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Khaya senegalensis augments the antinociceptive actions of piroxicam in murine models of hyperalgesia

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ABSTRACT

The increasing use of herbs alongside orthodox drugs, and the widespread use of *Khaya senegalensis* for its antinociceptive properties, necessitated investigation of the effect of the plant on piroxicam mediated antinociception in rodents. Carbohydrates, anthraquinones, tannins and saponins were found to be present, with the latter two being consistently present in plants with antinociceptive properties. At 200 and 400 mg/kg the ethanolic stembark extract of *Khaya senegalensis* exhibited antinociceptive properties ($p<0.05$). The effect of piroxicam was enhanced in the acetic acid, formalin and carrageenan models. In the acetic acid model, the co administration of the extract with piroxicam resulted to a slightly greater inhibition of writhing than piroxicam alone. In the formalin test, there was also enhanced analgesia in the group that received the extract and piroxicam in comparison to piroxicam alone. In the carrageenan induced inflammation model, the effect of the extract and piroxicam both alone and together produced significant ($p<0.05$) reduction in paw edema from the second hour in comparison with piroxicam. A similar effect was observed in the chronic inflammatory model in mice. The concurrent administration of *Khaya senegalensis* and piroxicam showed synergistic antinociceptive effects. New chemical entities with analgesic effects that may be combined with NSAIDS reducing propensity for current toxicities may be explored from the plant.

Keywords: *Khaya senegalensis*, piroxicam, drug interaction, antinociception

INTRODUCTION

Herbal medicine is the medicinal or therapeutic use of herbs or herbal products (Kunle et al, 2012). In the last decade there has been a revival of herbal medicine (Thomas et al, 2012), with resurgence in popularity even in Western countries (Tachjian et al, 2010). Of all the purposes for which herbal remedies are utilized, their use as antinociceptive agents is replete in the literature with several species having been studied. One of these species is Khaya senegalensis (Meliaceae), the African mahogany. Various studies have established the antinociceptive activity of extracts from the stem bark of Khaya senegalensis (Thioune et al, 1999; Lompo et al, 2007; Kolawole et al, 2013). While the problem of pain management remains, patients are increasing turning to herbs as sole therapy or in combination with traditional orthodox treatment (Wirth et al, 2005). This is because patients often use traditional and orthodox drugs concurrently (Pal and Shukla, 2003), and this they do in several disease conditions. This practice is not usually reported by patients and can lead to potentially harmful herb-drug interactions being missed or unrecognized (Tachijian et al, 2010). Herb-drug interactions may lead to increase or decrease in pharmacological actions of either component, or result in complications with long term therapy when synergistic effects are produced (Yaheya and Ismail, 2009). Being a well used plant for the treatment of pain, and following documented scientific evidence of its efficacy as antinociceptive, the current study was aimed at evaluating the effect of the ethanolic stem bark extract of Khaya senegalensis on the antinociceptive properties of piroxicam in murine models of hyperalgesia. Preliminary phytochemical and acute toxicological screenings were also carried out.

MATERIALS AND METHODS

Plant collection authentication and extraction: The stem bark of Khaya senegalensis was collected in June 2012 in Zaria Nigeria. Plant authentication was done by Umar Gallah, a Taxonomist in the Department of Biological Sciences, Ahmadu Bello University, Zaria-Nigeria, where a voucher specimen number (900181) was assigned. The collected stem bark was air dried away from sunlight in the Department of Pharmacology and Therapeutics, Ahmadu Bello University until fully dried to constant weight. The dried stem bark was pulverized before being extracted by maceration technique using 70% ethanol in distilled water over 48 hours. The filtrate was evaporated to dryness over a water bath at about 40°C and the yield was calculated.

Phytochemical Analysis: The phytochemical constituents of the plant material were determined using the methods as described by Evans, 1983. The crude extract was subjected to phytochemical analysis for classes of chemical

constituents including alkaloids, anthraquinones, carbohydrates, flavanoids, glycosides, tannins, saponins, steroids and triterpenes.

***Drugs and chemicals*:** Acetic acid (BDH), carrageenan (St Louis USA) and piroxicam (Hovid) were used. All dilutions and drug preparation were done using distilled water.The extract was also prepared freshly using distilled water before each experiment.

Experimental animals: Wistar rats and swiss mice of either sex (weighing 20-30 g and 190-220 g respectively) were used for the studies. The animals were maintained on standard rodent Feed and water ad libitum except when fasting was required. Animals were treated in accordance with the NIH guidelines, the CPCSEA 1986 and approved institutional guidelines.

