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RESEARCH ARTICLE

Evaluation of analgesic and anti-inflammatory effects of the crude methanol extract of the stem-bark of *Annona senegalensis* Pers.

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Abstract: *Annona senegalensis* Pers. (Annonaceae) is a plant widely used in ethnomedicine medicine (EM) for controlling and treating inflammatory conditions and pain. In this study, the analgesic and anti-inflammatory properties of the methanol stem-bark extract of *A. senegalensis* was evaluated using laboratory animal models. Analgesic activity of the extract was determined using the acetic acid-induced writhing and hot plate tests in mice, while the anti-inflammatory test was conducted using the egg albumin-induced paw oedema in rats and vascular permeability test in mice. The methanol extract of *A. senegalensis* (100, 400, 1000 $mg.kg^{-1}$; p.o.) produced significant ($p<0.05$) dose-dependent inhibition of writhes induced by acetic acid while also increasing the nociceptive reaction latency in the hot plate test. In respect of the anti-inflammatory test, *A. senegalensis* caused significant ($p<0.05$) dose and time-dependent decrease in the size of the paw oedema caused by egg-albumin and in a dose-dependent fashion also inhibited significantly ($p<0.05$) the permeability of blue dye into the peritoneal cavity of mice induced by acetic acid. The effects of the stem-bark extract were generally comparable to that of the standard drugs (pentazocine and piroxicam) used in this study. The findings in this study suggest that the methanol extract of the stem-bark of *A. senegalensis* possesses analgesic and anti-inflammatory properties possibly mediated through peripheral and central nervous system pathways. The results further justify the use of *A. senegalensis* extract in ethnomedical practice for the treatment of painful and inflammatory conditions.

Keywords: *Annona senegalensis*; analgesic activity; anti-inflammatory activity; acute toxicity test; phytochemical test; medicinal plant.

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most widely used agents for treating inflammatory and painful disease conditions (Herndon et al. 2008). Despite the efficacy of these drugs, they have serious toxic effects when administered for a very long time. Gastrointestinal ulceration leading to bleeding and renal disorders is among their adverse effects (Tapiero et al. 2002; Fujimori et al. 2010). Conversely, selective COX-2 inhibitors with little toxic effect on the gastro-intestinal tract have been associated with toxic cardiovascular effects (Dogné et al. 2005). The treatment and control of inflammatory painful conditions using NSAIDs continue to be challenging. Research to explore safer and more efficacious alternative treatments is therefore very important.

The use of plant drugs for the treatment of various health conditions has been documented. Over the last ten years the study of plant drugs has been of major interest in different fields of the biological sciences (Penna et al. 2003). About 11% of the 250 drugs considered by the World Health Organisation (WHO) to be essential are obtained from plants and a good number are semi-synthetic from plants (Rates 2001). There is therefore a high chance of getting lead compounds with anti-inflammatory effects in some traditional medicinal plants. A number of herbal drugs obtained from traditional medical plants have been shown to possess anti-inflammatory effect (Hosseinzadeh et al. 2002; Moura et al. 2005; Fernandez-Arche et al. 2010).

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Annona senegalensis Pers. (Annonaceae) is widely employed by tribal groups in different countries to treat different diseases. In Nigeria, the plant is commonly called "*Gwandar daji*" in Hausa language and is used traditionally to treat helminth infection and painful joint conditions (Dalziel 1955), snake bite (Nwude and Ibrahim 1980) and diarrhoea (Suleiman et al. 2008). Scientific research into the pharmacological activities of *A. senegalensis* had revealed the anthelmintic (Alawa et al., 2003), anti-trypanosomal (Igweh et al., 2002), antimalarial (Ajaiyeoba et al., 2006), antivenin (Adzu et al. 2003) and neuropharmacological properties of *A. senegalensis*.

In the present study, the analgesic and anti-inflammatory activity of the crude methanol extract of *Annona senegalensis* was investigated using established animal models.

Materials and methods

Plant collection and extraction

The stem-bark of *Annona senegalensis* was collected around Zaria, Nigeria. The plant was identified at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, and a voucher specimen number A798 was deposited. The peeled stem-bark of the plant was air-dried and made into powdered form using a mortar and pestle. Five hundred grams of the powdered plant material was extracted in a percolator using methanol as solvent and was mixed in the ratio of 1:5 of plant material and solvent, respectively. The mixture was allowed to stand for 48 hrs following which the tap of the percolator was opened to obtain the liquid extract. The whole extraction process was repeated 3 times. The extracts obtained during the extraction processes were pooled together and concentrated *in vacuo* at 50 ºC. The dark brown dried extract was dissolved in freshly prepared normal saline solution at a concentration of 50 mg/ml and refrigerated at 4 ºC until used.

