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Mechanistic Evaluation of Toxicity, Antinociceptive, Antiinflammatory and Antioxidant Actions of Leaf Extract of *Uraria picta*

Amole Olufemi O[1,*], Yemitan Omoniyi K[1], Akinyede Akinyinka A[2], Adebayo Ibitayo M[2].

[1]Department of Pharmacology, Therapeutics and Toxicology, Faculty of Basic Medical Sciences, Lagos State University College of Medicine, P.M.B. 21266, Ikeja, Lagos, Nigeria.
[2]Department of Pharmacology, Therapeutics and Toxicology, College of Medicine of the University of Lagos, Idi-Araba, P.M.B. 12003, Lagos, Lagos, Nigeria.

[*]***Author for Correspondence:***
Amole Olufemi O.
Email: femiamole@yahoo.co.uk

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SUMMARY

Objectives: This study was carried out to investigate the indicators of acute toxicity, anti-nociceptive, anti-inflammatory and phytochemical properties of the ethanol leaf extract of *Uraria picta* (UP) in rodents.

Methods: The acute toxicity indicators *via* oral and intraperitoneal routes as well as acute spontaneous motor toxicity in the open field test were conducted in mice. For the anti-nociceptive tests, the acetic acid-induced writhing and formalin tests were conducted in mice, while the carrageenan-induced paw oedema and, wet and dried cotton pellet-induced granuloma formation tests in rats were applied for the anti-inflammatory tests. Mechanisms of action of the anti-nociceptive actions were also explored using treatment interactions with naloxone, metergoline, glibenclamide, sulpiride and N^G–nitro-*L-arginine*.

Results: The acute oral toxicity test did not record any mortality in mice up to 5g/kg, but the i.p. administered UP produced LD_{50} of 812.83 mg/kg; moreover, the i.p., but not orally administered UP, from 500 mg/kg, produced dose-dependent significant ($p<0.05$) decrease in number of line crosses as well as rearing behaviour. For anti-nociceptive action, a significant ($P<0.05$) inhibition of acetic acid induced writhing, and reduced duration of paw-licking in the formalin induced test in mice was produced by UP (25-100 mg/kg, p.o.). In the anti-inflammatory activity, UP caused significant ($P<0.05$) dose-dependent inhibition of edema development in carrageenan induced inflammation and cotton-pellet induced granuloma formation in rats. Furthermore, pre-treatment of mice with naloxone, glibenclamide or N^G–nitro-*L-arginine* prevented UP-induced antinociception in the mouse writhing test.

Conclusion: The findings in this study suggest that the ethanol leaf extract of *U. picta* is orally safe, possesses anti-nociceptive action mediated *via* the opioid, ATP-sensitive K+ channels and nitric oxide mechanisms; as well as anti-inflammatory activities *via* COX-2 mediated stabilization of lysosomal membrane and inhibition of the migration of the inflammatory cells. These justify the use of the extract as an orally safe remedy in Traditional African Medicine for the treatment of pain and inflammation, and with antioxidant properties.

INTRODUCTION

The history of the therapeutic uses of plants dates back to the early man who had the crudest tool as his implement and used stones to start fire. The medicinal use of plants had been in existence long before the advent of orthodox medicine. However, countries like China and India held firmly to it and developed their traditional medicines to an international acceptance level e.g. acupuncture, aromatherapy, etc. Similarly, Nigeria is presently progressing in its own forms of traditional medicines, but advancement in this respect, leaves much to be desired.

Uraria picta (Fabaceae) is commonly known as 'Wizardry' or 'Slight-of-hand' herb; and it is a very important plant, especially as it relates to its usefulness as well as efficacy for some certain ailments in traditional medicine, compared with many other plants. It is an erect suffruticose herb or under-shrub up to 1.5meter tall, and it is commonly found in dry grassland, waste places and forests. In Nigeria, *Uraria picta* is locally known within local and international tribes as Alupayida (Yoruba) meaning the 'power of changing object or things',Obuodo dumbwada (Igbo), Wutsiyarkusu (Hausa), Mei Hua Li Wei Dou (Chinese), and Dhavni, Dabra, Pitvan or Shankaraja (Hindu). As a medicine, it is considered very effective as antiseptic, anti-malarial, bone fracture repair, anti-oxidant, analgesic and anti-inflammatory.

Pain is the most common reason for physician consultation and a major symptom in many medical conditions and can interfere with a person's quality of life and general functioning.[1] Therefore, it is almost impossible to imagine a world without pain relievers. Similarly, inflammation is a protective response that involves immune cells, blood vessels and molecular mediators, and the purpose

of inflammation is to eliminate the initial causes of cell injury, clear out necrotic cells and tissues damaged. Although the effective anti-inflammatory drugs are available, prolonged use may produce undesired adverse effects. The use of natural remedies has a long traditional history with minimal or no side effects, and are also known to play a crucial role in management of various inflammatory diseases, therefore naturally originated agents with such medicinal potentials are enviable to surrogate the use of chemical therapeutics.[2]

In view of these, this study was conducted to investigate the acute toxicity, analgesic and anti-inflammatory activities of the ethanol leaf extract of *Uraria picta*, using standard models, partly because no report of such study was found in the course of literature search.

MATERIALS AND METHODS

Plant material

Fresh leaves of *Uraria picta* were purchased from a traditional herbal practitioner in Mushin, Lagos State, Nigeria, in August 2015. The botanical identification and authentication of the plant was done by Mr. O.O. Oyebanji, a forestry expert of the Department of Botany Herbarium in the Faculty of Science, University of Lagos, Akoka, Lagos, Nigeria, where the herbarium voucher specimen (LUH 6606) was deposited for reference.

