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A Mechanistic Approach to Anti-nociceptive Potential of *Nymphaea lotus* Linn (Nymphaeaceae) in Rodents

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ABSTRACT

Background: *Nymphaea lotus* Linn. (Nymphaeaceae), commonly known as white water-lily, white lotus or Egyptian lotus, is an important and well-known medicinal plant, widely used in the Ayurveda and Siddha systems of medicine for the treatment of diabetes, inflammation, liver disorders, urinary disorders, fever, skin diseases, cancer, gonorrhoea, pain and bronchitis. **Objective:** The study was designed to explore anti-nociceptive potential of aqueous extract of *Nymphaea lotus* leaf, its possible mechanism of action, and antioxidant properties. **Methods:** The anti-nociceptive activity of *Nymphaea lotus* (50, 100 and 250 mg/kg) was explored using writhing, formalin, tail clip and hot plate tests, while formalin test was used to investigate the involvement of opioid, dopamine, serotonin, K^+ channel blocker, α_1-adrenergic and α_2- adrenergic systems. The antioxidant effect was carried out using DPPH, nitric oxide free radical scavenging activity and the reducing power effect. Total phenolic and flavonoids contents were also explored. **Results:** Oral administration of *N. lotus* in doses of 50, 100 and 250 mg/kg recorded a significant ($p<0.05$) dose dependent obstruction of nociception. A remarkable effect was recorded with the writhing and formalin tests and a significant effect was also observed in the tail clip and hot plate test, which suggests peripheral and central anti-nociceptive activity of the extract. The anti-nociceptive effect produced by *N. lotus* was significantly reversed by naloxone and yohimbine, suggesting the possible involvement of opioid and α_2–adrenergic systems in its anti-nociceptive activity. *N. lotus* also displayed a potent antioxidant activity. **Conclusion:** These findings justify the folkloric use of *N. lotus* in pain management.

Keywords: Antagonists, *Nymphaea lotus*, Opioid, Pain, Antinociception, Antioxidant.

INTRODUCTION

Pain, a sensation which is always noticeable in inflammatory responses, is due to stimulation of pain fibres and hyperalgesia. It poses discomfort, hurt, or distress in individuals [1]. The conventional analgesic drugs – opioids and non-steroidal anti-inflammatory drugs (NSAIDs), which have been used for centuries, were all derived from plants. These pharmaceuticals are useful for both acute and chronic types of pain due to trauma, arthritis, cancer and surgery [2]. The NSAIDs are both analgesics and anti-inflammatory, therefore, they constitute the appropriate choice in inflammatory-induced pain, since they possess the propensity to inhibit prostaglandin synthesis by blocking the activity of cyclooxygenase synthetase. Howbeit, they are equally effective in non-inflammatory pains [3].

The usefulness of conventional analgesics is being challenged by adverse reactions such as constipation, dizziness, respiratory depression, dependency and tolerance.

On the other hand, NSAIDs can cause stomach pain and heartburn, stomach ulcers, liver or kidney problems high blood pressure, allergy, among others [4]. Hence, there is a need to explore for more naturally available alternatives, so that their therapeutic values can be assessed and expanded.

Nymphaeaceae is a family of aquatic, flowering and rhizomatous herbs. Members of this family are commonly called water lilies and live in freshwater areas in temperate and tropical climates around the world [5] such as Rwanda, Gambia, Nigeria, Ethiopia, and Burkina Faso. *Nymphaea lotus* Linn (NPL) (Nymphaeaceae) is a water-plant with white flowers, generally wide spread in Tropical Africa. It is an aquatic, perennial flowering plant up to 17.7 inches (45 cm) tall, with lily pads, a spreading perianth floating or immersed in water. The leaves are green and mostly floating and the flowers, white and sometimes tinged with pink, up to 10 inches (25 cm) in diameter [6].

