the plant extract reduced dose dependently the diarrhoea when compared with the control group. Then in terms of protection, the dose 500 mg/ kg of *S. birrea* was comparable with the standard drug loperamide (table 1).

Magnesium sulphate induces diarrhoea by increasing the volume of intestinal content through prevention of reabsorption of water; it promotes the liberation of cholecystokinin from the duodenum which increases the secretion and motility of small intestine and prevents the reabsorption of sodium chloride and water [20, 21]. The extract of *S. birrea* reduces diarrhoea in this model, suggesting that it increases the absorption of water and electrolyte from the intestine.

Our results in castor oil induced fluid accumulation shows that the extract at higher dose (500 mg/ kg) reduced fluid accumulation and suggests probably the inhibitory effect on prostaglandins because it was reported that the enteropooling assay was developed to test the diarrhoea producing property of prostaglandins [22].

Loperamide, a standard antidiarrhoeal component was used in this study because of its antagonized effect in diarrhoea induced by castor oil [23]. Its therapeutic effect is due to its antimotility and antisecretory activity [24]. In our models studies it protects mice against diarrhoea.

The *in vitro* results extend our previous studies on *S. birrea* properties in normotensive rat blood pressure [25].

We now show that *S. birrea* aqueous extract produces relaxant effects of rat aortic rings without endothelium precontracted by NE, an α_1 agonist and 5-HT. It is interesting to observe that this antagonism is reversible.

The plant extract shifted the NE curves to the right without suppression of the maximum effect, indicating a competitive or specific antagonism. Then that induced by 5-HT is displaced to the right with suppression of the maximum effect, pointing a non-competitive inhibition.

Working on endothelium-denuded aortic rings, the results suggested that *S. birrea* aqueous extract induced its effect against contraction through mechanisms contained in vascular smooth muscle cells.

The accumulation of Ca^{2+} into the sarcoplasmic reticulum in smooth muscle is accomplished by a Ca^{2+}-ATPase. The Ca^{2+}-ATPase is the target of action of the blocker cyclopiazonic acid [26, 27], which did not inhibit the vasorelaxant effect of *S. birrea*. It suggested that Ca^{2+}-ATPase may not be involved in the observed effects.

Our result is in accordance with that of Baragatti et *al*. (2002) [28] who showed that the methanolic extract of *Gentiana kokiana* induced a vasorelaxation which is not mediated by Ca^{2+}-ATPase.

The contraction of smooth muscle is dependent upon an increase in the cytoplasmic free calcium, which activates the contractile elements [29].

In vascular smooth muscle cells, NE induced calcium release *via* IP_3 receptors on sarcoplasmic reticulum [30]. Indeed, NE stimulates α_1 adrenergic receptors leading to convert phosphatidylinositol to inositol 1, 4, 5-trisphosphate (IP_3) which increases Ca^{2+} release from intracellular stores.

The increase in intracellular calcium occurs either *via* an influx through voltage dependent calcium channels or its release from intracellular stores in the sarcoplasmic reticulum.

In the sarcoplasmic reticulum of vascular smooth muscle cells, there are two kinds of receptors from which the calcium is released from sarcoplasmic reticulum: an inositol trisphosphate receptor (IP_3R) and a ryanodine receptor (RyR).

A blocking action on the IP_3 sensitive Ca^{2+} channels with a 2-APB and a ryanodine sensitive channel with a ryanodine (20μM) induced a relaxation by the plant extract. This showed that these receptors channels could account for the mechanism of action of the plant extract.

Indeed, the use of 2 – APB and ryanodine did not impede the relaxation by the extract, suggesting that the extract provoked a reduction on the sarcoplasmic reticulum calcium release by a possible IP_3 and ryanodine signalling interference. Our results are in accordance with those reported by other investigators [31, 32].

On the other hand, *S. birrea* provoked at higher doses a depression of the cumulative concentration response curves of NE; this suggests that its activity is probably linked to more than one mechanism, for example involvement of a cyclic nucleotide pathway (cGMP) [33] or association with adrenoceptors blockage [34].

In previous studies, phytochemical screening of the leaf extract of *S. birrea* had led to the isolation of the flavonoids [3]. The vasorelaxation observed here could be due to these flavonoids. Indeed, Satoh and Nishida (2004) [35] reported that flavonoid (quercetin) induced relaxation on rat aortic rings by inhibition of calcium channels and PKC.

The fact that *S. birrea* caused endothelium-independent action suggests that its mode of action involves smooth muscle cells. Indeed, smooth muscle cells possess potassium channels whose opening causes hyperpolarization which induces relaxation. The involvement of these types of channels cannot

be ruled out in our experiments because of their importance reported in previous studies by other investigators [36, 32, 37].

5. Conclusion

The *in vivo* studies show that the extract of *S. birrea* possesses antidirrhoeal and antisecretory properties in mice in our models studies.

Our *in vitro* results suggest that the aqueous extract of the plant induces relaxation in rat aortic rings through an endothelium-independent pathway indicating that endothelium-derived relaxing factors such as NO, prostacyclin or endothelium-derived hyperpolarizing factor (EDHF) are not involved. Our results demonstrate for the first time that *S. birrea* can be a source of vasoactive components that might account for its use as antihypertensive therapy in traditional medicine. Further experiments are in progress in order to isolate the main vasorelaxant and antihypertensive components contained in the plant leaves.

6. Acknowledgments

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