Preparation of plant extract

The dried leaves were milled into coarse powder and 1 kg of the material was loaded into a percolator. Extraction was done with 2 L of absolute ethanol for 72 h. After filtration, the residue was discarded and the final filtrate was concentrated in a rotary evaporator (40°C,under vacuum). The yield was 6.96% w/w. The greenish solid extract obtained was always reconstituted in distilled water to appropriate concentrations before administration to experimental animals.

Experimental animals

Albino mice (20–25g) and Wistar rats (180–200g) of either sex used in this study were obtained from the Laboratory Animal Centre of the College of Medicine, University of Lagos, Lagos, Nigeria. The animals were kept in well-ventilated and hygienic compartments, maintained under standard environmental conditions and fed with standard feed (Livestock Feed PLC, Lagos, Nigeria) and water *ad libitum*. The experimental procedures adopted in this study were in accordance with the United States National Institutes of Health Guidelines for Care and Use of Laboratory Animals in Biomedical Research.[3]

Acute toxicity tests

Median Lethal Dose (LD_{50}) Determination

Five groups of mice (n=5) were fasted for12h prior to the experiment, and were administered UP orally at doses of 500, 1000, 2000, and 5000 mg/kg. Animals in the different groups were observed for 2h post-treatment for immediate signs of toxicity. Mortality observed in each group within 24h was recorded. Animals that survived were observed for signs of delayed toxicity for a further fourteen days. The LD_{50} was estimated by the Log Dose–Probit analysis method. [4,5]

Acute spontaneous motor toxicity in the open field test

Each mouse was placed in an open field box (68 × 68 × 45 cm) equipped with a video camera. Observations over a 30 minutes period were recorded for assessment later.[6] The spontaneous motor (exploratory) activities of the mice were measured. Three parameters namely locomotion (line crossing), rearing and grooming were recorded. Five groups of 12-h fasted mice (n=5) were administered UP orally at doses of 500, 1000, 2000, and 5000 mg/kg. One hour later, animals in the different groups were tested for changes in locomotor and exploratory behaviours, compared to control. To another set of groups of mice, UP (500, 1000, and 2000 mg/kg) were given intraperitoneally. The number of line crosses and rearing behavior were determined in the mice.

Analgesic activity

Mouse writhing test

Mice fasted overnight were divided into five groups of five animals each. The animals were then treated with distilled water (10ml/kg, *p.o.*); UP (25, 50,100 & 200, mg/kg, *p.o.*); and diclofenac (100mg/kg, *p.o.*). Sixty minutes later, mice were administered with acetic acid(0.6%, v/v in saline, 10ml/kg, *i.p.*). The number of writhes (characterized by contraction of the abdominal musculature and extension of the hind limbs) was then counted for 30min. at 5 min. interval.[7]

$$\text{Inhibition (\%)} = \frac{\text{Number of Writhes [Control]} - \text{Number of Writhes [Treatment]} \times 100}{\text{Number of Writhes [Control]}}$$

Formalin test

Mice fasted overnight for 12 h were divided into five groups of five animals each. The different groups of animals were treated with distilled water (10ml/kg, p.o.), UP(25, 50 &100 mg/kg, *p.o.*); and morphine (15mg/kg, *s.c.*). Sixty minutes after administration for the oral route or thirty minutes for the subcutaneous route, formalin (20µL of 1% solution) was injected subcutaneous into the right hind paw of each mouse. The time (in seconds) spent in licking and biting responses of the injected paw, indicative of pain, was recorded for each animal. The responses of the mice were observed for 5min (first phase) and 15–30min (second phase) post-formalin injection.[8,9]

Elucidation of mechanism *U. picta*-induced anti-nociceptive in mice.

To investigate the mechanism by which UP produces antinociception in acetic acid-induced writhing, animals were pre-treated with antagonist of receptors implicated in pain. The choice of the doses was based on previous studies. The acetic acid writhing test was chosen based on its sensitivity in the transmission of pain.

Involvement of opioid, pre-treatment with naloxone

To investigate the role of opioid system in UP-induced anti-nociceptive effect, mice were pre-treated with naloxone (5 mg/kg, *s.c*, non-selective opioid receptor antagonist) or vehicle and after 15 min, UP (25 mg/kg, *p.o*) or vehicle (10 mg/kg, *p.o*) was given. One hour later, acetic acid 0.6%v/v in saline (10 ml/kg, *i.p*) was administered.

Involvement of nitric oxide, pre-treatment with N^G-nitro-*L*-arginine

To investigate the role played by nitric oxide synthase inhibitor pathway in the anti-nociceptive effect of UP was evaluated, mice were pretreated with N^G-nitro-*L-arginine* (10mg/kg, *i.p*; nitric oxide synthase inhibitor), after 15min, the animal received UP (25 mg/kg, *p.o*) or vehicle (10 mg/kg, *p.o*). One hour after treatment, acetic acid (10 mg/kg, *i.p*) was administered.

Involvement of serotonergic pathway, pre-treatment with metergoline

In another study, the possible participation of serotonergic pathway in the anti-nociceptive effect of UP was evaluated, mice were pre-treated with metergoline (4mg/kg, *i.p*; nitric oxide synthase inhibitor), after 15min, the animal received UP (25mg/kg, *p.o*) or vehicle (10 mg/kg, *p.o*). One hour after treatment acetic acid (10 mg/kg, *i.p*) was administered.

Involvement of dopaminergic pathway, pre-treatment with N^G-nitro-*L*-arginine

Also, the possible participation of non-selective dopaminergic pathway, particularly the D_2 and D_3 in the anti-nociceptive effect of UP was evaluated, mice were pretreated with metergoline (50 mg/kg, *i.p*; nitric oxide synthase inhibitor), after 15min, the animal received UP (25mg/kg, *p.o*) or vehicle (10mg/kg, *p.o*). One hour after treatment acetic acid (10 mg/kg, *i.p*) was administered.