Experimental animals

Adult Swiss albino mice (19-22 g) and wistar rats (180-210 g) of both sexes were used for this study. The animals were sourced from the Animal House, Department of Pharmacology and Therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria. Animals were kept in cages and pre-conditioned for two weeks in the laboratory (25 ± 2 ºC and 12 hrs light/dark cycle). Wood shavings were used as beddings and changed every week throughout the period of the experiment. Animals were fed with standard diet and allowed free access to water. The Animal Ethics Review Committee for Animal Experimentation of Ahmadu Bello University, Zaria approved all experimental protocols described in this study (U05VM1017-51).

Phytochemical analysis

The methanol extract of *A. senegalensis* was screened for presence of carbohydrates, glycosides, flavonoids, tannins, saponins, steroids and triterpenes using standard techniques (Tiwari et al. 2011).

Acute toxicity study

The LD_{50} as a measure of establishing the safety of the extract was determined using the method described by Lorke (1983) with slight modification. Briefly, 12 Swiss albino mice were deprived of feed and water for 24 and 12 hours, respectively and randomly divided into 4 groups of 3 mice each. Animals in groups 1, 2 and 3 received the extract orally at doses of 10, 100 and 1000 mg/kg, respectively. Animals were observed continuously for 1 hour after the treatment, intermittently for 4 hours, and thereafter over a period of 24 hours. The mice were further observed for up to 14 days following treatment for any sign of toxicity.

In the second phase of the trial, 9 mice were randomly divided into 3 groups of 3 animals each. Mice in groups 1, 2, 3 were given the extract at 1600, 2900 and 5000 mg/kg, respectively. All treatments were given through the oral route. Animals were observed as described earlier.

Anti-inflammatory test

Egg albumin-induced paw oedema in rats

The rat paw oedema for testing anti-inflammatory agents as described by Winter et al (1962) was used. Twenty five rats were randomly divided into 5 groups of 5 animals each. Groups 1, 2, 3 received 100, 400 and 1000 mg/kg of the extract of *A. senegalensis*, while groups 4 (treated control) and 5 (non-treated control) were given piroxicam at 10 mg/kg and 5 ml/kg of normal saline, respectively. All treatments were administered by oral route. Thirty minutes later, oedema was induced by injecting 100 µL of fresh egg albumin into the right plantar surface of the hind paw of each rat. The paw volume was measured before and at 20 minutes interval for two hours after induction of oedema. Inflammation was assessed as the difference between the zero time volume of the treated paw (V_0) and the volume at the various times (V_t) after the administration of the inflammatory agent. Acute inflammation was measured in respect to change in volume of the rat hind paw (Backhouse et al. 1996) induced by sub-plantar injection of egg albumin (Okoli et al. 2007).

Vascular permeability test

The method of Whittle (1964) with slight modification was used to evaluate the effect of the extract on vascular permeability in mice. Twenty five Swiss albino mice were randomly divided into 5 groups of 5 animals each. Groups 1, 2, 3 received 100, 400 and 1000 mg/kg of the extract of *A. senegalensis*, respectively, while groups 4 (positive control) and 5 (non-treated control) were given piroxicam at 10 mg/kg and 5 ml/kg of normal saline, respectively. All treatments were given through the oral route. One hour after, 100 µL of blue dye (0.25 % in normal saline) was given to each mouse intravenously through the tail vein. Thirty minutes later, each mouse was given 100 µL of acetic acid (0.6%, v/v) through the intraperitoneal route. All the animals were then sacrificed 30 minutes after the administration of acetic acid and their peritoneal cavity was individually washed with 3 mL of normal saline into heparinized tubes and centrifuged at 1500 *g* for 3 minutes. The supernatant was decanted into a clean test tube and the concentration of the dye contained in the supernatant was measured spectrophotometrically at a wavelength of 610 nm using Thermo Helious Zeta UV/VIS Spectrophotometer (serial number UV-164617).

