TOCOLYTIC EFFECT OF *ACANTHUS MONTANUS* IN RAT UTERUS

Harquin Simplice Foyet[1*], Emmanuel Acha Asongalem[2], Paulin Nana[1], Gabriel Ngossong Folefoc[3], Pierre Kamtchouing[1]

[1] Department of Animal Biology and Physiology, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon, P.O. Box 812, Yaounde
[2] Pharmacology and Toxicology Unit, Department of Physiological Sciences, Faculty of Medicine and Biomedical Sciences, University of Yaounde 1, Yaounde, Cameroon, P.O. Box 8283, Yaounde
[3]Department of Organic Chemistry, Faculty of Science, University of Yaounde 1, Yaounde, Cameroon
∗Corresponding author: fharquins@yahoo.fr

Summary

The objective of this study was to investigate the tocolytic efficacy of *A. montanus* (Nees) T. Anderson (Acanthaceae) as a possible remedy for threatened abortion and dysmenorrhoea. Aqueous and organic extracts and fractions of dried leaves prepared with different solvents (hexane, ethyl acetate and methanol) were tested *in vitro* for relaxant activity on rat uterus. Aqueous and organic extracts exhibited relaxant properties with the EC_{50} values of 3.0 and 0.27 mg/ml respectively. Each extract was then fractionated on silica gel column, eluted with hexane (hex), mixture of hexane and ethyl acetate (Hex- EtOAc), mixture of ethyl acetate and methanol (EtOAc-MeOH) ant finally methanol (MeOH). Each fraction was examined using thin layer chromatography and tested for relaxant activity in an *in vitro* rat uterus. The fraction eluted with hexane (Hex and Hex- EtoAc) provided a strong dose-dependant relaxation with the EC_{50} between 0.13 and 0.31 mg/ml. These results indicated that the active compounds are of relatively non polar nature. Aqueous extract also antagonised prostaglandin ($PGF_{2\alpha}$), acetylcholine, oxytocin, serotonin, dihydroergotamine, noradrenalin and $BaCl_2$-induced uterine contraction. Evidence was obtained that *A. montanus* extracts exhibited tocolytic activity in rat uterus.

Keywords: *Acanthus montanus*, tocolytic effect, antagonist.

Introduction

Acanthus montanus (Acanthaceae) is a plant traditionally used in Cameroon for various illnesses. This includes diarrhoea, threatened abortion, dysmenorrhoea, pains, heart troubles and epilepsy (1, 2). Chemical constituents of Acanthus species includes flavonoids, alkaloids, saponins, tannins, phenols, triterpenoids and sterols (3). Extracts from this species possess relaxant properties on intestinal smooth muscles (4) and antiinflammatory, analgesic and antipyretic actions (5). Premature delivery is one of the tribulations faced by pregnant women in malaria endemic areas like Cameroon (6) and originates from different causes, culminating into uterine contractions. Current treatment includes use of synthetic drugs (terbutaline, salbutamol or diazoxide), herbal drugs or both. Previous work has shown *A. montanus* to be a nonspecific intestinal smooth muscle relaxant but no information on its effect on uterus is available. The present study aimed to extract the leaves of *A. montanus*, purify the extract to some degree and study the relaxant activity in uterine smooth muscle.

Materials and methods

Plant collection and identification

Fresh leaves of *Acanthus montanus* were harvested in Nsimeyong area of Yaounde, Cameroon in October 2004 and identified in the National Herbarium, Yaounde where a voucher specimen has been deposited under number1652SRFCAM.

Preparation of extracts

The organic and aqueous extracts were prepared by maceration of a kilogram of sun-dried pulverized leaves of the plant in 5 L of methanol-methylene chloride (1:1) for a week and 3.8 kg in 10 L of boiled distilled water for 24 h respectively. The extracts were later filtered and the solvents eliminated by concentration in a rotor evaporator to give 65 g (6.5% w/w) of organic and 400 g (10.5% w/w) of aqueous extracts.