Involvement of ATP-sensitive potassium channel pathway, pre-treatment with glibenclamide

The possible contribution of ATP-sensitive potassium channel pathway was also determined, mice were pre-treated with glibenclamide (10mg/kg, *i.p*) and 15min later, they received UP (25 mg/kg, *p.o*) or vehicle (10 ml/kg, *p.o*) and 1hr post treatment the acetic acid writhing test was carried out.

Anti-inflammatory activity

Carrageenan-induced paw oedema

Rat used in this experiment were divided into five groups of five animals each, and the respective groups were treated with distilled water (10ml/kg, *p.o*), UP (25, 50, & 100mg/kg, *p.o.*), and diclofenac (10mg/kg, *p.o.*). One hour later, oedema was induced by injection of carrageenan (0.1ml, 1%, w/v in saline) into the sub-plantar tissue of the right hind paw [10]. The linear paw circumference was then measured using the cotton thread method [11]. Measurements of paw circumference were done immediately before injection of the phlogistic agent and at 1h interval for 6h.

$$\text{Inhibition (\%)} = \frac{\text{Increase in paw oedema (control)} - \text{Increase in paw oedema (treated)}}{\text{Increase in paw oedema, control}} \times 100.$$

Cotton pellet-induced granuloma formation in rats.

The pellets of adsorbent cotton wool (20 mg) were sterilized in a hot air oven (model 600, Memmert, Germany) at 120˚C for 2 h. Two pellets were implanted subcutaneously, one on each side of the abdomen of the animal under light ether anesthesia and sterile technique. Then, UP at 25, 50, and 100mg/kg, and Celecoxib (20mg/kg) and distilled water were orally administered into 5 groups of 6 rats (180-200g) each, respectively, every 24 h for 72 hours. One hour after the last administration, the rats were anaesthetized using chloral hydrate (400 mg/kg). Cotton pellets implantation was done subcutaneously one on each side of the abdomen. Then oral administration was continued till the 10th day. Rats were sacrificed, the cotton wool was carefully removed out from the surrounding tissue and weighed immediately, then wrapped inside a foil paper which was then dried inside the oven at 40°C for 24h, after which the dried weight were determined.[12]

The transudative weight, granuloma weight and the percent granuloma inhibition of the test substance were calculated.

Quantitative phytochemical analysis

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay

The free radical scavenging capacity of *U. picta* was determined using the Stanley free radical DPPH.[13] The extract was mixed with 95% ethanol to prepare a stock solution (5mg/ml). Then, DPPH solution (0.004%,w/v) was placed in test tubes and UP was added followed by serial dilution (25µg to 150µg) in every test tube so that the final volume was 3ml. After 10min., the absorbance was read at 515nm using a spectrophotometer (HACH 4000 DR UV-Visible Spectrophotometer, USA). Ascorbic acid was used as a reference standard and dissolved in distilled water to prepare a stock solution with the same concentration (5mg/ml).

A control sample of the same volume was prepared without any extract and reference ascorbic acid. A solution of 95% ethanol served as a blank. The % scavenging of the DPPH free radical was measured using the following equation.

$$\text{\%Scavenging activity} = \frac{\text{Absorbance of the control x Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100.$$

The IC_{50} value is the concentration of the sample required to inhibit 50% of the radical,[14] served as the blank. Antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Determination of total flavonoid content

The total flavonoid content was determined using a method previously.[15] One millilitre of UP in ethanol (200µg/ml) was mixed with one millilitre of aluminium trichloride in ethanol (20mg/ml) and a drop of acetic acid, and then diluted with ethanol to 25ml The absorption at 415nm was read after 40min. blank samples were prepared from 1 ml of UPand a drop of acetic acid and then diluted to 25mL with ethanol. The total flavonoid content was determined using a standard curve for quercetin (12.5–100µg/ml and was expressed as mg of quercetin equivalent (QUE/g of extract)

Determination of total phenol content

Total phenol content in the UP was determined with Folin-Ciocalteu reagent. Then UP (200µg/ml) was mixed with 400µl of the Folin-Ciocalteu reagent and 1.5ml of 20 % Sodium Carbonate. The mixture was shaken thoroughly and was topped to 10ml using distilled water. The mixture was allowed to stand for 2h. The absorbance was read at 765nm. The total phenol content of UP was then determined as the mg of gallic acid equivalent, using equations that were obtained from a standard gallic acid graph.[16]

Determination of total tannin content

Five hundred milligram of the dried powdered samples was boiled in 20ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black coloration, which signified the presence of tannin.

Drugs and chemicals

The chemicals used were: Acetic acid (May & Baker Ltd., Dagenham, England); Formaldehyde (Griffin & George, Leics, England); Diclofenac (Total Healthcare, Parwanoo, India); Morphine (Martindale Pharma®, Essex, United Kingdom); Carrageenan (Sigma Chemical Company, USA).

Data Analysis

The results from the experiment were expressed as mean ± standard error of the mean (S.E.M.). Statistical comparisons between groups were analyzed by using two-way analysis of variance (ANOVA).

RESULTS

Toxicity tests

Acute toxicity test

Acute oral administration of UP, up to 5000 mg/kg did not produce any mortality (Table 1). Intraperitoneally, a mean lethal dose of 813 mg/kg was derived for UP (Figure 1).

Acute toxicity of *U. picta* on spontaneous motor activity in the open field test.

At 5000 mg/kg, orally-administered acute doses of UP did not produce any significant toxic changes in locomotor and exploratory behaviours compared to control. However when given intraperitoneally, UP (500, 1000, and 2000 mg/kg) produced toxic manifestations as significant ($p<0.05$) decreases in number of line crosses and rearing behavior in comparison to control (Table 2; Figure 1).