Analgesic test

Acetic acid-induced writhing test

The acetic acid induced writing test as describe by Koster et al (1959) was used. Twenty five Swiss albino mice were randomly divided into 5 groups of 5 animals each. Groups 1, 2, 3 received 100, 400 and 1000 mg/kg of the extract of *A. senegalensis*, while groups 4 (positive control) and 5 (non-treated control) were given pentazocine at 10 mg/kg and 5 ml/kg of normal saline, respectively. All treatments were given through the oral route. Thirty minutes later, all animals were given 0.06% acetic acid intraperitoneally. Five minutes after acetic acid injection, mice were placed in an individual case and the number of abdominal contractions exhibited by each mouse was counted for 10 minutes.

Hot plate test

The hot plate latency assay was based on the method of Eddy et al (1950). Twenty five Swiss albino mice were randomly divided into 5 groups of 5 animals each. Groups 1, 2, 3 received 100, 400 and 1000 mg/kg of the extract of *A. senegalensis*, while groups 4 (positive control) and 5 (non-treated control) were given pentazocine (10 mg/kg) and normal saline (5 ml/kg), respectively. All treatments were given *per os*. Animals were placed individually on an enclosed copper hot plate maintained at 55 $\pm$ 0.5 ^{o}C and the time between placement of a mouse on the hot plate and occurrence of either a hind-paw lick or jump-off the surface was recorded as hot plate latency. The hot plate latencies were determined at 0, 60, 120 and 240 minutes after treatment.

Statistical analysis

Data were expressed as mean ± S.E.M and then analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The analyses were done using Graphpad Prism Version 4.0 for Windows from Graphpad Soft-

ware, San Diego, California, USA. Values of $p<0.05$ were considered significant.

Results

Extract yield and phytochemical test

A percentage yield of 22.7% was recorded after concentration of the extract to solid dry form. Glycosides, tannins, saponins, flavonoids, sterols, alkaloids and reducing sugars were detected in the extract of *A. senegalensis*.

Acute toxicity studies

The extract administered at doses of 10, 100, and 1000, 1600 and 2900 mg/kg did not produce any sign of toxicity or mortality in the tested animals. However, when given at a dose of 5000 mg/kg, the extract caused death of two animals. The LD_{50} of the extract was calculated by taking the geometric means of the highest dose that did not cause death (2900 mg/kg) and the lowest dose (5000 mg/kg) that produce mortality (Lorke 1983) using the equation below:

$$LD_{50} = (2900 \times 5000)^{½} = 3807.9 \text{ mg/kg}$$

Effect of extract on egg albumin-induced paw oedema

Sub-plantar injections of fresh egg albumin provoked marked, time-related, progressive increases in the hind paw diameters of the 'untreated control rats. Although pedal inflammation (oedema) was evident within 5-8 minutes following fresh egg albumin injection, maximal swelling and/or oedema occurred approximately 90 minutes following fresh egg albumin administration. The extract of *A. senegalensis* produced a significant ($p<0.05$) dose and time-dependent decrease in the size of the paw oedema caused by egg-albumin (Table 1). The activity of the extract was highest at 1000 mg/kg after 3 hours and is comparable to piroxicam (standard anti-inflammatory agent; 10 mg/kg).

Table 1: Effect of the crude methanol extract of stem-bark of *Annona senegalensis* on egg albumin-induced acute inflammation

Treatment	Dose (mg/kg)	Average paw size (mm) ± S.E.M.									
		0 minute	20 minutes	40 minutes	60 minutes	80 minutes	100 minutes	120 minutes	140 minutes	160 minutes	180 minutes
A. senegalensis	100	2.85 ± 0.07	4.49 ± 0.10[a]	4.10 ± 0.14 [a]	5.21 ± 0.08 [a]	5.06 ± 0.33 [a]	4.87 ± 0.18 [a]	4.37 ± 0.11 [a]	4.38 ± 0.09 [a]	5.82 ± 0.05 [a]	5.68 ± 0.11 [a]
A. senegalensis	400	2.80 ± 0.12	4.05 ± 0.12[a]	4.32 ± 0.11 [a]	4.27 ± 0.28 [b]	4.03 ± 0.13 [b]	3.68 ± 0.09 [b]	3.58 ± 0.16 [b]	3.31 ± 0.06 [b]	3.15 ± 0.02 [b]	3.09 ± 0.02 [b]
A. senegalensis	1000	2.56 ± 0.17	3.39 ± 0.19 [b]	3.09 ± 0.09 [b]	4.18 ± 0.06 [b]	4.03 ± 0.05 [b]	3.55 ± 0.10 [b]	3.35 ± 0.01 [b]	3.22 ± 0.01[b]	3.11 ± 0.01 [b]	3.07 ± 0.04 [b]
Piroxicam	10	2.66 ± 0.13	3.49 ± 0.46 [b]	3.12 ± 0.12 [b]	3.30 ± 0.27 [c]	3.52 ± 0.27 [c]	3.22 ± 0.23 [b]	3.94 ± 0.02 [b]	3.76 ± 0.08 [b]	3.01 ± 0.0 [b]	3.38 ± 0.19 [b]
Normal saline	5 ml/kg	2.60 ± 0.07	5.52 ± 0.15 [a]	5.8 ± 0.06	5.77 ± 0.17 [a]	6.13 ± 0.16	5.63 ± 0.14	5.68 ± 0.15	5.86 ± 0.07	5.83 ± 0.13 [a]	5.74 ± 0.11 [a]