Acute toxicity studies: Acute toxicity was conducted in female rats and mice using the OECD 420 Limit test protocol (OECD, 2001). This method in brief consisted of administration of a single oral dose of 2000 mg/kg of the extract to overnight fasted animals, followed by initial acute observation and subsequent observation for 14 days for signs of toxicity and death. Observations included behavioral and external signs including any changes on skin and defecation. Histological examination of organs liver, kidney, stomach, small intestine, heart, brain, lungs and spleen were also conducted at the end of the 14 day period, following euthanasia with chloroform. The protocol was repeated in a second phase following the outcome of the first phase in which there was neither death nor severe observable adverse consequences resulting from administration of the extract.

Experimental design and animal grouping: Animals were grouped into six groups (consisting of five or six animals). The first group served as a physiological control group receiving normal saline (10 ml/kg) while the second group received 20 mg/kg piroxicam as standard. The third and fourth groups received Khaya senegalensis at 200 and 400 mg/kg, respectively.

The fifth and sixth groups received similar treatment as the third and fourth, but received 20 mg/kg of piroxicam in addition. Both the extract and control drug were administered orally.

Acetic acid induced abdominal writhing: Acetic acid induced writhing; a method of evaluation of peripherally mediated analgesia was used based on the method of Collier et al (1968). This consists of administration of 10 ml/kg of 0.6% acetic acid solution intraperitoneally to swiss albino mice following a thirty minutes pretreatment with the individual group treatments. Number of writhes consisting of abdominal constrictions and stretching of limbs were counted for a cumulative fifteen-minute duration.

Carrageenan induced paw edema: This was conducted as previously described (Winter et al., 1962). The method in summary consists of administration of 0.1 ml of 1% w/v carrageenan solution to the plantar tissue of the left hind paw of rats, sixty minutes following drug pre-treatment (Badilla et al. 1999). Level of inflammation was determined by measuring paw inflammation over five hours duration using vernier calipers.

Formalin induced pain: The method of Dubbuison and Dennis 1977 was adopted in this experiment. This method consists of the administration of 25 µl of formalin solution to the plantar surface of the hind limb of mice following a thirty minute pretreatment time with the respective group treatments. An early (five minutes) and a late (forty-five) minute phase were evaluated for antinociceptive behavior.

However for scoring of antinociceptive effect, the protocol of Tjolsen et al (1992) was adopted. This consisted of a graded pain score ranging from 0 to 3. With 0 being no pain while 3 was recorded when the animal chewed or savored its paw.

Formalin induced chronic inflammation: The method used was as described by Han et al, 2012. This consists of administration of 0.05 ml of 5% formalin solution into the right hind limb paw of the mice. Formalin administration was done one hour after the various drug treatments. Drug treatment was continued daily for eight consecutive days as per the experimental protocol. Level of inflammation was determined by measuring the paw diameter using a vernier caliper from day 3 where the maximum inflammation was observed.

Data analysis: Data from the study was analyzed with One Way Analysis of Variance followed by Tukey or Dunnett's multiple comparison test. Kruskall Wallis and Mann Whitney tests were used where applicable. P values equal to or less than 0.05 were considered statistically significant.

RESULTS

The yield of the crude extract was 18.56%. Results of phytochemical analysis carried out showed the presence of anthraquiniones, carbohydrates, flavonoids, glycosides, steroids, tannins, saponins and triterpenes (Table 1). Oral acute toxicity studies using the OECD Limit test in both rats and mice at 2000 mg/kg showed that the extract had an LD50 above 2000 mg/kg.

This formed the basis for the doses selected for the main experiments. Relative weight of the spleen of rats differed significantly from the control group ($p<0.05$), while that of other organs did not significantly differ from the controls (Table 2). The histological studies conducted on organs 14 days after the single 2000 mg/kg dose of the extract showed varying degrees of histopathological changes particularly on the liver, kidney, lungs and spleen. These were however slight and other organs including the heart, brain, stomach and small intestine showed no distortion in histological architecture (Table 3). In the acetic acid induced writhing in mice, the extract showed statistically significant reduction in the number of writhes caused following the intraperitoneal administration of acetic acid $p<0.002$). In the presence of the extract, the effect of piroxicam was enhanced ($p<0.005$, Table 4). In the formalin induced acute pain model, there was no statistically significant difference between groups in the phase I of the study. In the second phase however there was statistically significant difference between the groups, with all treatment groups showing significant antinociceptive effect ($p<0.005$) in comparison with saline control. The higher dose of 400 mg/kg in combination with piroxicam produced better analgesia than the group that received piroxicam alone (Table 5). In the carrageenan induced paw edema in rats, the extract also showed a dose dependent inhibition of carageenan induced inflammation. The combination also produced a better effect than that of piroxicam alone (Table 6).