Tradomedicinal uses of the plant include treatment of fever, skin diseases, cancer, gonorrhoea, pain and bronchitis[5]. Myricetin-3-0-rhamnoside, Nympholide A and B, Myricitrin, 1, 2, 3, 4, 6 pentagalloyl glucose as well as nupharine, nympheine, nelombine and nupharidine have been reportedly isolated from the plant [5, 6]. Furthermore, its antioxidant and antiviral potentials have been reported [7]. *Nymphaea lotus* has

also been shown to possess antibacterial [8] and antidiabetic [9] properties. The effect of *N. lotus* on pain has not been investigated, therefore, the study was set to explore the effect of *N. lotus* on pain as well as the possible mechanism(s) of action, using standard *in-vivo* anti-nociceptive models. Its anti-oxidant and phytochemical constituents were also evaluated.

MATERIALS AND METHODS

Drugs and Chemicals

Acetic acid (BDH Chemicals Ltd., Poole, England), formalin, (Sigma-Aldrich chemical company, United Kingdom), acetylsalicylic acid ASA (Dispirin®; Reckitt & Coleman Ltd., Pakistan), morphine, naloxone, metoclopramide and glibenclamide (Evans Medical Ltd., Leatherhead, England), yohimbine, prazosin and cyproheptadine (Koch-Light Laboratories Ltd., Suffolk, England). Folin-Ciocalteau reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin, Gallic acid and potassium acetate (BDH, Frankfurter, Germany).

Plant Collection and Extraction

Fresh leaves of *Nymphaea lotus* were collected from a flowing river in Idera, Ikorodu, Lagos, Nigeria. Identification and authentication were carried out by Prof J.D. Olowokudejo, in the Department of Botany, University of Lagos, Lagos-Nigeria, where a voucher specimen (LUH 7590) was deposited. The leaves were dried at room temperature until a constant weight was obtained within three consecutive days. The dried leaves (150 g) were pulverized and macerated for 72 h in a 1L distilled water, with intermittent swirling to facilitate extraction. Refrigeration maintained freshness of the mixture, which was filtered using Whatman's filter paper (25 mm). The filtrate was evaporated to dryness in an oven (Gallenhamp^R England) at 40 °C.

Animals

Healthy albino mice, 15-20 weeks old (18-20 g) used in the body were obtained from National Agency for Food and Drugs Administration and Control (NAFDAC), Yaba, was used in this study, and they were maintained under standard laboratory conditions, as approved by the United States National Institute of Health (NIH) guide for Care and Use of laboratory animals and recommendation of IASP.[10] The mice, fed on rodent diet (Livestock Feeds PLC, Ibadan, Oyo-State, Nigeria) and given free access to water, were made to acclimatize for a period of one week. They were fasted for 12 h before using for the study.

Acute toxicity

Lorke's (1983) [11] method was employed, with a slight modification.

Nine mice were divided into three groups (n=3), and each group dosed intraperitoneal (i.p) with 10, 100 and 1000 mg/kg NPL. The animals were placed under close observation for an initial two hours period to score the possible drug effect on behavioural parameters as well as drug lethality, and thereafter monitored until 24 hours post therapy. The check on the animals for any delayed toxicity continued for 14 days.

The same protocol was repeated, but via oral administration of 1600, 2900 and 5000 mg/kg doses of NPL.

Then the LD_{50} was calculated using the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,

D_{100} = Lowest dose that produced 100 % mortality.

Phytochemical Analysis of the Leaf Extracts of *Nymphaea lotus*

A measured weight of the dry solute was suspended in a known volume of distilled water, and 100 mg/ml of the mixture was used for phytochemical screening using standardized protocols [12] extract was suspended in distilled water and 100 mg/kg used for phytochemical screening, using standard methods [12].

Estimation of total phenolic content

The method of Wolfe *et al.*, (2003) [13] was employed with slight modification using a reference standard (gallic acid). The extract of *N. lotus* (0.5 mL) was added to the standard reagent (Folin-Ciocalteau; 0.1 mL; 0.5 N); mixed and incubated for 15 min. Afterwards, sodium carbonate solution (2.5 ml; 7.5% w/v) was added and the mixture was further incubated for 30 min at room temperature. The absorbance was spectrophotometrically estimated at 760 nm. The gallic acid equivalent (GAE) (mg/g of dry mass) was used to express the concentration of total phenolic content.