Fractionation of extracts

The organic (60 g) and aqueous extracts (380 g) were subjected to flash chromatographic fractionation on a silica gel column, eluted with hexane followed by gradient mixtures of hexane-ethyl acetate-methanol. Several fractions were obtained and combined based on their thin layer chromatography (Sigma-silica gel precoated TLC plates) resemblance. Fifteen major fractions were obtained from the organic extract: F_{o1} (Hexane 100%); F_{o2} (hexane–EtOAc 90:10); F_{o3} (hexane–EtOAc 85:15); F_{o4} – F_{o8} (hexane–EtOAc 80:20); F_{o9} (hexane–EtOAc 60:40); F_{o10} (hexane–EtOAc 55:45); F_{o11} (hexane–EtOAc 50:50); F_{o12} (hexane–EtOAc 25:75); F_{o13} (EtOAc-MeOH 95:05); F_{o14} (EtOAc-MeOH 90:10) and F_{o15} (MeOH 100%). The aqueous extract yielded 9 fractions: F_{A1} (Hexane 100%); F_{A2} (hexane–EtOAc 90:10); F_{A3} (hexane–EtOAc 50:50); F_{A4} (hexane–EtOAc 25:75); F_{A5} (EtOAc 100); F_{A6} (EtOAc-MeOH 90:10); F_{A7} (EtOAc-MeOH 75:25); F_{A8} (EtOAc-MeOH 50:50); F_{A9} (MeOH 100%). The organic extract and the non polar fractions were dissolved in the Tween 80 (1%) while the aqueous extract and polar fractions were prepared in the De Jalon solution.

Reagents

Prostaglandin $F_{2\alpha}$ Tris ($PGF_{2\alpha}$), Acetylcholine hydrochloride, Dihydroergometrine methanesulphonate, Oxytocin acetate, Serotonin hydrochloride, Prazosin hydrochloride, Atropine sulphate and Verapamil hydrochloride Terbutaline were acquired from Sigma-Aldrich Chemie GmbH, Kappelweg 1, Germany. Calcium chloride, Barium chloride and Potassium chloride were products of British Drug House. Chemicals used in the preparation of the physiological solution were of reagent grade.

Animals and isolated tissue preparation

Thirty adult Wistar non-pregnant rats (150-220 g) were used. They were raised in the Animal House of the Faculty and fed with standard laboratory chow with water made *ad libitum*. The animals were handled based on US NIH publication #85-23, revised in 1985. Following clearance from the Institutional Ethical Committee, the rats were sacrificed by CO_2 asphyxiation, the uterine horns was isolated, cleaned of connective and cut in strips of about 2 cm long. Uterine strips were suspended vertically in 10 ml organ bath chamber containing de Jalons solution of the following composition (g/l): NaCl 9.0; KCl 0.43; $CaCl_2.2H_2O$ 0.08; $NaHCO_3$ 0.5; D-glucose 0.5. The physiological solution was maintained at 30 ± 0.5°C and continuous gassed with 100% O_2. Before starting the experiments, the uterine strips were equilibrated for 45 min under a resting tension of 0.7g. During this period, the solution was changed every 15 min. Muscle contraction were measured using isometric transducer (Ugo Basile cat. 7010) coupled to an Ugo Basile 2-channel Gemini 7070 recorder.

Relaxing effects

After the equilibration period, contraction were recorded for 10 min and used as 100% initial spontaneous contractions. Cumulative concentrations of organic (0.02 – 1.28 mg/ml) and aqueous (0.2 – 6.4 mg/ml) extracts, fractions (0.02 – 1.28 mg/ml) were then added in the incubation medium. The speed of the paper was 2 mm/min. Each experiment was performed at least six times (7). In another experiment, the uterine strips were precontrated with KCl (60 mM). After the contraction was steady for at least 3 min, the amplitude was consider as 100% initial contraction. Aqueous extract (0.2 – 6.4 mg/ml) was added cumulatively to assess its uterine relaxation. The effect of the extract was observed for 5 min. The amplitude of contraction at the end of the effect of each concentration was expressed as the percentage of initial amplitude (8).

Inhibitory effects

The uterine contractions induced by various agonists, namely, $PGF_{2\alpha}$ (0.02 – 20.5 ηM), acetylcholine (5.0 ηM – 1.1 µM), dihydroergotamine (0.02– 3.9 µM), oxytocin (0.05 - 3.9 µM), serotonin (0.02 – 4.9 µM), Calcium chloride (5.0 – 631.0 µM, in Ca^{2+} - free De Jalons solution) and Barium chloride (50.0 µM – 1.5 mM) were recorded according to the method of Okpo and Adeyemi (8). The tonic effects of these drugs were antagonized with aqueous extract (0.2 – 6.4 mg/ml) and antagonists, namely atropine (2 µM), prazosin (4 µM), phentolamine (10 µM) and verapamil (2 µM). The effect of each agonist concentration was expressed as a percentage of a reference contraction. For each drug, the experiment was performed at least six times. Effect of terbutaline on spontaneous uterine contraction was also evaluated.