The formalin induced inflammation model in mice also showed that both doses of the extract significantly ($p<0.001$) reduced inflammation in comparison with the control. Both doses when individually combined
with piroxicam also significantly reduced inflammation, with the inhibitory effect of the combination in both cases better than that of piroxicam alone (Table 7).

Table 1: Results from phytochemical screening

Chemical Test/Reagent	Constituent	Observation
Dragendorff, Mayer, Wagner	Alkaloids	Absent
Free anthraquinones	Anthraquinones	Present
Molisch	Carbohydrates	Present
Shinoda	Flavonoids	Present
Fehling A + B	Glycosides	Present
Frothing Hemolysis	Saponins	Present
Salkowski	Sterioids	Present
Ferric chloride, lead subacetate	Tannins	Present
Libermann-Buchard	Triterpenes	present

Table 2: Effect of single dose 2000 mg/kg of ethanolic stem bark extract of *Khaya senegalensis* on relative weights (%) of selected organs in wistar rats and albino mice

Organs:	Brain	Liver	Spleen	Stomach	Kidney	Lungs	Heart
$Control_R$	0.8±0.1	2.8±0.5	0.6±0.1	0.6±0.1	0.3±0.0	0.7±0.1	0.3±0.0
Phase 1_R	0.7±0.1	2.8±0.1	0.3±0.0*	0.6±0.1	0.3±0.0	0.7±0.1	0.3±0.0
Phase 2_R	0.9±0.0	3.4±0.2	0.4±0.1	0.7±0.1	0.3±0.0	0.7±0.1	0.3±0.0
$Control_M$	1.4±0.2	7.2±1.1	1.4±0.2	1.1±0.1	1.0±0.2	1.9±0.3	0.5±0.4
$Phase1_M$	1.4±0.2	6.8±0.9	1.2±0.1	1.0±0.0	0.7±0.1	1.6±0.1	0.8±0.1
$Phase2_M$	1.5±0.3	6.0±1.0	1.1±0.2	1.1±0.2	0.8±0.1	1.8±0.3	0.8±0.2

Data are Mean ± SEM, n=3, * $p<0.05$ significant. (One-way ANOVA followed by Dunnett's Post hoc test). $_R$ =Rats, $_M$=Mice. *=P<0.05 compared with control

Table 3: Histopathological findings on selected organs two weeks after single oral dose of 2000 mg/kg of the extract

	Organs							
Treatment	Liver	Kidney	Stomach	Small intestine	Heart	Brain	Lungs	Spleen
Saline rats	NPO	NPO	NPO	NPO	NPO	NPO	NPO	NPO
Phase 1_{Rats}	Slight venous and sinusoidal congestion	Lymphocyte hyperplasia with slight tubular necrosis	NPO	NPO	NPO	NPO	Alveolar congestion and necrosis	Slight lymphocyte hyperplasia
Phase 2_{Rats}	Slight venous and sinusoidal congestion	Lymphocyte hyperplasia with slight tubular necrosis	NPO	NPO	NPO	NPO	Alveolar congestion and necrosis	Slight lymphocyte hyperplasia
Saline mice	NPO	NPO	NPO	NPO	NPO	NPO	NPO	NPO
$Phase1_{Mice}$	Sinusoidal congestion, Lymphocyte and kupfer cell hyperplasia	Lymphocyte hyperplasia with slight glomerular and tubular necrosis	NPO	NPO	NPO	NPO	Alveolar congestion and necrosis	Intense lymphocyte hyperplasia
$Phase2_{Mice}$	Sinusoidal congestion, Lymphocyte and kupfer cell hyperplasia	Lymphocyte hyperplasia with slight glomerular and tubular necrosis	NPO	NPO	NPO	NPO	Alveolar congestion and necrosis	Intense lymphocyte hyperplasia

Table 4: Effect of the ethanolic stem bark extract of *Khaya senegalensis* on piroxicam mediated inhibition of acetic acid induced writhing in mice

Group	Treatment	Number of writhes	Percentage inhibition	P value (vs control)
I	Saline	22.20 ± 3.27	0	-
II	Piroxicam 20 $mgKg^{-1}$	8.25 ± 2.35	62.83	0.005
III	KS 200 $mgKg^{-1}$	8.00 ± 1.54	63.96	0.002
IV	KS 400 $mgKg^{-1}$	7.80 ± 2.24	64.86	0.002
V	KS 200 $mgKg^{-1}$+ Pir 20 $mgKg^{-1}$	9.00 ± 1.78	59.45	0.005
VI	KS 400 mg^{-1}+ Piroxicam 20 $mgKg^{-1}$	7.60 ± 2.15	65.76	0.002