Estimation of total flavonoid content

The method described by Chang *et al.*, (2002) [14] was used with slight adjustment. Quercetin was a reference standard and the results were expressed as total quercetin equivalent (QE). *N. lotus* (1 ml; 100 µg/mL) was mixed with methanol (3 ml), 10% $AlCl_3$ (0.2 mL), potassium acetate (0.2 ml; 1 M). For 30 min (at room temperature) the mixture was incubated, after which the absorbance of the mixture was measured at 415 nm.

Anti-nociceptive models

Mouse writhing test

Overnight fasted mice were divided into five groups (n = 5) and administered orally the test substance as follows: Group 1: distilled water (10 ml/kg), Groups 2, 3 and 4 NPL 50, 100 and 250 mg/kg, Group 5: ASA (Acetylsalicylic acid) 100 mg/kg. All treatments were through oral administration. Sixty minutes after treatment, acetic acid (0.6 % v/v in saline, 10 ml/kg *i.p*) was administered. The number of writhes (characterized by contraction of the abdominal musculature and extension of the hind limbs) were counted for 30 minutes [4, 15].

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

Formalin test

Adult mice (18-20 g) fasted overnight were divided into five groups (n = 5). The animals were dosed treated accordingly: distilled water (10 ml/kg), NPL (50, 100, and 250 mg/kg), morphine 10 mg/kg subcutaneously (s.c.*)*. Sixty minutes post administration (oral) and thirty minutes (s.c), formalin (20 µL of 1 % solution) was injected into sub plantar tissue of 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 5 minutes (first phase) and 15-30 minutes (second phase) post formalin injection [1].

$$\text{Inhibition (\%)} = \frac{\text{Reaction Time (Control)} - \text{Reaction Time (Treatment)}}{\text{Reaction Time (Control)}} \times 100$$

Hot plate test

The animals employed in this study were initially screened by placing them on hot plate (II-39, Ugo Basile, Italy) maintained at 55 °C ± 0.5 to induce thermal pain and animals that failed to lick the paw or jump out of the plate in 10 seconds were rejected [16]. Animals that showed reaction to pain, were divided into five groups (n = 5) and treated as follows: Group 1: distilled water (10 ml/kg), Groups 2, 3 and 4 were treated with NPL at 50, 100 and 250 mg/kg respectively, Group 5: ASA 100 mg/kg *s.c*. The times in seconds to lick the paw or jump out of the plate were taken as the reaction time, which was recorded after oral administration of NPL at 30, 60, 90, 120, and 150 minutes. A post treatment cut-off time of 30 s was used [1].

$$\text{Inhibition (\%)} = \frac{\text{(Post- treatment Latency)} - \text{(Pre -treatment Latency)}}{\text{(Cut-off Time} - \text{Pretreatment Latency)}} \times 100$$

Tail clip test

As usual, the animals were first screened to determine their suitability by fixing a metal artery clip (RS-7440-35, Roboz surgical store, Gaithersburg, United State) to the root of the tail to induce pain [1]. Animals that failed to attempt to dislodge the clip in 10 seconds were discarded. Mice found suitable, were divided into five groups of five animals each. The pre-treatment reaction time of all mice to clip were determined after which the animals were treated as follows: Group 1: distilled water (10 ml/kg), Groups 2, 3 and 4 were treated with NPL 50, 100, 250 mg/kg Group 5: morphine 10 mg/kg *s.c*. Reaction time of each mouse was determined 60 min post treatment for oral administration and 30 mins post treatment for subcutaneous administration [1]. A post-treatment cut-off time of 30s was used.

$$\text{Inhibition (\%)} = \frac{\text{(Post- treatment Latency)} - \text{(Pre -treatment Latency)}}{\text{(Cut-off Time} - \text{Pretreatment Latency)}} \times 100$$

Mechanism of antinociceptive potential of *Nymphaea lotus*

Opioidergic mediation

Overnight fasted albino mice were divided into three groups (n = 5) and each group was pre-treated with naloxone (opioid receptor antagonist; 5 mg/kg, s.c.), and 15 min thereafter administered the vehicle (10 ml/kg, p.o.), *N. lotus* (250 mg/kg, p.o.) and morphine (10 mg/kg, *s.c*.). Forty-five minutes post treatment, mice were subjected to formalin test [17].

