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Mechanism of the Anti-inflammatory Activity of *Khaya senegalensis* A. Juss. (Meliaceae)

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Abstract: In the present study, the antipyretic, analgesic and antiphospholipase A_2 properties were investigated to explain the antiinflammatory effect of the stem barks aqueous extract. Yeast-induced hyperthermia in rat test was used to evaluate the antipyretic effect; writhing response induced by acetic acid in mice and rat tail-flick tests were used for antinociceptive effect. The effect of extract on the release of Arachidonic Acid (AA) and Oleic Acid (OA) in P388D1 cells was also investigated for the inhibitory activity of Phospholipase A_2. It was found that 1 g kg^{-1} of extract inhibited significantly Yeast-induced hyperthermia about 100% only 1 h after administration. The extract inhibited significantly the writhing response. The ED_{50} of extract was 157.821 mg kg^{-1} wile ED50 for Aspirin was 65.09 mg kg^{-1}. The reaction time to thermal stimuli was prolonged significantly ($p<0.05$) in dose-dependant manner in rats treated with 500 mg kg^{-1} (5.93 sec) and 750 mg kg^{-1} (7.08 sec) versus the control (4.32 sec) at 60 min. The extract inhibited the release of arachidonic (59.69%) and oleic acid (27.63%) and so inhibited Phospholipase A_2 activity in P388D1 cells.

Key words: *Khaya senegalensis*, aqueous extract, antiinflammatory, antipyretic, antinociceptive, anti-phospholipase A_2, P388D1 cells

INTRODUCTION

In the previous study (Lompo, 1998), using croton oil-induced mice ear oedema test, we found that aqueous extract from the stem barks of *Khaya senegalensis* commonly used in Burkina Faso folk medicine showed anti-inflammatory effect. Inflammation is a disorder involving localized increase in the number of leukocytes and a variety of complex mediator molecules (Gupta *et al*., 2006). It is a process characterized by the redness (rubor), beat (calor), pain (dolor) and swelling (tumor) (DeRuiter, 2002). In this anti-inflammatory effect, we evaluated only one parameter (antiphlogistic parameter). The other parameters like pain, hyperthermia and the release of arachidonic acid a precursor inflammatory mediators like prostaglandin (Michiels *et al*., 2002) were not yet studied. Prostaglandins are ubiquitous substances that indicate and modulate cell and tissue responses involved inflammation (Gupta *et al*., 2006). Thus prostaglandins and their therapeutic application include drugs which stabilize cell membranes and block the liberation of arachidonic acid. The aim of the present study was to investigate antipyretic, antinociceptive and antiphospholipase A_2 actions of this extract in order to explain the mechanism of its anti-inflammatory effect and to give a support of the folk medicinal use of the plant.

MATERIALS AND METHODS

The present study was carried out during 2005 and 2006 at Institut de Recherche en Sciences de la Santé of Ouagadougou, Burkina Faso and at the Free University of Brussels in Belgium.

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Plant and material extraction: Stems barks of *Khaya senegalensis* were collected at Sapone (Province of Bazega) in Burkina Faso and identified by the Institute of Natural Products Research in Ouagadougou. The barks were air-dried in the shade and powdered. One hundred and fifty gram of the powder was macerated with 800 mL of distilled water during 24 h and filtrated. The extract was then centrifuged 15 min at 3000 rpm and lyophilized giving 11 g of residue.

Animals: Wistar rats and Swiss albino N.M.R.I. mice were purchassed from C.I.R.D.E.S (Centre International pour la Recherche-Développement sur l'élevage en zone Sub-humide). All animals were acclimatized for at least 1 week in a room at 22-25°C before the beginning of experiments.

Testing for the inhibition of Ca or Ca and ATP-induced phospholipase A_2 activation (release of arachidonic acid or oleic acid in P388D1 Cells)

P388D1 Cells culture and labeling conditions (Balsinde *et al.*, 2000): P388D1 cells (MAB clone) were maintained at 37°C in a humidified atmosphere of 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units mL^{-1}) streptomycin (100 μg mL^{-1}) and nonessential amino acids. P388D1 cells were plated at 10^6/well, allowed to adhere overnight and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium.

Radiolabeling of the P388D1 cells was achieved by including 0.5 μCi mL^{-1} [^{3}H]AA (Arachidonic Acid) or 0.5 μCi mL^{-1} [^{3}H]OA (Oleic Acid) during the overnight adherence period (20 h).