Analgesic Tests

Acetic acid-induced writhing test

As shown in Table 3, intraperitoneal injection of acetic acid elicited writhing in control mice with 46.2 ± 2.8 writhes counted within 30 minutes. A significant ($p<0.05$) reduction in the number of writhes with peak effect (61.9%) was produced by the extract at the lowest dose of 25mg/kg. This effect was though, less than (70.56%), but not statistically significantly different from that produced by 10mg/kg diclofenac.

Formalin-induced nociceptive test

In the first phase injection of formalin into the sub-plantar tissue of the right hind paw of control mice produced nociceptive response of biting and licking of the treated paw with a total duration of 31.2±32.7s. A significant ($p<0.01$) inhibition of nociceptive reaction with peak effect (83.34%) produced at the lowest dose of 25mg/kg UP. This effect was comparable to that produced bymorphine 5 mg/kg (83.34%). In the second phase the total duration of nociceptive reaction in the control group was 31.2 ± 32.71 s. The effect of UP in inhibiting the biting and licking response was significant ($P<0.0001$) and dose-dependent. However the highest inhibition (70.93%) was produced at the highest dose of 100 mg/kg. The effect of morphine was less comparable and not significantly different ($P>0.05$) from that of the effective dose (Table 4).

Table 1: Acute toxicity determination of the ethanol extract of *U. picta* administered orally in mice.

Treatment & Dose (mg/kg)	Log Dose	Mortality	% Mortality	Probit
U. picta 1000	3.000	3/3	100	8.79
U. picta 500	2.3979	0/3	0	0

Table 2: Effect of *U. picta* locomotor activity in open field test.

Treatment (mg/kg)	Line crossing	Rearing	Grooming
Control	47.00±10.44	16.00±3.46	6.33±0.88
UP 5000(p.o)	53.33±6.43	20.00±2.08	6.66±0.88
UP 2000(i.p)	31.66±2.08*	17.33±0.88	6.33±0.33
UP 1000(i.p)	30.00±2.64*	15.00±1.00	6.00±1.15
UP 500(i.p)	19.33±0.88***	10.33±0.66*	3.33±0.88

Values are mean ± SEM (n=5); ***P <0.001; *P<0.05 versus vehicle treated, Control; Statistical level of significance analysis by two way ANOVA followed by Turkeys multiple comparison test. UP = Ethanol leaf extract of *Uraria picta*.

Table 3: Effect of *U. picta* on acetic- acid induced writhing in mice.

Treatment	Dose (mg/kg)	Total number of Writhes	Inhibition (%)
Vehicle	10ml/g	46.2 ± 2.8	-
U. picta	25	17.60 ± 3.2***	61.9
U. picta	50	23.00 ±2.18**	50.21
U. picta	100	24.40 ±2.9***	47.19
Diclofenac	10	13.60 ±1.2***a	70.56

Values are mean ± SEM (n=5); ***P <0.001 versus vehicle-treated, Control; p< 0.05 versus *U. picta* (100mg/kg). Statistical level of significance analysis by one way ANOVA followed by Turkeys multiple comparison test.

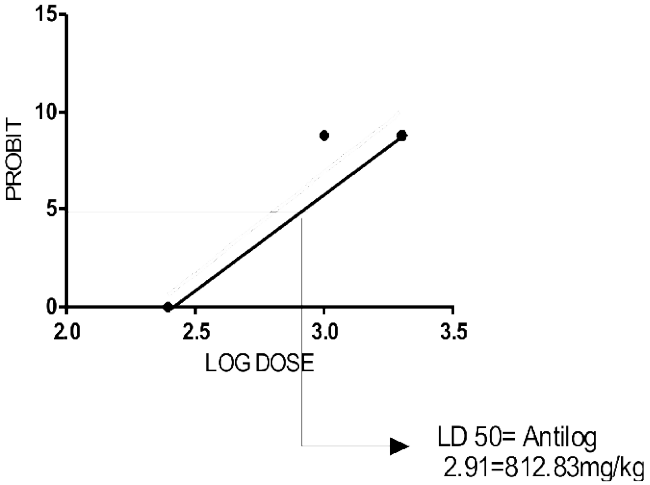

Figure 1: Acute toxicity test (intraperitoneal route)

Table 4: Effect of *U. picta* on formalin-induced pain in mice.

Treatment	Dose (mg/kg)	0-5 min		15-30 min	
		Response duration(s)	Inhibition (%)	Response duration(s)	Inhibition (%)
Vehicle	10mg/l	312.2±32.7		203.20 ± 33.2	
U. picta	25	52.00 ± 12.1**	83.3	59.00 ± 12.5***	70.9
U. picta	50	67.00 ± 16.2**	79.0	73.60 ± 21.0***	63.7
U. picta	100	71.20 ± 13.1*	77.2	78.80 ± 23.1***	61.2
Morphine	5	56.40 ± 12.2**	82.3	70.20 ± 24.7***	65.4

Values are mean ± SEM (n=5); *P <0.05; **P<0.01; ***P<0.001 versus vehicle-treated, control; P < 0.05 versus U. picta (100mg/kg). Statistical level of significance analysis by one way ANOVA followed by Turkeys multiple comparison test.

Involvement of metergoline on anti-nociceptive effect of *U. picta*

Figure 2 shows that the pre-treatment of mice with metergoline (4 mg/kg, i.p) prevents the anti-nociceptive effect elicited by UP in the mouse writhing assay. Two-way ANOVA revealed significant differences of treatments UP [F(1,16)=142.3,P<0.0001], but not pre-treatment of mice with metergoline [F(1,16)=2.763,P=0.1160] and metergoline pre-treatment × UP treatment interaction [F(1,16)=1.868,=0.1906]. Post-hoc analysis indicated that the effect of UP was not blocked by metergoline pre-treatment.