Serotonergic mediation

Overnight fasted mice were treated as before but pre-treated with cyproheptadine (non-selective serotonin receptor antagonist 5 mg/kg, s.c.) [18].

Dopaminergic (D_2-receptor) mediation

Animals were pre-treated with metoclopramide (dopamine (D_2) receptor antagonist;5 mg/kg, s.c.), and processed as above stated [17].

ATP K^+ Sensitive Channel Mediation

The same protocol described above was repeated after pre-treatment with glibenclamide (K^+ channel blocker; 5 mg/kg, s.c.) [19].

Alpha1-adrenoceptor mediation

Prazosin (alpha1-adrenoceptor antagonist; 62.5 mg/kg) pre-treatment and the formalin test [19].

Alpha2-adrenoceptor mediation

Yohimbine (alpha2 adrenoceptor antagonist;1 mg/kg) pre-treatment and the formalin test [19].

Total antioxidant capacity

DPPH radical scavenging activity assay

Free radical scavenging activity of *N. lotus* was estimated using DPPH according to established procedure [20]. NPL (0.5 ml) in ethanol (95 %) at different concentrations (25, 50, 75, 100 µg/ml) was added to 2.0 mL DPPH. The control contained only DPPH solution in place of the sample while methanol was used as the blank. The mixture was kept at room temperature after shaken vigorously. The absorbance was measured spectrophotometrically at 517 nm after 30 min. The scavenging effect was calculated using the expression:

$$\text{\% inhibition} = \frac{[Ab_0 - Ab_1]}{Ab_0} \times 100$$

Where Ab_0 is the absorbance of the blank sample and Ab_1 is the absorbance of the extract.

Nitric oxide scavenging activity assay

Four millilitre of *N. lotus* in different concentrations (25, 50, 75, 100 µg/ml) in different test tubes and 1 mL of sodium nitroprusside (5 mM in phosphate buffered saline) solution was added into the test tubes. For 2 h the mixture was incubated (30 °C). Two millilitres of the mixture

were added with 1.2 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4). The absorbance will be measured at 550 nm using spectrophotometer [21]. (T80 spectrometer, PG Instrument Ltd, Leicestershire, United Kingdom).

Reducing power assay

The reducing power was determined according to established procedure [20]. Various concentrations of *N. lotus* extracts (25, 50, 75, 100 µg/ml) was mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 minutes. Afterward, 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 650 rpm for 10 min. The upper layer will be mixed with 2 ml deionized water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 517 nm.

Statistical analysis

Results obtained were expressed as mean ± SEM. The data were analyzed using one-way ANOVA or by two- way ANOVA followed by Tukey's multiple comparison test GraphPad Prism 6 (GraphPad Software Inc., CA, USA). Results were considered significant when $p<0.05$

RESULTS

Physico-chemical Properties

The aqueous leaf extract of NPL was dark brown, fine powder with pungent odour. It has a pH of 7.2.

Acute Toxicity

Aqueous leaf extract of NPL was found to be safe orally, as 4000 mg/kg did not cause any mortality; the LD_{50} when administered intraperitoneally was estimated to be 22.36 mg/kg.

Acetic acid-Induced Writhes

Acetic acid elicited the writhing syndrome in control mice with 73.0 ± 2.5 writhes counted in 30 minutes. NPL produced a significant dose-dependent ($p< 0.05$) reduction in the number of writhes with peak effect (24.20 ± 2.35; 67.12%) produced at the highest dose of 250 mg/kg (Figure 1). The inhibition of pain produced by NPL was lesser but comparable with that of acetyl salicylic acid (21.40 ± 2.37; 71.2 %).