Measurement of the released arachidonic acid or oleic acid in P388D1 cells: After the 20 h for radiolabeling, labeled AA or OA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 0.5 mg mL^{-1} albumin.

For [^{3}H]AA or [^{3}H]OA release measurements the cells were placed in serum-free medium for 30 min before the addition of Ca 0.5M or Ca 0.5M + ATP 1 mM or EGTA 0.5 mM without Ca or EGTA 0.5 mM + ATP 1 mM without Ca in the presence of 0.5 mg mL^{-1} bovine serum albumin. Extract (100 μg) was added to the cells 30 min prior to addition of the stimulus (Ca and ATP). The supernatants were removed, cleared of detached cells by centrifugation and assayed for radioactivity by liquid scintillation counting using LKB WALLAC 1211 MINIBETA Liquid Scintillation Counter.

Data presentation: Assays were carried out in triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments. The percentage of inhibition is calculated:

$$I\ \% = 100\times(\text{Control value-Value with Extract})/\text{Control value}$$

Antipyretic testing: Antipyretic property of *K. senegalensis* was tested in rats in which hyperthermia had been induced following the method described by Colot (1972). Initial rectal temperatures of the rats were recorded using an electric thermometer (Physitemp, Model BAT-12) connected with probes (Model SENSORTEK N.J. 07013 USA). Rats were made hyperthermic by subcutaneous injection of 20% yeast suspension at a dose of 1 mL/100 g body weight. When the temperature was at peak (16 h after yeast injection) the rectal temperature were again recorded. Those animals that showed a rise in rectal temperature of more than 1.2°C were used. Test substances and control vehicle were given orally and the rectal temperature of animals were recorded at 1 h intervals for 5 h following the administration of drug or plant extract.

Antinociceptive testing

Acetic acid induced writhing test: The method described by Veerappan *et al.* (2005) and Sawadogo *et al.* (2006) was used to investigate the analgesic effects of the plant extract.

The plant extract, i.e., lyophilized aqueous extract from *Khaya senegalensis* stem barks was orally administered to the mice at 100, 400 and 500 mg kg^{-1}. The reference (acetylsalicylic acid) was orally administered at 37.5, 75 and 100 mg kg^{-1}. The control mice received only water. After 1 h 0.6% acetic acid solution was injected intraperitoneally (15 mL kg^{-1}). Each animal tested was housed individually in a plastic cage. Nociception was evaluated 15 min after acetic acid injection by counting the number of abdominal constrictions for a period of 5 min as described by Bentley *et al.* (1983).

Tail-flick test: Male rats weighing 180-200 g, 5 in each group, were used. The method was assessed using a tail-flick apparatus following the method of D'Amour and Smith (1941) as modified by Gray *et al.* (1970). The light intensity was adjusted to give a normal reaction time of 2-5 sec. A 10 sec cutt-off time was used in order to prevent tissue damage. Two control readings, taken 30 min apart, were averaged and constituted the control reaction time. The extract or drug was administered (p.o) immediately after this step and 30, 60, 120 and 180 min later, the post-drug reaction time was measured. The

parameter evaluated was the latency time for tail-flicking responses after exposure on light beam. The increase of latency time in relation to the control was taken as an index of analgesic activity (Ravi kanth and Diwan, 1999).

Statistical analysis: Data were processed to obtain mean and standard deviation (SD) values. One-way analysis of variance followed by student's t-test from PHARM/PCS (Pharmacologic Calculation System) version 4.2 software was used to compare the mean values of different groups.

RESULTS

Testing for the inhibition of Ca or Ca and ATP-induced phospholipase A_2 activation: release of arachidonic acid in P388D1 cells: Figure 1 shows that in all the conditions, 100 μg of the lyophilised aqueous extract of *Khaya senegalensis* stem barks in a final volume of 500 μL inhibited significantly at $p<0.01$, the ATP-induced AA release in the presence of calcium. The same activity is observed when in P388D1 cells were Ca^{2+}-depleted by incubation with 1 mM EGTA.

The results of AA release decreased from 357.23 cpm for the calcium (control) to 144 cpm (59.69% of inhibition); and from 1436.63 cpm for ATP + Ca (control) to 781.17 cpm (45.62% of inhibition) when extract was used.