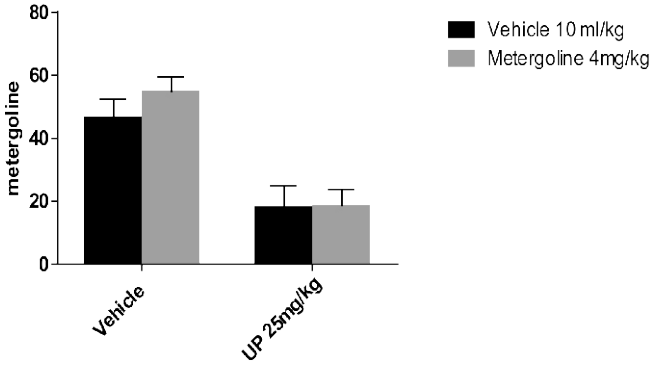

Figure 2; The effect of L-nitro arginine in *U. picta* induced anti-nociceptive in the acetic acid test. Values are expressed as mean ± SEM (n=5).no significant between vehicles. Metergoline, using two way ANOVA followed by Tukey *post- hoc* multiple comparison.

Involvement of naloxone on anti-nociceptive effect of *U. picta*

The results depicted in Figure 3, showed that the pre-treatment of mice with naloxone (5 mg/kg, s.c., non-selective opioid receptor antagonist), prevented the anti-nociceptive effect elicited by UP (25 mg/kg) in writhing test. Two-way ANOVA revealed significant differences of UPtreatment [F(1,16)=31.01,P<0.0001], naloxone pre-treatment [F(1,16)=3.528, P=0.0787], and naloxone pre-treatment × UP treatment interaction [F(1,16)=14.86, P=14.86]. Similarly, Tukey post hoc test showed that the anti-nociceptive effect of UP in writhing test was significantly (P<0.001) blocked by naloxone pre-treatment.

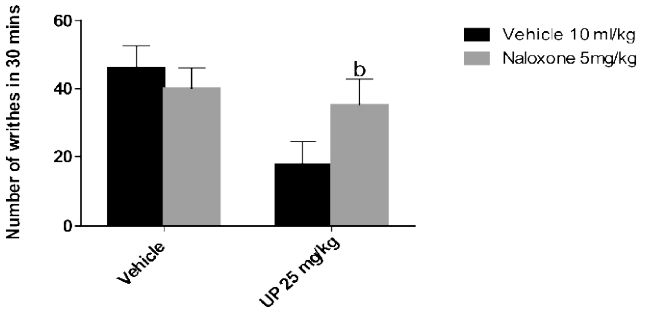

Figure 3 : The effect of L-nitro-arginine in *U. picta* induced antinociceptive in the acetic acid test. Values are expressed as mean ± SEM (n=5). [b]P<0.01 vs. vehicle; using two way ANOVA followed by Tukey *post-hoc* multiple comparison.

Involvement ofATP-sensitive K^+ channels pathways on anti-nociceptive effect of *U. picta*

Pre-treatment of mice with glibenclamide (10 mg/kg, ATP-sensitive K^+ channels blocker, i.p.), prevents the anti-nociceptive effect elicited by UP in the mouse writhing assay. Two-way ANOVA revealed significant differences of UP treatments [F(1,16)=27.30,P<0.0001], pre-treatment of mice with glibenclamide [F(1,16)=37.66,P<0.0001] and glibenclamide pre-treatment × UP treatment interaction [F(1,16)=11.99, P=0.0032] . Similarly, Tukey post hoc test showed that the anti-nociceptive effect of UP in writhing test was significantly (P<0.001) blocked by glibenclamide pre-treatment (Figure 4).

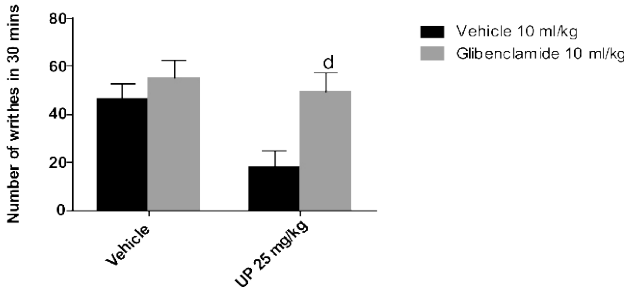

Figure 4: The effect of L-nitro arginine in *Uraria picta* induced antinociceptive in the acetic acid test. Values are expressed as mean ± SEM (n=5) [d]P<0.001, vs. vehicle; using two way ANOVA followed by Tukey *post-hoc* multiple comparison.

Involvement of non-selective dopamine antagonist (D_2 and D_3) on anti-nociceptive effect of *U. picta.*

Figure 5, shows that the pre-treatment of mice with sulpiride (10 mg/kg, non-selective dopamine antagonist i.p.), prevents the anti-nociceptive effect elicited by UP in the

mouse writhing assay. Two-way ANOVA revealed significant differences of UP treatments [F(1,16)=64.86, P<0.0001], but not pre-treatment of mice with sulpiride [F(1,16)=2.458,P=0.1301] nor sulpiride pretreatment × UP treatment interaction [F(1,16)=6.558,=0.0209]. *Post-hoc* analysis indicated that the effect of UP was not blocked by sulpiride pre-treatment.