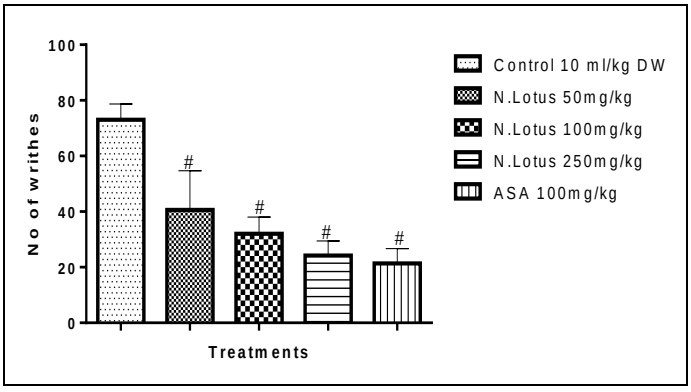

Figure 1: Effect of NPL on acetic acid induced writhing test.

Control: Distilled water, ASA: Acetyl salicylic acid,

Result represented as mean ± S.E M. (n=5); $^{\#}p< 0.05$ statistically significant compared to control (two-way ANOVA followed by Tukey's multiple comparison test).

Formalin-Induced Pain

Formalin test is biphasic, that is first phase (0-5 min) and second phase (15-30 min). 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 paw with duration of 104.0 ± 16.3 seconds. NPL produced a significant ($p<0.05$) dose dependent inhibition of nociceptive reaction with peak effect (15.8 ± 4.8; 84.80 % inhibition) produced at 250 mg/kg (Figure 2). The inhibitory effect was less than but compared effectively to that produced by 10 mg/kg morphine (11.2 ± 7.8; 89.2% inhibition). In the second phase, the duration of nociceptive reaction in the control group was 94.38 ±10.4 seconds. In this case, NPL significantly ($p<0.05$) inhibits the biting and licking response in a dose-dependent manner with peak effect (10.00 ± 3.56; 89.39 %) produced at 250 mg/kg, while the standard drug, morphine recorded (2.8 ± 1.9; 97.03% inhibition).

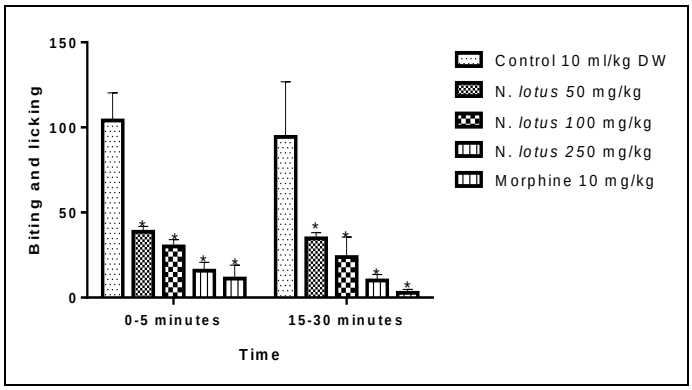

Figure 2: Effect of NPL on formalin induced pain test

Control: Distilled water, Result represented as mean ± S.E M. (n=5); $^{*}p< 0.05$ statistically significant compared to control (two-way ANOVA followed by Tukey's multiple comparison test).