Values are expressed as mean ± S.E.M. Means within the same column and having the same superscript letters are not significantly ($p<0.05$) different

Effect of extract on vascular permeability

The extract of *A. senegalensis*, at increasing doses, inhibited the permeability of blue dye into the peritoneal cavity of mice induced by acetic acid in a dose-dependent fashion (Figure 1). The extract (1000 mg/kg) showed greater inhibition of the membrane permeability to blue dye than piroxicam (10 mg/kg). Results from all the treated groups were compared with the non-treated (normal saline) control.

Effect of extract on acetic acid-induced writhes

The mean number of writhes were significantly ($p<0.001$) reduced in groups treated with the extract at 400 and 1000 mg/kg (Figure 2). Similarly, there was significant ($p<0.05$) reduction in the mean number of writhes between the group treated with the extract at 100 mg/kg and the non-treated control group.

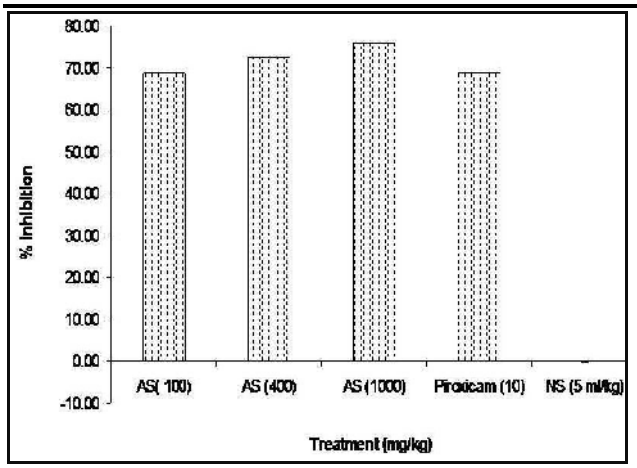

Figure 1: Inhibitory effect of extract on acetic acid-induced vascular permeability in mice.

Effect of extract on hot plate-induced nociception

The methanol extract of *A. senegalensis* significantly increased the reaction (latency) time of mice to heat nociception in a dose-related manner (Table 2). The extract at 1000 mg/kg produced the highest latency period of 23.00 ± 1.53 seconds after 60 minutes of pain induction and is not significantly different from that produced by pentazocine (standard drug).

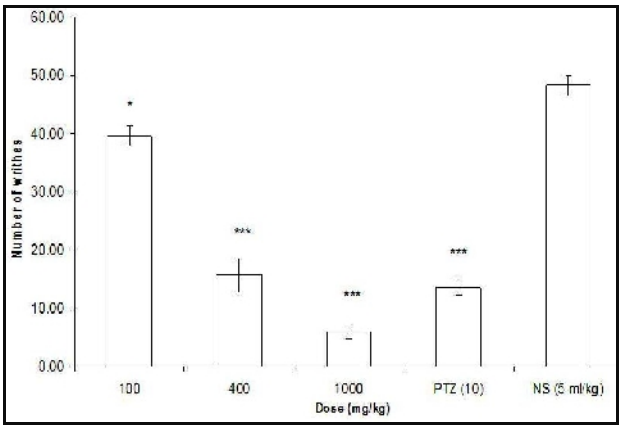

Figure 2: Effect of different doses of *A. senegalensis* on acetic acid-induced writhes in mice. Control group was dosed with normal saline at 5 mL/kg. Values are mean ± S.E.M. ***p < 0.001 and *p < 0.05 show significant difference when compared with normal saline-treated control group.