AA release decreased from 313.5 cpm in Ca^{2+}-depleted cells (+0.5 mM EGTA) to 143.37 cpm (54.27 % of inhibition) and from 1088.33 cpm for ATP (+ 0.5mM EGTA) (control) to 700.07 cpm (35.67% of inhibition) when extract was used.

Testing for the inhibition of Ca or Ca and ATP-induced phospholipase A_2 activation: release of oleic acid in P388D1 cells: Figure 2 shows that in all the conditions, 100 μg of the lyophilised aqueous extract of *Khaya senegalensis* stem barks in a final volume of 500 μL inhibited significantly at $p<0.01$, the ATP-induced AA release in the presence of calcium. The same activity is observed when P388D1 cells were Ca^{2+} -depleted by incubation with 1 mM EGTA.

The results of OA release decreased from 5473.67 cpm for the calcium (control) to 3961.33 cpm

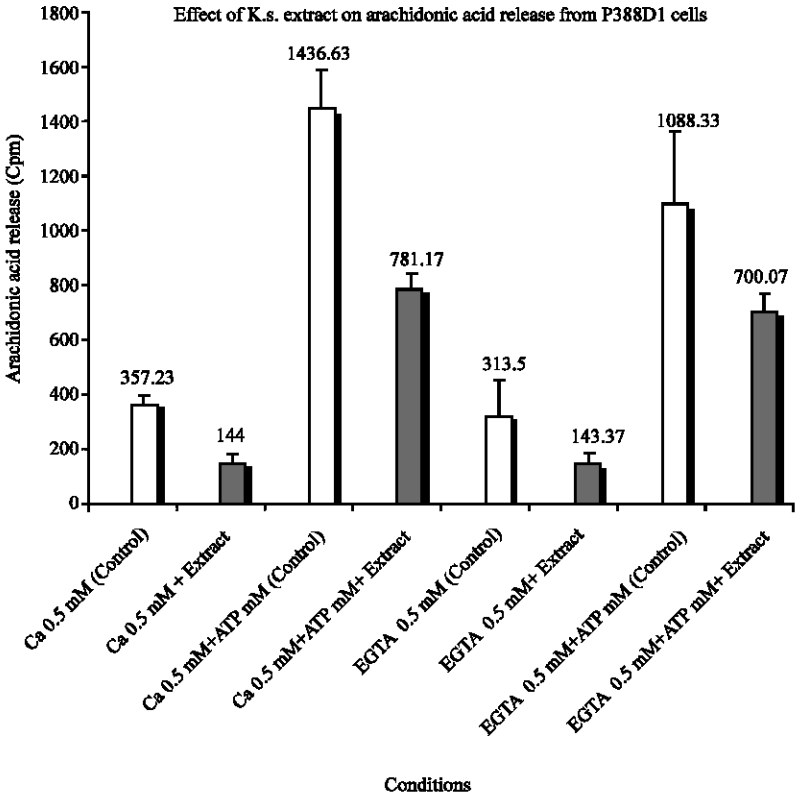

Fig. 1: Arachidonic acid release from P388D1 cells

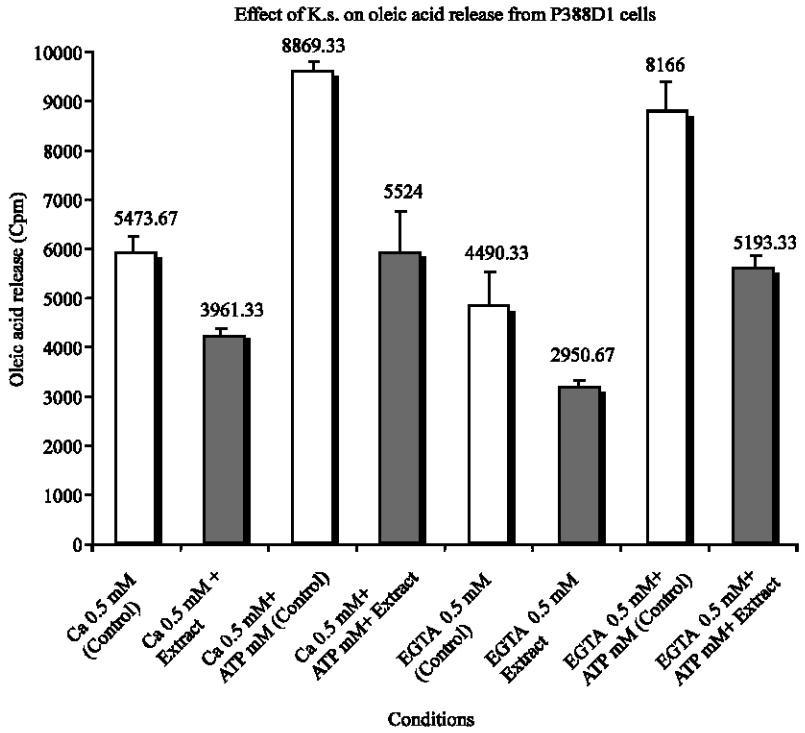

Fig. 2: Oleic acid release from P388D1 cells

Table 1: Effect of lyophilised aqueous extract of *K. Senegalensis* stem barks on yeast-induced hyperthermia in rats

		Rectal temperature (°C)					
Groups	Dose	0 h	1 h	2 h	3 h	4 h	5 h
Vehicle	5 (mL kg^{-1})	37.29 (±0.30)	37.58 (±0.26)	37.54 (±0.39)	37.7 (±0.32)	37.64 (±0.34)	37.9 (±0.45)
Brewer yeast	(Control)	38.73 (±0.31)	38.86 (±0.39)	38.6 (±0.44)	38.39 (±0.53)	38.51 (±0.22)	38.65 (±0.22)
K. senegalensis	1000 mg kg^{-1} after yeast	38.51 (±0.20)	37.53* (±1.09)	37.44* (±0.69)	37.4* (±0.69)	37.31* (±0.77)	37.71* (±1.01)

Drugs were given orally 16 h after yeast injection. Each value represents mean±SEM, (n = 8).*($p<0.01$), compared with control values for the corresponding h

Table 2: Effect of the lyophilised aqueous extract of the stem barks from *K. senegalensis* and aspirin on acetic acid-induced writhing in mice

Groups	No. of writhes	Inhibition (%)	ED_{50} (mg kg^{-1})
Control	60.17±1.34	-	
Aspirin, 37.5 (mg kg^{-1})	50.00±1.53	16.90	