Statistics

Data are expressed as mean ± SEM. Significant differences among the mean values of multiple groups were evaluated by ANOVA repeated-measures followed by Student t-test. The effective mean concentrations (EC_{50}) were obtained using both the linear regression and Litchfield and Wilcoxon methods.

Results

Effects of organic extract and organic fractions

The organic extract of *Acanthus montanus* at lower dose (0.02 mg/ml) produced slight but insignificant contraction. However, higher doses (0.04-1.28 mg/ml) produced dose- related inhibition of the spontaneous contractions. The EC_{50} of the extract was 0.27 mg/ml (0.23 – 0.32). The recovery of the tissue after washing was prolonged (Figure 1).

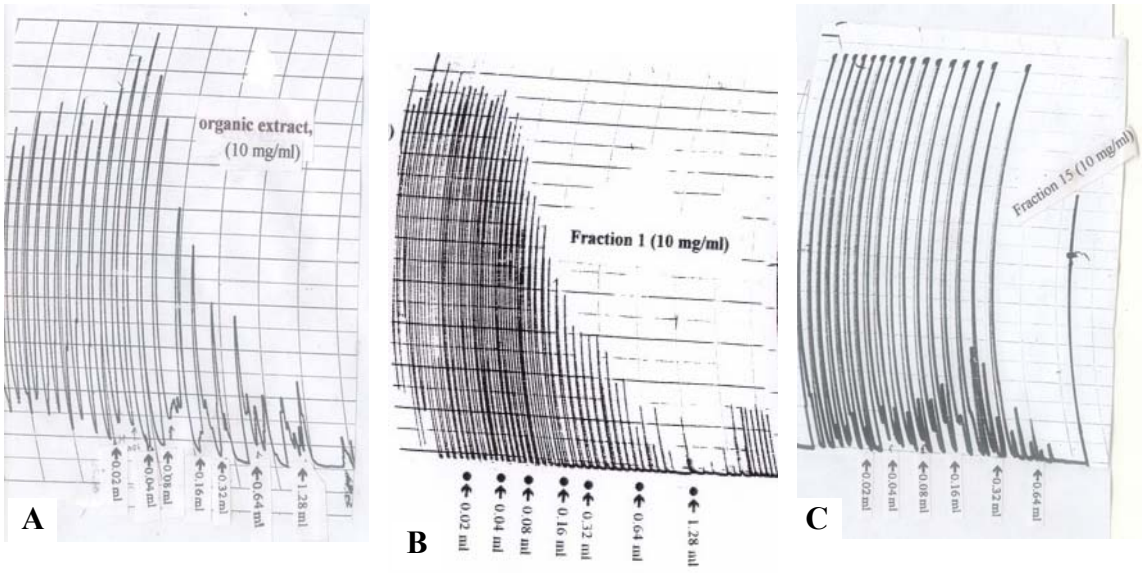

Figure1: Tracing showing the effect of organic extract (A) and organic extract fractions, F_{o1} (B) and F_{o15} (C) on rat uterine spontaneous contractions.

Fractions F_{o1} - F_{o12} had their relaxation patterns similar to that of organic extract, whereas relaxations with fractions F_{o13} - F_{o15} occurred abruptly at higher doses (Figure 1). The regain of spontaneous contractions by the tissue varied; F_{o1} and F_{o2} showed prompt and prolonged recoveries, respectively. Addition of fractions F_{o13} –F_{o15} to the tissue after refractory period, resulted into contractions.

Table 1 shows variable EC_{50s} of the fractions. The non polar fractions (F_{o1} to $F_{o6)}$ were more potent (EC_{50s} between 0.13 and 0.31 mg/ml), whereas F_{o8} to F_{o15} had increased EC_{50s}.