Table 5: Effect of *Khaya senegalensis* on piroxicam mediated analgesia in the formalin induced hyperalgesia model in mice

Group	Treatment	Mean rank Phase I	Mean rank Phase II
I	Sal	15.5	27.0
II	Pir 20	15.5	16.0**
III	Ks 200	15.5	19.5*
IV	Ks 400	15.5	10.0**
V	Ks 200+Pir	15.5	12.5**
VI	Ks 400+Pir	15.5	8.0**

Values presented are mean group ranks. Data was analyzed using Kruskall Wallis Test, followed by Mann Whitney Test for between group significance. *=P values ≤ 0.05; **=P values ≤ 0.005 compared to control. Pir = piroxicam; Ks 200/400 = *Khaya senegalensis* 200 and 400 mg/kg

Table 6: Effect of the ethanolic stem bark extract of *Khaya senegalensis* on the antinociceptive effect of piroxicam in carrageenan induced inflammation in rats

Group treatment	Mean hind paw diameter (millimeters)						
	0hr	0.5hr	1hr	2hr	3hr	4hr	5hr
Sal	2.26±0.06	3.29±0.16	4.09±0.04	6.27±0.14	7.09±0.28	6.06±0.19	5.60±0.25
Pir 20	2.23±0.04	3.17±0.16	3.32±0.18*	4.00±0.37**	4.36±0.32**	4.30±0.26**	3.74±0.22**
Ks 200	2.14±0.02	2.98±0.03	3.51±0.13*	5.14±0.23**	5.84±0.41*	5.67±0.42	4.92±0.31
Ks 400	2.23±0.03	2.90±0.22	2.98±0.10**	2.74±0.07^{**b}	2.71±0.09^{**b}	2.81±0.02^{**b}	2.71±0.02^{**a}
Ks 200+Pir	2.30±0.03	3.11±0.08	3.47±0.08*	3.97±0.08**	4.50±0.18**	4.11±0.07**	3.86±0.12**
Ks 400+Pir	2.13±0.05	2.51±0.07^{**a}	2.75±0.09**	2.70±0.07^{**b}	2.75±0.09^{**b}	2.63±0.05^{**b}	2.56±0.05^{**b}

*= p≤0.05, **= p≤0;005 compared with saline control; ^{a}= p≤0.05, ^{b}= p ≤0.005 compared with piroxicam treated. Data was analyzed with One way ANOVA followed by Tukey multiple comparison test. P <0.05 were considered significant. sal=saline; pir=piroxicam 20 $mgKg^{-1}$; Ks=*Khaya senegalensis* (200/400 $mgKg^{-1}$ respectively)

Table 7: Effect of *Khaya senegalensis* on piroxicam mediated anti inflammatory activity in the chronic formalin inflammatory model in mice

		Paw diameter readings at various days in (mm)					
Group	**Treatment**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**Day 8**
I	Sal	2.70±0.15	2.54±0.10	2.47±0.13	2.24±0.08	2.46±0.19	2.27±0.16
II	Pir 20	2.69±0.14	2.65±0.12	2.2±0.10*	1.90±0.09**	1.92±0.07**	1.93±0.06**
III	KS 200	2.41±0.03	2.39±0.09	2.40±0.12	2.27±0.12	2.19±0.18*	2.05±0.18**
IV	KS 400	2.72±0.07	2.85±0.12	2.60±0.17	2.09±0.11*	1.98±0.15**	2.18±0.14*
V	Pir + KS 200	2.74±0.16	2.32±0.11*	2.14±0.10**	1.82±0.06**	1.79±0.04**	1.73±0.06**
VI	Pir + KS 400	2.71±0.21	2.30±0.10*	2.26±0.08**	1.75±0.05**	1.76±0.06**	1.64±0.05**

Values are mean ± SEM of 4 - 5 observations, with the first reading serving as control for each group. Data was analyzed using One Way ANOVA followed by Dunnett's post hoc test. $P \leq 0.05$ was considered significant. Sal = saline; pir = piroxicam 20 mg/kg; KS 200/400 = *Khaya senegalensis* 200/400 mg/kg.*=p<0.05; **=p<0.001

DISCUSSION

The presence of secondary metabolites in plants is usually responsible for the biological actions produced (Kensa and Yasmin, 2011). Some of the phytochemical constituents present in the plant extract have been previously associated with antinociceptive effects. The constituents detected were largely corroborative of the work of Ibrahim et al, (2006). Tannins and flavonoids have been reported in the literature to be present in plants exhibiting antinociceptive properties (Hasan et al, 2011). The presence of these phytochemical constituents in the plant extract thus aligns with this previously documented evidence.