Hot Plate-Induced Pain

The placement of the mice on the hot plate maintained at 55 ± 0.5 °C in the control group elicited reactions towards heat with the post-treatment latency being 3.61±0.83s, 3.53±0.56 s, 2.45±0.26 s and 2.43±0.46 s measured at 60 minutes, 90 minutes, 120 minutes and 150 minutes respectively with a pre-treatment latency of 4.61±0.83s (Table 1). The extract of NPL produced a significant ($p< 0.05$) dose dependent increase in reaction latency time with peak effect (68.96 % inhibition) at 60 minutes post-treatment at 250 mg/kg. Morphine 10 mg/kg also produced highest inhibitory effect (82.87 % inhibition) at 60 minutes

Table 1: Effect of aqueous leaves extract of *N. lotus* on hot plate-induced pain

Dose		Post-treatment (minutes)				
Treatment	**mg/kg(minutes)**	**Pre-treatment 60**		**90**	**120**	**150**
Control	10 ml/kg	4.61 ± 0.83	3.61 ± 0.34	3.53 ± 0.53	2.45 ± 0.26	2.43 ± 0.46
N. lotus	50	4.24 ± 0.70	13.84 ± 1.75[a] (37.26)	14.37 ± 0.71[a] (39.32)	10.09 ±1.23[a] (22.72)	4.76 ± 0.97 (2.05)
	100	4.07 ± 0.46	16.76 ± 1.18[a] (47.70)	18.24 ± 0.81[a] (53.52)	13.47 ± 0.82[a] (36.25)	4.74 ± 0.46 (2.58)
	250	4.26 ± 0.23	22.01 ± 2.32[a] (68.96)	16.28 ± 1.55[a] (46.68)	7.95 ± 0.82[a] (14.33)	4.71 ± 0.42 (1.82)
Morphine	10	3.30 ± 0.34	25.48 ± 3.42[a] (82.87)	21.22 ± 2.51[a] (66.56)	17.28 ± 1.94[a] (52.36)	7.84 ± 1.02[a] (17.00)

Control: Distilled water

Result represented as mean ± S.E M. (n=5); [a]p< 0.05 statistically significant compared to control (two-way ANOVA followed by Tukey's multiple comparison test).

The values in parenthesis indicate percentage inhibition of pain

Haffner's Tail Clip-Induced Pain

Application of the metal artery clip unto the tail of animals in the control group produced reactions towards clip removal with the post-treatment latency being 1.94±0.31s, 1.89±0.33s, 1.79±0.33s and 1.29±0.15s measured at 60 minutes, 90 minutes, 120 minutes and 150 minutes respectively with a pre-treatment latency of 2.23±0.47s (Table 2). The extract of NPL produced a significant (p<0.05) dose dependent increase in reaction latency time with peak effect (75.08 % inhibition) at 60 minutes post-treatment by the highest dose of 250 mg/kg. The effect produced by morphine 10 mg/ kg (100 % inhibition) is also significant (p<0.05) compared to control. At all the doses of the NPL (50 mg/kg, 100 mg/kg, 250 mg/kg), the highest inhibition was produced at 60 minutes post-treatment, while that of standard was produced at 90 minutes post treatment.

Table 2: Effect of aqueous leaves extract of *N. lotus* on haffner's tail clip-induced pain

	Dose		Post-treatment (minutes)			
Treatment	**mg/kg**	**Pre-treatment (minutes)**	**60**	**90**	**120**	**150**
Control	10 ml/kg	2.23 ± 0.47	1.94 ± 0.31	1.89 ± 0.34	1.79 ± 0.33	1.29 ± 0.15
N. lotus	100	3.08 ± 1.07	18.76 ± 3.08[a] (58.35)	14.19 ± 1.14[a] (41.44)	10.39 ± 0.85[a] (27.37)	6.12 ± 0.39 (11.55)
	250	2.61 ± 0.48	23.01 ± 3.19[a] (75.08)	15.22 ± 1.11[a] (45.98)	10.77 ± 0.95[a] (29.81)	8.82 ± 0.58[a] (22.72)
Morphine	10	2.21 ± 0.43 (89.49)	27.08 ± 1.64[a] (100.00)	30.00 ± 0.00[a] (88.00)	26.80 ± 1.33[a] (74.24)	23.12 ± 1.31[a]

Control: Distilled water

Result represented as mean ± S.E M. (n=5); [a]p< 0.05 statistically significant compared to control (two-way ANOVA followed by Tukey's multiple comparison test).