Involvement of N^G-nitro-*L*-arginine nitric oxide synthase inhibitor on anti-nociceptive effect of *U. picta* system

Figure 6 shows that the pre-treatment of mice with N^G-nitro-*L*-arginine (10 mg/kg, nitric oxide synthase inhibitor i.p), prevents the anti-nociceptive effect elicited by UP in the mouse writhing assay. Two-way ANOVA revealed significant differences of *U. picta* treatments [F(1,16)=12.26, P=0.0030], but not pretreatment of mice with N^G-nitro-*L*-arginine [F(1,16)=38.16,P<0.0001] nor N^G-nitro-*L*-arginine pretreatment × UP treatment interaction [F(1,16)=60.08,<0.0001]. Tukey *post-hoc* analysis indicated that the effect of UP was blocked by N^G-nitro-*L*-arginine pre-treatment.

Anti-inflammatory tests

Carrageenan-induced paw edema

Injection of carrageenan into the sub-plantar tissue of the right hind paw of rats in the control group caused edema development, which peaked (7.48±0.21cm)as increase in paw circumference at 4h post-phlogistic agent injection. The effect of UP was dose-dependent from the 4th to the 6th hour with peak inhibitory effect (82.26 %) produced at the lowest dose of 25mg/kg at the 6th hour. This effect was less, but not significantly different(p>0.05) from that produced by 10 mg/kg diclofenac (83.11%).

Effect of *U. picta* onwet transudative cotton pellet granuloma

Figure 7 shows that the ethanol leaf extract of UP exhibited a significant and dose-related inhibition of the wet weight of cotton pellet granuloma. The inhibitory values for 25, 50, 100 mg/kg of the extract were 69.18%, 59.89% and 32.21% (P< 0.01), respectively. Celecoxib (20mg/kg) produced a value of 75.13%, comparable to 25mg/kg of the extract.

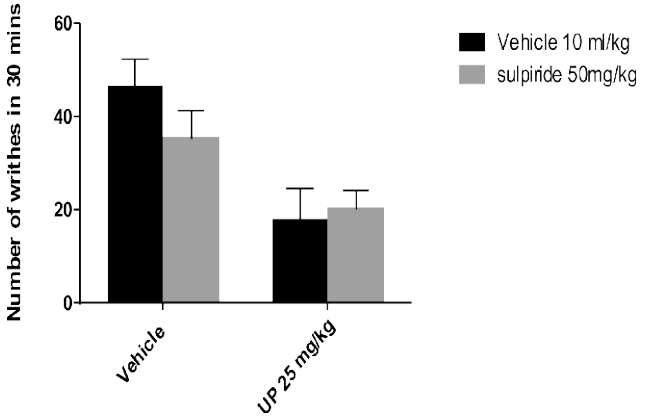

Figure 5: The effect of L-nitro arginine in *U. picta* induced antinociceptive in the acetic acid test. Values are expressed as mean ± SEM (n=5). No significant between vehicle vs. Sulpiride, using two way ANOVA followed by Tukey *post-hoc* multiple comparison.

Effect of dried transudative cotton pellet in *U. picta.*

Figure 8 shows that the ethanol leaf extract of UP exhibited a significant and dose-related inhibition of cotton pellet granuloma. The inhibitory values for 25, 50, 100mg/kg of the extract of the extract were 69.18%, 59.89% and 32.21% (P< 0.01), respectively. Celecoxib (reference drug) value of 75.13%, a slightly higher value than that recorded with doses of UP.

Quantitative phytochemical tests

The results of total tannin contents (TTC) assay showed that UP exhibited 3.80±0.05 mg/100g GAE. The total phenolic and flavonoid contents assay revealed that UP exhibited 3.90± 0.15 mg and 26.43 ± 0.36 mg/100g GAE, respectively (Table 2).

1,1-Diphenyl-2-picrylhydrazylfree radical scavenging assay

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical was used to determine hydrogen-donating ability of UP. The free radical reacts with hydrogen donors (free radical scavengers) to yield a stable product 1, 1-Diphenyl-2-picrylhydrazine resulting in a colour change from purple to yellow. As shown in Fig. 9, the values for IC50 obtained for DPPH assay are 18.96 µg/ml, 39.50 µg/ml gallic acid and UP, respectively.

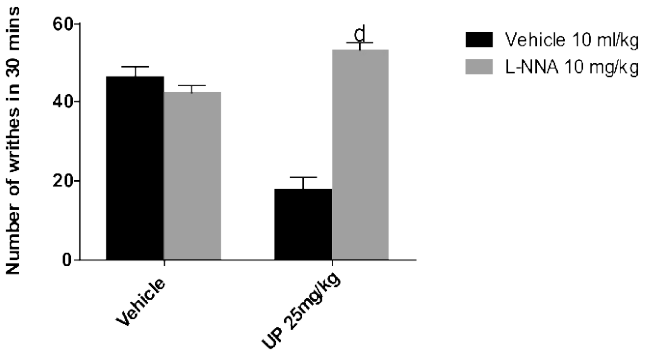

Figure 6; The effect of L-nitro arginine in *U. picta* induced antinociceptive in the acetic acid test. Values are expressed as mean ± SEM (n=5). ^{d}P <0.001 vs. vehicle; using two way ANOVA followed by Tukey *post-hoc* multiple comparison.

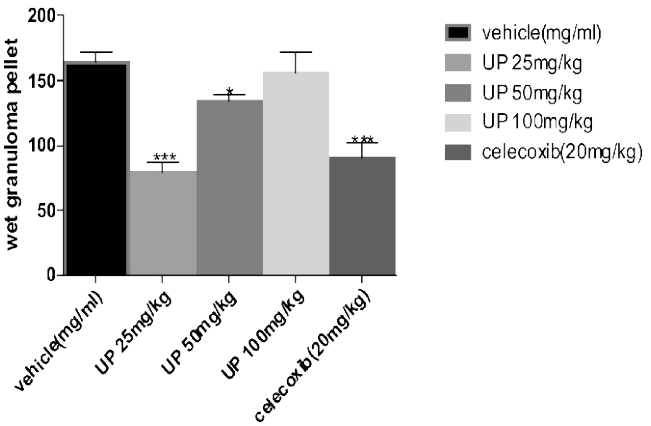

Figure 7: Values are mean ± SEM (n=5); ***P<0.001;*P<0.05 versus vehicle treated, Control. Statistical level of significance analysis by one way ANOVA followed by Tukey's multiple comparison test.