Table 2. Effect of crude methanol extract of *A. senegalensis* on heat nociception

Treatment (mg kg^{-1})	Latency period (second)			
	0 minutes	**60 minutes**	**120 minutes**	**240 minutes**
A. senegalensis (100)	7.33 ± 0.88	16.33 ± 0.33*,^{a}	15.30 ± 0.67*,^{a}	13.33 ± 1.76*,^{b}
A. senegalensis (400)	7.67 ± 1.20	22.31 ± 0.33**,^{a}	18.67 ± 0.88**,^{b}	17.97 ± 0.81**,^{b}
A. senegalensis (1000)	7.33 ± 0.88	23.00 ± 1.53**,^{a}	21.67 ± 1.20***,^{a}	22.33 ± 1.20***,^{a}
Pentazocine (10)	7.22 ± 0.67	25.33 ± 0.89**,^{a}	25.67 ± 0.33***,^{a}	12.67 ± 0.22*,^{b}
Normal saline (UC) (5 mL kg^{-1})	7.01 ± 0.33	5.33 ± 0.67	6.67 ± 0.33	5.01 ± 0.31

Values are mean ± S.E.M (n=5) seconds. *p < 0.05, **p < 0.01 and ***p < 0.001 versus untreated control (UC) group. Means within the same row and having the same superscript letters are not significantly ($p<0.05$) different. Similarly, means within the same column with different number of asterisk differ significantly ($p<0.05$).

Discussion

The present global interest in ethnomedicine has led to studies of herbal drugs used by different tribes of the world for treating different disease conditions. About 80% of the population living in developing countries relies on herbal medicine for their basic health care needs. Despite the widespread use, few scientific studies have been done to determine the safety and efficacy of traditional remedies (Tahraoui et al. 2007). To determine the safety of drugs and plant products for human and animal use, toxicological study is first done on any potential drug candidate in order to establish its safety. In the present study, the calculated LD_{50} of methanol extract of *A. senegalensis* is 3807.9 mg/kg. This value falls within the practically non-toxic range (Loomis 1978). Doses of up to 2900 mg/kg were found to be practically safe, causing no toxic sign or death in the test animals. Based on the above result, doses of 100, 400 and 1000 mg/kg were selected for the pharmacological screening tests.

Egg albumin-induced inflammation model is a significant predictive test for anti-inflammatory activity (Akah and Nwambie 1994). Egg albumin-induced oedema is characteristic of an acute inflammatory process and progresses with time. The inhibition of oedema caused by egg albumin suggests that the extract may suppress the early phase of the acute inflammatory response (Akuodor et al. 2011). The early phase which is immediate is characterized by mast cell degranulation leading to histamine and serotonin release. The second phase is followed by bradykinin release and pain and usually occurs after 1-2 hours from the start of inflamma-

tion. At the last stage of acute inflammation, which occurs during the third or fourth hour from the start of inflammation, eicosanoids are produced (Goetzl 1980). The results of the present study demonstrate that the methanol extract of the stem-bark of *A. senegalensis* possesses anti-inflammatory activity possibly by blocking the release of these inflammatory mediators.

It has been reported that vascular permeability increases due to contraction and separation of endothelial cells at their boundaries which exposes the basement membrane and becomes freely permeable to plasma proteins and fluid (Brown and Roberts 2001). Increased vascular permeability leads to exudation of fluids rich in plasma proteins including immunoglobulins (antibodies), coagulation factors (Cotran et al. 1999) and cells (Burt and Smith 2001) into the injured tissues (with subsequent oedema at the site). Exudation which is a consequence of increased vascular permeability is considered a major feature of acute inflammation. Mediators of inflammation (histamine, serotonin, bradykinin and eicosanoids) increase vascular permeability at different times after injury. Acetic acid induces vascular permeability that is immediate and is sustained over 24 hours (Okoli et al. 2007). The extract produced a dose-dependent inhibition of vascular permeability produced by acetic acid. Perhaps, this suggests that the extract may effectively suppress the exudative phase of acute inflammation.