Aspirin, 75 (mg kg^{-1})	21.67±1.25	63.99	65.09
Aspirin, 100 (mg kg^{-1})	15.83±2.11	73.69	
K.s. Extract, (100 mg kg^{-1})	35.50±1.72	41.00	
K.s. Extract, (400 mg kg^{-1})	21.50±1.38	64.27	157.82
K.s. Extract, (500 mg kg^{-1})	13.83±1.34	77.02	

Each value represents mean ± SEM(n = 6)

Table 3: Effect of lyophilised aqueous extract of the stem barks from *K. senegalensis* on the tail-flick test in rats

		Tail-flick reaction times (min)			
Groups	Dose	30	60	120	180
Control	-	4.42±0.50	4.32±0.45	4.24±0.56	4.40±0.5
K.s. Extract,	250 (mg kg^{-1})	4.49±1	5.40±0.81	3.90±1.41	4.60±1.19
	500 (mg kg^{-1})	6.54±1.96	5.93±0.81*	5.08±0.67	4.67±0.68
	750 (mg kg^{-1})	5.55±0.95	7.08±1.23*	5.04±1.14	5.25±0.85

*$p<0.05$ vs control; Student's t-test. Values correspond to mean±SEM (n = 5)

(27.63% of inhibition); and from 8869.33 cpm for ATP + Ca (control) to 5524 cpm (37.72 % of inhibition) when extract was used.

OA release decreased from 4490.33 cpm in Ca^{2+}-depleted cells (+0.5 mM EGTA) to 2950.67 cpm (34.29 % of inhibition) and from 8166 cpm for ATP (+ 0.5 mM EGTA) (control) to 5193.33 cpm (35.40% of inhibition) when extract was used.

Antipyretic activity: Yeast-induced hyperthermia in rats: Subcutaneous injection of yeast suspension markedly increased the rectal temperature.

As shown in Table 1, subcutaneous injection of yeast suspension markedly elevated the rectal temperature 16 h after administration. The difference between vehicle and control is significant at $p<0.01$ using Students't-test. The lyophilised aqueous extract of *Khaya senegalensis* stem barks has significantly decreased the yeast-induced elevation of rectal temperature at the dose of 1 g kg^{-1} b.w. After an hour, the extract decreased completely the hyperthermia since the comparison between the values of vehicle and the extract at 1h has shown no significant difference using student's t-test.