Table 5: Effect of *U. picta* on carrageenan induced paw oedema.

Treatment	Dose (mg/kg)	Time (h) 1	2	3	4	5	6
Vehicle % inhibition	10mg/l	8.27±0.23 (-)	8.04±0.29 (-)	7.6±0.24 (-)	7.48±0.21 (-)	7.97±0.15 (-)	7.13 ±0.28 (-)
U. picta % inhibition	25	6.44±0.17[c] 51.78%	6.39±0.17[c] 53.42%	5.92±0.14[c] 64.07%	5.79±0.61[c] 65.83%	5.51±0.11c 75.52%	5.12±0.18[c] 82.86%
U. picta %inhibition	50	6.95±0.47[b] 54.25%	7.68±0.19[b] 30.43%	7.27±0.30[b] 41.81%	7.15±0.23[b] 38.07%	6.73±0.21[b] 56.71%	6.82 ±0.14[b] 38.49%
U. picta % inhibition	100	7.44±0.14 35.06%	8.408±0.47 40.50%	7.65±0.33 19.18%	7.80±0.37 9.80%	7.36±0.23 31.64%	6.95±0.15 25.79%
Diclofenac % inhibition	10	6.53 ±0.15[c] 55.06%	6.47±0.10[c] 52.08%	6.10 ±0.15[c] 60.26%	6.08±0.27[c] 66.01%7	5.68 ± 0.16[c] 6.71%8	5.27±0.16[c] 3.11%

Values are mean ± SEM (n=5); [a]P< 0.001; [b]P < 0.01; [c]P < 0.0001 versus vehicle-treated, control; Statistical analysis by one way ANOVA followed by Tukey's multiple comparison test.

Table 6. Antioxidant properties of *U. picta*

Total Phenolic content (mg GAE/g)	Total Flavonoid content (mg QUE/g)	Total Tannin content (mg QUE/g)
90±0.15	26 ± 0.15	3.80 ± 0.05

Values are expressed as Mean ± SEM (n=2) [QUE- Quercetin equivalent; GAE- Gallic acid equivalent).

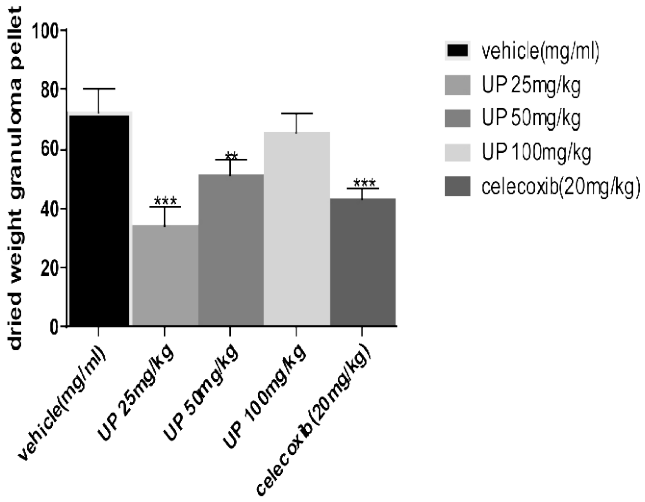

Figure 8: Values are mean ± SEM (n=5); ***P<0.001; **P<0.01; versus vehicle treated, Control. Statistical level of significance analysis by one way ANOVA followed by Tukey's multiple comparison test.

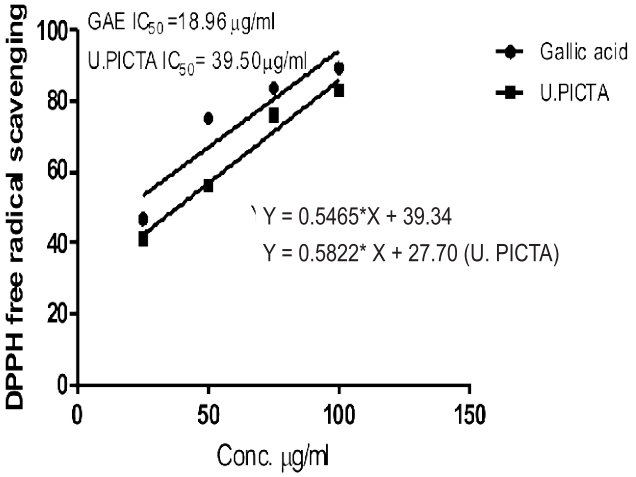

Figure 9: Graph of percentage inhibition of DPPH free radical generation against varying concentration of *U. picta* ethanol leaf extract and gallic acid. Values are expressed as mean of percentage inhibition.

DISCUSSION

In this study, the ethanol leaf extract of *Uraria picta* was investigated for its safety when used acutely, *via* the oral route, for which up to 5000mg/kg did not produce any mortality in mice. It has been suggested that above 5.0 g/kg no toxicity relationship should be ascribed to a substance.[17]. This is an indication that the extract is safe when used orally in this manner. Despite lack of necessity for the intraperitoneal administration of the extract, nevertheless, for the purpose of toxicity characterization, a median lethal dose, LD_{50} of 812.83mg/kg was derived for the i.p. route; moreover, the i.p., but not orally administered extract, from 500 mg/kg, produced dose-dependent manifestations as significant ($p<0.05$) decrease in number of line crosses as well as rearing behavior in the acute spontaneous motor toxicity in the open field test. Drugs that depress, or cause sedation of the central nervous system inhibit locomotion, grooming and rearing behaviour [18]. The significant level of inhibition of the spontaneous motor activity of mice by *U. picta*, as recorded in this study, especially as it relates to locomotor and rearing, suggests that it could cause considerable depression of central nervous system during use.