Piroxicam reduces inflammation by inhibiting prostaglandins synthesis and/or production (Rang et al. 2003). The drug suppresses inflammation induced by phlogistic agents in these experimental animal models. Although the study is not conclusive, it tends to suggest that methanol extract of the stem-bark of *A. senegalensis* leaf aqueous extract may probably exert anti-inflammatory effects by inhibiting the release, synthesis and/or production of inflammatory cytokines and mediators, including: prostaglandins, histamine and polypeptide kinins. Flavonoids have been shown to inhibit eicosanoid biosynthesis (Damas et al. 1985). Eicosanoids, such as prostaglandins, are involved in various inflammatory responses (Moroney et al. 1988) and are the end products of the cyclooxygenase and lipoxygenase pathways. Another anti-inflammatory property of flavonoids is their suggested ability to inhibit neutrophil degranulation. This is a direct way to diminish the release of arachidonic acid by neutrophils and other immune cells (Nijveldt et al. 2001). *A. senegalensis* contains flavonoids and it is possible that the flavonoids present in this plant may act in a similar fashion to produce the observed anti-inflammatory response.

The writhing test has long been used as a screening tool for the assessment of analgesic properties of new substances (Collier et al. 1968). Acetic acid causes inflammatory pain by inducing capillary permeability (Amico-Roxas et al. 1984), while hot plate-induced pain indicates narcotic involvement (Besra et al. 1996). The effect of the extract on these two types of pain induction indicates that its analgesic effect may probably due to its anti-inflammatory neurogenic and narcotic properties.

The acetic acid-induced writhing test was used because of its high sensitivity and its ability to detect antinociceptive effects of substances at dose levels that may appear to be inactive in other analgesic tests (Bentley et al. 1981). The intraperitoneal injection of acetic acid produces abnormal writhing response due to sensitization of chemo-sensitive nociceptors by prostaglandins. Pain sensation in acetic acid-induced writhing method is elicited by triggering localized inflammatory responses resulting release of free arachidonic acid from tissue phospholipid (Ahmed et al. 2006) via cyclooxygenase (COX), and prostaglandin biosynthesis (Duarte et al. 1988). In other words, the acetic acid induced writhing has been associated with increased level of PGE_2 and $PGF_{2\alpha}$ in peritoneal fluids as well as lipoxygenase products (Derardt et al. 1980). The increase in prostaglandin levels within the peritoneal cavity then enhances inflammatory pain by increasing capillary permeability (Zakaria et al. 2008). The acetic acid-induced writhing method was found effective to evaluate peripherally acting analgesic. The analgesic effect of the extract may therefore, be due to either its action on the visceral receptors sensitive to acetic acid, or to inhibition of the production of algesic substances (bradykinin) or inhibition of transmission of painful messages (Magaji et al. 2008). The extract produced a dose dependent-decrease of writhing produced by acetic acid in this study.

To confirm the involvement of central mechanism(s) in the analgesic activity of *A. senegalensis*, the hot plate test was used based on the fact that centrally acting analgesic drugs elevate the pain threshold of rodents towards pressure and heat. The hot plate test involves the spinal reflex (Pini et al. 1997) and measures the complex response to a non-inflammatory, acute nociceptive input (Zakaria et al. 2008). The activity of the extract in the hot plate test indicates its central antinociceptive effect.

The methanol extract of the stem bark of *A. senegalensis* contains carbohydrates, glycosides, saponins, flavonoids, tannins, alkaloids and sterols/triterpenes. One or a combination of these

phytoconstituents may be responsible for the observed anti-inflammatory activities of *A. senegalensis* in this study. Saponins and tannins (Larkins and Wynn 2004), alkaloids (Reanmongkol et al. 1994), phenols and flavonoids (Calixto et al. 2000) have been reported to possess both analgesic and anti-inflammatory activities. In addition, flavonoids have been shown to act as antioxidants and also have analgesic and anti-inflammatory effects due to their inhibitory action on enzymes involved in the production of the chemical mediators of inflammation (Ahmadiani et al. 2000; Owoyele et al. 2005). A number of drugs used as analgesics are quite useful as anti-inflammatory agents because of their ability to block the synthesis of chemical mediators of inflammation (Pal et al. 1999). Similarly, in another study, terpenoids demonstrated anti-inflammatory activity (Neukirch et al. 2005). The secondary metabolites present in the extract of *A. senegalensis* may act either singly or in concert to produce the observed pharmacological effect in this study.

In conclusion, the experimental findings in this study suggest that the stem-bark methanol extract of *A. senegalensis* possess anti-inflammatory and analgesic activities. The results obtained justify the use of the plant extract in the treatment of pain and inflammation. Further work to isolate, identify, characterize, and elucidate the structure of the phytoconstituents responsible for the observed pharmacological activities in this study are ongoing in our laboratory.

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