Effect of aqueous extract and aqueous fractions

Unlike the organic extract, a dose dependent depolarizing relaxant effect was observed with the aqueous extract (Figure 2A). The absolute refractory period was at least 20 min with loss of tissue spontaneity. The EC_{50} was 3.0 mg/ml (2.3 – 3.7). Aqueous fractions obtained from solvents containing methanol showed contractions each time the tissue was exposed, followed by relaxation. The amplitude of these contractions reduced as the extracting solvent became less polar. F_{A3} and F_{A4} (EC_{50} = 0.10±0.002) mg/ml had the maximum relaxing effects. Terbutaline – a well known β_2-adrenergic uterine smooth muscle relaxant had a similar pattern like that of organic extract and hexane fraction on the tissue spontaneous contraction (Figure 2B). Hexane organic fraction was more potent (EC_{50} = 0.13 ± 0.08 mg/ml) than that of the hexane aqueous fraction (EC_{50} = 0.32 ± 0.02 mg/ml).

Table 1. EC_{50s} of fractions from extracts of *Acanthus montanus* on rat uterine smooth muscle.

Organic fractions			Aqueous fractions		
Fractions	**Solvent ratio**	**EC_{50} (mg/ml)**	**Fractions**	**Solvent ratio**	**EC_{50} (mg/ml)**
F_{o1}	Hexane (100%)	0.13±0.08	F_{A1}	Hexane (100%)	0.32±0.02
F_{o2}	Hexane-EtOAc (90:10)	0.13±0.06	F_{A2}	Hexane-EtOAc (90:10)	0.20±0.005
F_{o3}	Hexane-EtOAc (85:15)	0.13±0.07	F_{A3}	Hexane-EtOAc (50:50)	0.10±0.002
F_{o4}	Hexane-EtOAc (80:20)	0.16±0.06	F_{A4}	Hexane-EtOAc (25:75)	0.10±0.002
F_{o5}	Hexane-EtOAc (80:20)	0.16±0.04	F_{A5}	EtOAc (100%)	0.30±0.006
F_{o6}	Hexane-EtOAc (80:20)	0.17±0.04	F_{A6}	EtOAc-MeOH (90:10)	0.80±0.007
F_{o7}	Hexane-EtOAc (80:20)	0.37±0.05	F_{A7}	EtOAc-MeOH (75:25)	0.13±0.01
F_{o8}	Hexane-EtOAc (80:20)	0.31±0.06	F_{A8}	EtOAc-MeOH (50:50)	0.12±0.02
F_{o9}	Hexane-EtOAc (60:40)	0.37±0.07	F_{A9}	MeOH(100%)	0.15±0.02
F_{o10}	Hexane-EtOAc (55:45)	0.26±0.05			
F_{o11}	Hexane-EtOAc (50:50)	0.25±0.06			
F_{o12}	Hexane-EtOAc (25:75)	0.37±0.04			
F_{o13}	EtOAc-MeOH (95:05)	0.39±0.06			
F_{o14}	EtOAc-MeOH (90:10)	0.47±0.06			
F_{o15}	MeOH(100%)	0.49±0.05			

n = 6-7; EtOAc = Ethylacetate; MeOH = Methanol.

Effects of some standards drugs

The extract showed dose dependent relaxations of $PGF_{2\alpha}$ and oxytocin-induced contractions. At very low doses, the extract (2 mg/ml) inhibited $PGF_{2\alpha}$– induced contractions by 50-56% as compared to oxytocin which required 16 – 64 mg/ml (Figure. 3A and 3B). The aqueous extract also inhibited the contractions induced by dihydroergotamine, noradrenalin and $BaCl_2$, with no significant changes in their EC_{50s} but the amplitude of contraction (Emax) reduced dose-dependently. On acetylcholine, serotonin, $CaCl_2$ and KCl-induced contractions, the inhibition responses were dose-dependant. On KCl- precontraction, the extract caused dose dependant relaxations. The extract was very weak blocker of Ca^{2+} and Ba^{2+} but not of KCl- induced contractions.