Triterpenes have also been reported to possess antinociceptive effects (Freire et al, 1991), and saponins have been consistently associated with antinociceptive activities. Significant antipyretic and peripheral analgesia was also reported while evaluating an extract that tested positive for saponins, tannins and flavonoids (Nisar et al, 2007). Thus the presence of these groups in this extract not only establishes reason for their antinociceptive actions but also a strong support towards possible isolation of novel compounds with antinociceptive activities.

Using the OECD method of oral acute toxicity, the extract was seen to possess an LD50 greater than 2000 mg/kg, establishing the relative safety of the extract and supporting its wide use in folkmedicine. With a relatively large LD50 pure compounds that may be obtained from the extract may be relatively safe, which is in line with current focus to obtain safer analgesic agents. Following single dose exposure of the extract, the histopathological investigations on the 14th day revealed some degree of pathological lesions particularly in the kidney, liver, spleen and lungs. Although these were slight in most cases, these findings suggest the need for further investigations and closer monitoring of the affected organs during prolonged use of the plant. This is more so due to effects on organs of detoxification and excretion namely the liver and kidney that were affected. However, it is not certain if the separation/elucidation of the compounds responsible for the antinociceptive effects may alleviate the observed pathological findings.

Of all organs weighed, only the spleen of the rats differed significantly which was in consonance with the histological findings following the single dose exposure in the acute toxicity studies. The reduction in the weight of the spleen shows a possible toxicological consequence of the extract, and is likely to impact on immunological functions, as reduction in spleen size has been associated with immunosuppression (Hoffman et al, 2009). However, the doses used in the main study were lower than 2000 mg/kg and hence may not constitute the observed risk at therapeutic doses.

The non steroidal anti-inflammatory drugs are a pharmacological group to which piroxicam belongs, and act by inhibition of cyclooxygenase (COX) enzymes. These drugs are amongst the most widely used drugs in therapeutics for pain and inflammation (Danhardt and Kiefer, 2001). The non selective NDAIDS inhibit COX 1 and also COX 2 (involved in the so called "house keeping") thus resulting in untoward effects associated with them (Stillman and Stillman, 2007). In vitro and ex vivo data shows that both traditional and COX 2 selective inhibitors all inhibit both COX 1 and COX 2 enzymes to varying degrees (Knights et al, 2010). Pharmacological evidence for the antinociceptive properties of the stem bark of Khaya senegalensis is replete in the literature, establishing its peripherally mediated analgesia and use for its anti inflammatory effects. This thus places Khaya senegalensis as eliciting its antinociceptive effects at least in part by COX inhibition. The concurrent medicinal use

of the plant in treating pain alongside orthodox drugs is therefore considered by patients as being beneficial.

However majority of synthetic drugs currently used as antinociceptive agents are known to have several side effects and toxic properties (Sateesh et al, 2013), and many of the existing analgesic drugs have side effects which tend to place restrictions on their use (Okusada et al, 2011). With the extract showing synergistic effect in all the models used, the traditional use of this herb in conjunction with piroxicam may not be without benefits. Besides, as the NSAIDs are known for their characteristic side effects, these may be reduced if the dose of piroxicam is reduced while taking the combination. However the non existence of co recognition/integration of traditional medicine in the Nigerian health system may pose a challenge to further exploiting this potential. The ability to obtain a synergistic analgesic effect in the presence of a herbal remedy as seen with Khaya senegalensis and piroxicam in this study enhance anti-inflammatory therapy and analgesic prognosis. While deliberate use of herbs and orthodox drugs is often practiced by patients, apparent deliberate adulteration of orthodox drugs to enhance antinociception has been reported (Vannacci et al, 2009). This may be undesirable and with unknown toxicological consequences. However with the demonstration of some level of synergy in the antinociceptive effects of Khaya senegalensis and piroxicam, structural elucidation of the active compound may also result in the production of an analgesic with better safety profile than the non steroidal anti-inflammatory drugs which in combination may be more effective and tolerable.

CONCLUSION

The outcome of this study shows that the concurrent use of the stembark extract of *Khaya senegalensis* and piroxicam produces synergistic analgesic effects, thus representing enhanced therapeutic benefit from herb-drug use. However the safety of this combination requires future investigation.

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