The decrease in rectal temperature still existed when assessment was made 2, 3, 4 and 5 h after test drug administration.

Antinociceptive activity

Acetic acid-induced writhing in mice: *K. senegalensis* extract at the doses of 100, 400 and 500 mg kg^{-1} b.w. and Aspirin at the doses of 37.5, 75 and 100 mg kg^{-1} b.w. induced a significant ($p<0.01$) decrease in the number of writhes when compared to the control untreated group (Table 2). The effective dose 50% of *Khaya senegalensis* extract (ED50 = 157.82 mg kg^{-1} b.w.) and Aspirin (ED50 = 65.09 mg kg^{-1} b.w.) were calculated according to the Method of Litchfield and Wilcoxon I: Confidence Limits of ED50 using PCS software.

DISCUSSION

PLA_2-mediated hydrolysis of glycerophospholipids results in the release of AA, which may either exert direct effects or serve as substrate for the generation of other lipid messengers such as the prostaglandins and leukotrienes (Balsinde *et al*., 1997; Balboa and Balsinde, 2002). PLA_2 plays a key role in cellular signaling by generating a wide array of biologically active lipid mediators. In this study of the mechanism of the anti-inflammatory effect of *Khaya senegalensis* stem barks, we found that lyophilised aqueous extract inhibited the release of arachidonic acid and oleic acid in presence of calcium in P388D1 cells or in Ca^{2+}-depleted P388D1 cells. It means that the extract exhibited an anti-phospholipase A_2 activity since Phospholipases A_2 (PLA_2) build up a class of enzymes which catalyse the release of AA and other unsaturated fatty acids by hydrolysing phospholipids on the sn-2 position (Glaser *et al*., 1993; Mayer and Marshall, 1993). The inhibited PLA_2 may be a calcium-indepedent PLA_2.

Since antipyretic activity is commonly mentioned as a characteristic of drugs or compounds which have an inhibitory effect on prostaglandin-biosynthesis (Vane, 1987), the yeast-induced hyperthermia in rat model was employed to investigate the antipyretic activity of the aqueous extract of *Khaya senegalensis* stem barks. It was found that the aqueous extract caused a significant decrease in rectal temperature. It seems to support the view that the extract has some influence on prostaglandins-biosynthesis because prostaglandin is believed to be a regulator of body temperature (Milton, 1982).

The writhing response of the mouse to an intraperitoneal injection of noxious chemical is used to screen for both peripherally and centrally acting analgesic activity (Trongsakul *et al*., 2003). Acetic acid causes algesia by liberating endogenous substances and many others that excite pain nerve endings (Raj, 1996). When compared the ED_{50} (Table 2), *Khaya senegalensis* extract exhibited a significant antinociceptive activity (ED_{50} = 157.82 mg kg^{-1} b.w.), but this activity is about 2.4 times lower than that of aspirin (ED_{50} = 65.09 mg kg^{-1} b.w.). This difference in writhing response can be due to the fact that the extract is a crude extract and not purified like aspirin. The mechanism of analgesic action of the extract could probably be due to the blockage of the effect or release of endogenous substances that excite pain nerve endings like aspirin and NSAIDs.

The tail-flick test is widely used to investigate the centrally acting analgesic activity. The tail-flick response appears to be a spinal reflex, which is modulated by a supraspinal inhibitory mechanism (Trongsakul *et al*., 2003). The reaction time (Table 3) to thermal stimuli was prolonged signicantly ($p<0.05$) in dose-dependant manner in rats treated with 500 mg kg^{-1} (5.93 sec) and 750 mg kg^{-1} (7.08 sec) versus the control (4.32 s) at 60 min.

The results obtained from the writhing response and tail-flick test suggest that the extract possess an analgesic effect that is mediated via a peripheral mechanism and also via the central nervous system which is suggested to be the hypothalamic region (Vane, 1987).

In summary, in order to explain the anti-inflammatory effect described previously, the study has shown that the lyophilised aqueous extract of the stem barks from *Khaya senegalensis* exhibited an anti-PLA_2 effect. This effect is calcium independent. The extract had an antipyretic effect and an antinociceptive activity which is mediated via a peripheral mechanism and also via the central nervous system. That was the aim of this study.

Attempt is under way to further examine what kind of PLA_2 did this extract inhibit since Balboa and Balsinde (2002) revealed that at present, 14 different PLA_2 groups have been identified.

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