The values in parenthesis indicate percentage inhibition of pain

Mechanisms of Action of *N. lotus*

In the mechanism of action of NPL, naloxone (opioid receptor antagonist; 5 mg/kg, *s.c.*), cyproheptadine (non-selective serotonin receptor antagonist; 5 mg/kg *s.c.*), metoclopramide (dopamine (D_2) receptor antagonist; 5 mg/kg, *s.c.*), glibenclamide (K^+ channel blocker; 5 mg/kg, s.c.), prazosin (alpha1-adrenoceptor antagonist; 62.5 mg/kg) and yohimbine (alpha2 adrenoceptor antagonist; 1 mg/kg) were investigated.

Naloxone and yohimbine significantly (p<0.05) reversed the antinociceptive effect of NPL at 250 mg/kg dose level employed. Metoclopramide, glibenclamide and cyproheptadine insignificantly (p>0.05) curtailed its anti-nociceptive effect, while prazosin did not exert any observable effect on its activity (Figures 3-8).

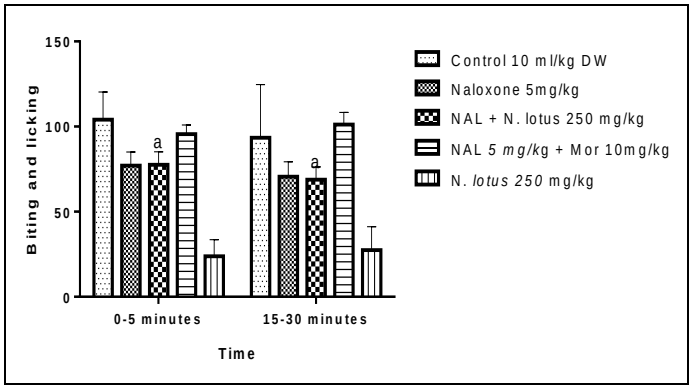

Figure 3: Effect of naloxone on NPL using formalin induced pain. NAL: naloxone, Mor: Morphine.

Control: Distilled water, Result represented as mean ± S.E M. (n=5); [a]p< 0.05 statistically significant compared to NPL (250 mg/kg) (two-way ANOVA followed by Tukey's multiple comparison test).

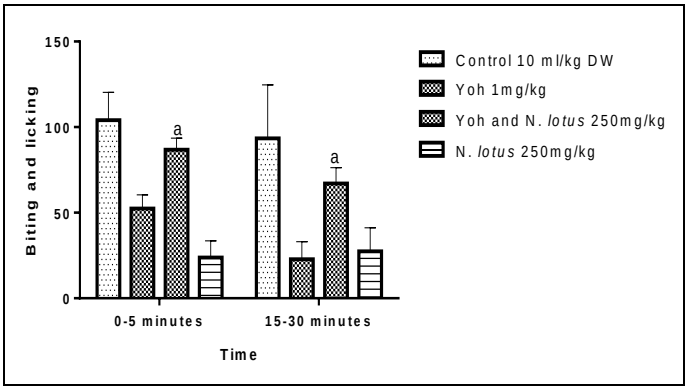

Figure 4: Effect of yohimbine on *N. lotus* using formalin induced pain. Yoh: yohimbine, DW: Distilled water

Control: Distilled water, Result represented as mean ± S.E M. (n=5); [a]$p < 0.05$ statistically significant compared to NPL (250 mg/kg) (two-way ANOVA followed by Tukey's multiple comparison test).