Analgesic activity using the mouse writhing and formalin test models. The intraperitoneal injection of acetic acid elicited writhing. The writhing test is simple, reliable and affords rapid evaluation of analgesic activity.[7] The induction of writhing by chemical substances injected intraperitoneally results from sensitization of nociceptors by prostaglandins,[19] and the test is useful for mild analgesic anti-inflammatory drugs.[20] The inhibition of writhing in the mouse, as demonstrated by *U. picta* suggests a peripherally-mediated analgesic activity based on the association of the model with stimulation of peripheral receptors especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity (Zakaria *et al.*, 2008). In other to confirm the peripheral mechanism of action and establish any possible central involvement, another model of analgesic activity evaluation, the formalin test was used. This test is commonly used to study the anti-nociceptive and anti-inflammatory properties of drugs.[21] The early phase (0-5min) has been associated with direct effect of formalin on nociceptors, while the late phase (15-30min) is said to involve inflammatory process.[22] According to Chan and co-workers, centrally-acting drugs such as morphine inhibit both phases of the formalin test while peripherally-acting drugs (e.g. diclofenac) inhibit the late phase only [23].

In the formalin test, *U. picta* inhibited both phases, thereby suggesting some central as well as peripheral analgesic mechanisms. Furthermore, that the effect of *U. picta* in the second phase was greater than that produced in the first phase suggest greater involvement of peripheral mechanism in its anti-nociceptive action. Interestingly, the anti-nociceptive effect was more evident at lower dose of 25mg/kg than at 100mg/kg. This observation therefore calls for further studies for a possible explanation.

The anti-inflammatory activity of *U. picta* was evaluated in this study using the carrageenan-induced paw oedema and cotton wool implantation tests. Inflammation induced by carrageenan is acute, non-immune, highly reproducible and can be quantified by the increase in paw size.[24] It has frequently been used to access the anti-inflammatory effect of natural products.[25] Furthermore, carrageenan-induced edema is a biphasic event, with the involvement of several inflammatory mediators: In the first phase- during the first 2h after carrageenan injection, chemical mediators such as histamine and serotonin play a role, whereas in the second phase, which approximates about 3–5hours after carrageenan injection, kinins and prostaglandins are involved.[26]

In this study, *U. picta* showed significant inhibitory effect on rat paw edema development in the early, and more pronouncedly, in the late phase of carrageenan inflammation, suggesting possible inhibition of serotonin, histamine, kinins and prostaglandin release. Also in the model, the anti-inflammatory effect was more evident at 25mg/kg than at 100 mg/kg, similar to observation in the anti-nociceptive models.

The response to subcutaneously implanted cotton pellet in rats is an experiment to investigate the ability of an agent to inhibit the proliferative component of the sub-chronic and chronic inflammatory process; and has been divided into transudative and proliferative phases, respectively. The transudative phase is defined as the increase in the wet weight of the granuloma whereas the proliferative phase is defined as the increase of dry weight of the granuloma. Even the non-steroidal anti-inflammatory drug, Celecoxib, is reported to only show a slight inhibition in this model.[27] In the present investigation, it was observed that the extract at 25mg/kg exhibited significant, peak inhibitory effects on both phases. The migration of leukocytes to the injury site occurs during chronic inflammation. Leukocytes accumulation leads to the release of lysosomal enzymes and oxygen radicals at inflammatory site.[28] In cotton pellet-induced granuloma formation, the activity of lysosomal enzymes is markedly elevated on the 7th day after implantation,[29] and can therefore be reliably suggested that *U. picta* at 25mg/kg normalized the reaction through the stabilization of lysosomal membrane and inhibition of the migration of the inflammatory cells into the inflammatory sites.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is widely used as the model system to investigate the scavenging activities of several natural compounds.[30] It is scavenged by antioxidants through the donation of proton, forming the reduced DPPH which can be quantified by its decrease of absorbance.[31] Radical scavenging activity increased with increasing percentage of the free radical inhibition; and DPPH radical as shown in the results indicated potent radical scavenging activities- an essential antioxidant property indication by *U. picta*, which offers protective effect against oxidative stressors.

Mechanistically, the local peripheral anti-nociceptive effect of *U. picta* in the acetic acid test was prevented by naloxone, *L*-nitro arginine, and glibenclamide, but not by sulpiride and metergoline. These results suggest that the anti-nociceptive effects induced by U. picta are mediated by the opioidergic system involving activation of δ and κ, and probably μ, opioid receptors at the periphery. Based on the results, therefore, it is suggested that *U. picta* activates the NO– cyclic GMP-ATP-sensitive K^+ Channels pathway in order to produce its local peripheral anti-nociceptive effect in the acetic acid test.

CONCLUSION

The experimental findings in this study indicate that the ethanol leaf extract of *Uraria picta* is not acutely toxic, orally, and that it possesses analgesic and anti-inflammatory activities possibly mediated *via* peripheral and central mechanisms involving the activation of opioidergicreceptors; also involved is NO–cyclic GMP-ATP-sensitive K^+ channels pathways, but not serotonergic and dopaminergic receptors. This study also demonstrates the antioxidant activities of *U. picta* which may be attributed to the presence of phenolic compounds, tannins and flavonoid content. Therefore, there is justification for the use of *Uraria picta* extract in Traditional African Medicine as an orally safe remedy for the treatment of pain and inflammatory conditions, with antioxidant properties, but with sedative side effects.

Conflict Interest

The Authors declare no conflict of interest in this study.

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