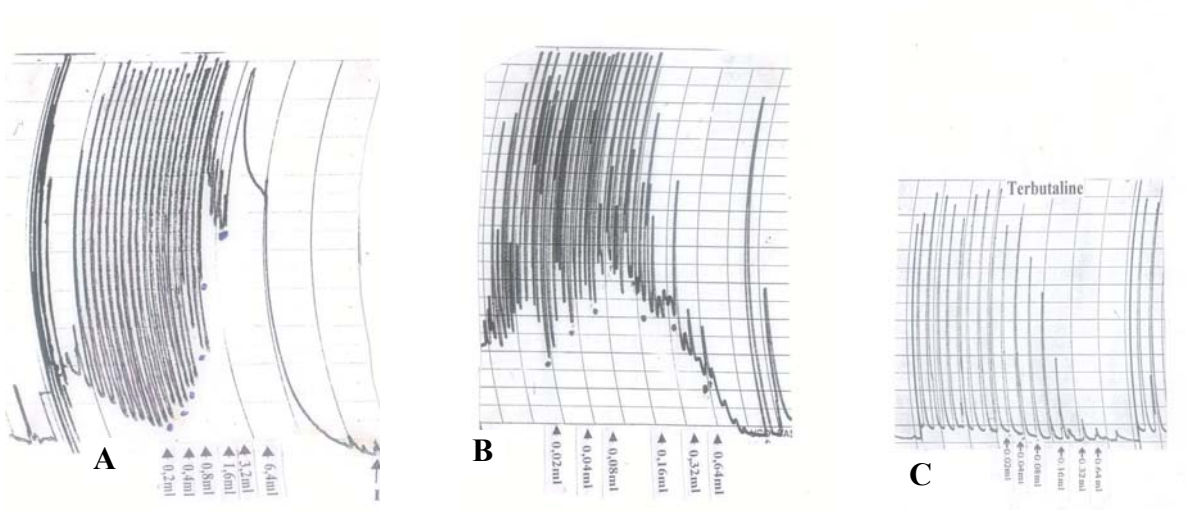

Figure 2: Tracing showing the effect of aqueous extract (A), its fraction (F_{A4}), and terbutaline (C) on rat uterine spontaneous contractions.

Discussion

Tocolytic effect of many medicinal plants has been tested on the uteri of rat (9); guinea pig (10) and rabbit (7). The organic and aqueous extracts of *A. montanus* caused prolonged relaxation of spontaneous uterine contractions. The organic extract had a non-depolarizing pattern, whereas the aqueous extract was depolarizing in nature. This difference could be attributed to highly water soluble stimulating substance (s) in the aqueous extract. Stimulation prior to relaxations by fractions of methanol and/or ethyl acetate revealed the presence of such substance(s). The relaxant property of methanolic extract of this plant had earlier been shown (4). Organic extract (EC_{50} = 0.27 mg/ml) relaxed more potently than the aqueous extract (3.0 mg/ml). Fractions from less polar solvents (e.g. hexane and ethyl acetate) showed more potent inhibitory actions when compared to polar solvent (methanol) derived fractions.

Since the solvent used by the traditional practitioners is water, the aqueous extract was used to antagonize some standard uterine agonists. The aqueous extract reversed the contractions induced by cumulative concentrations of $PGF_{2\alpha}$, acetylcholine, oxytocin, serotonin, histamine and calcium chloride in a dose- related manner. These inhibitions showed parallel and graded rightward shift of concentration-response curves. Antagonism of dihydroergotamine, noradrenalin and $BaCl_2$ contractions by the extract was dose independent but their EC_{50}s remained almost the same, despite geometric progression in dose.

The aqueous extract strongly inhibited the response of the rat uterus to autacoids of which serotonin was the least sensitive compared to $PGF_{2\alpha}$. Prostaglandin has been implicated in the genesis of many adverse conditions including oligospermia, antifertility, abortion and dysmenorrhea (11). The inhibition of $PGF_{2\alpha}$ and oxytocin-induced contractions by the extract could play an important role in the prevention of abortion. A relationship exists between oxytocin-induced uterine contractions and prostaglandin

synthesis. When oxytocin first interacts with membrane-bound receptors, it results in prostaglandin biosynthesis and calcium influx which culminates into contraction (12).

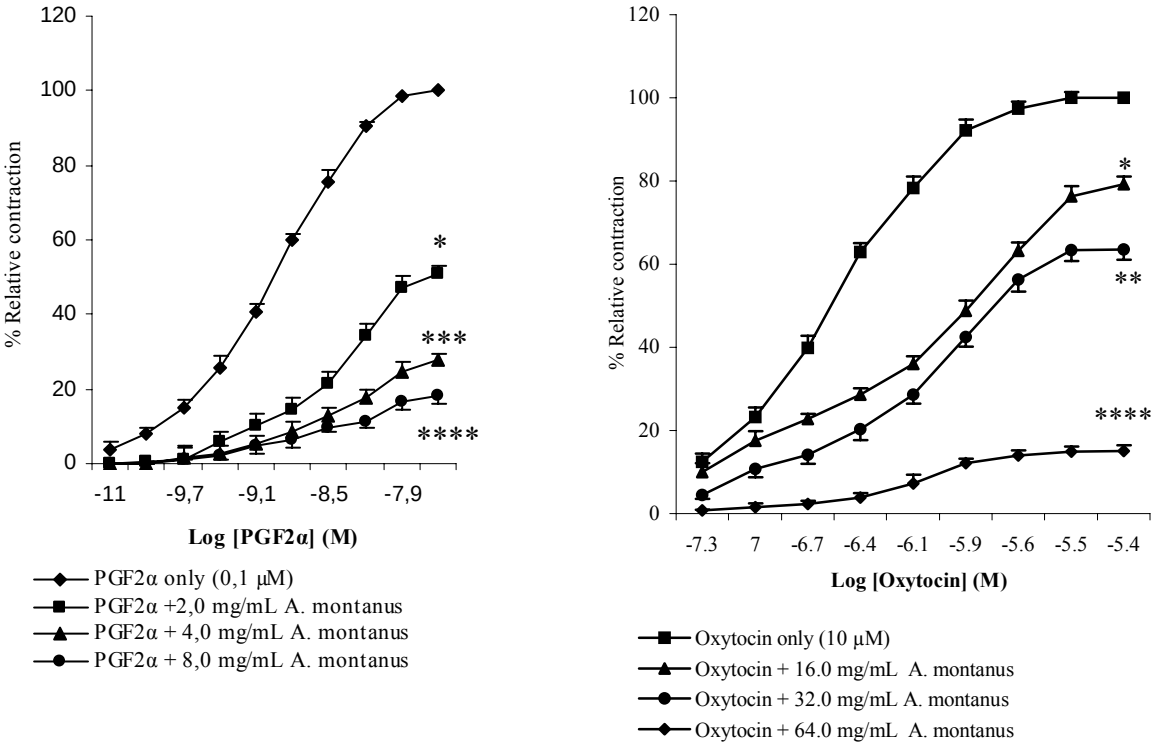

Figure 3: Cumulative dose-response curves of $PGF_{2\alpha}$ (A) and oxytocin (B) in the presence and absence of an aqueous extract of *A. montanus.*
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n = 6-7 experiments.

The aqueous extract was non-specific antagonists. This property might have given rise to the use of the plant to treat ailments such as false labour, dysmenorrhoea, pains and epilepsy. The non-specificity of the plant activity implied interaction with muscarinic, adrenergic, histaminergic, serotonergic, oxytocic (12) and prostanoid (13) receptors located on the uterus. These interactions could lead to reduction in intracellular calcium through several molecular mechanisms such as ion channels (14), second messengers and intracellular calcium stores (15).

The poor inhibition of Ca^{2+} and Ba^{2+} -induced contractions by aqueous extract was substantiated by the fact that Ba^{2+} enters into the cell through voltage sensitive Ca^{2+} channels (16) and any blockade will affect both. The aqueous extract was also assayed on the phasic component of contraction induced by KCl. This phasic contraction is due to direct calcium influx through L-type voltage dependant channels (17). The aqueous extract significantly inhibited the phasic contractions induced by KCl. These results suggested that the extract may have inhibitory effect on L-type voltage dependant calcium channels or reduces the sensitivity of contractile system to calcium. However, Ca^{2+} blockade was not the main second messenger involved in the relaxation. It had earlier suspected that the relaxant properties of methanolic extract of *Acanthus montanus* were completely inhibited by 10^{-4}M methylene blue (a nitrous oxide inhibitor) and 10^{-3}M procaine (a sodium ion pump inhibitor) (4). The non selectivity of the extract may lead to many side effects in case of prolonged treatment or over dosage, taking into account its pharmacokinetics. The potent inhibitory activity of the extract on $PGF_{2\alpha}$, serotonin and

histamine has buttressed the earlier findings that the extract had anti-inflammatory, peripheral analgesic and antipyretic properties (5).

The results substantiate the ethnomedical use of *A. montanus* to treat false labour and dysmenorrhoea and provide indirect evidence that the tocolytic effect of this plant is due to non polar components. Further research on other pharmacological profile of these extracts as well as the bioactive component (s) is currently in progress in our laboratory.

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