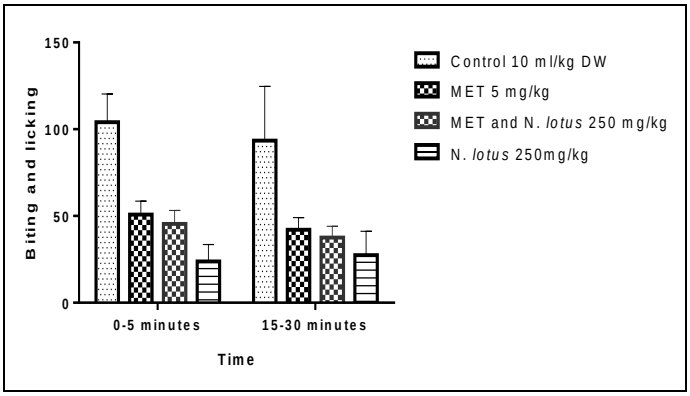

Figure 5: Effect of metoclopramide on *N. lotus* using formalin induced pain. MET: metoclopramide, DW: Distilled water Control: Distilled water, Result represented as mean ± S.E M. (n=5)

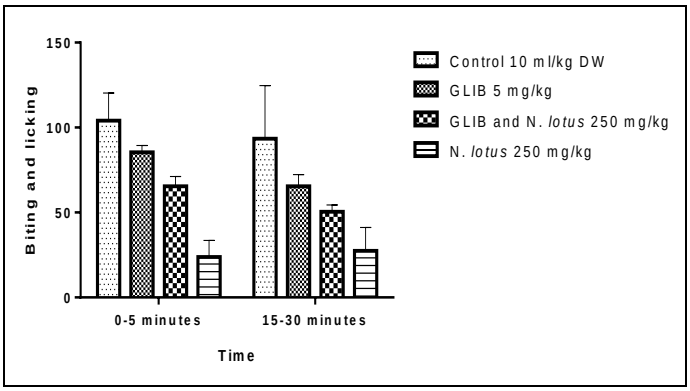

Figure 6: Effect of glibenclamide on NPL using formalin induced pain. GLIB: glibenclamide, DW: Distilled water Control: Distilled water, Result represented as mean ± S.E M. (n=5)

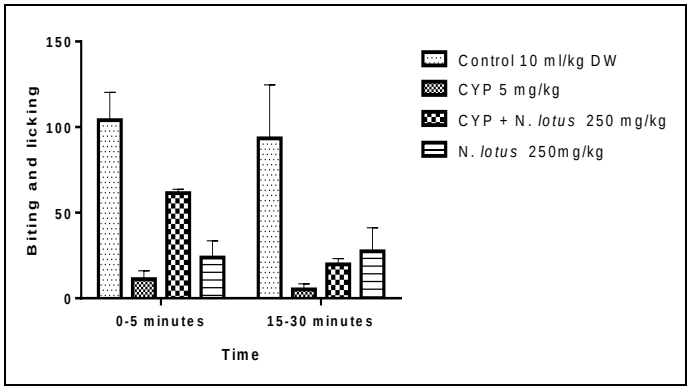

Figure 7: Effect of Cyproheptadine on NPL using formalin induced pain. CYP: Cyproheptadine, DW: Distilled water Control: Distilled water, Result represented as mean ± S.E M. (n=5)

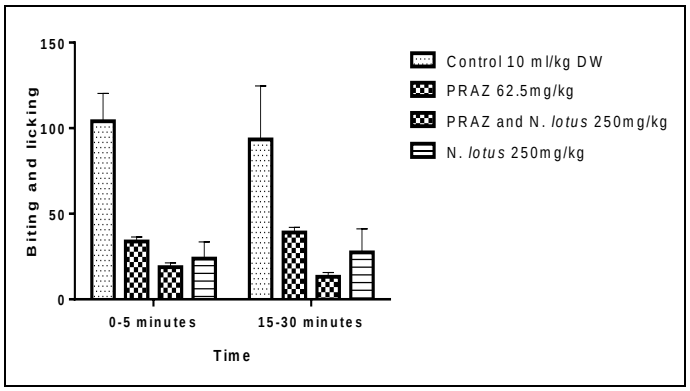

Figure 8: Effect of prazosin on NPL using formalin induced pain. PRAZ: prazosin, DW: Distilled water Control: Distilled water, Result represented as mean ± S.E M. (n=5)

Total Phenolic and Flavonoid Contents

The total phenolic and flavonoid contents present the aqueous extract of NPL was estimated to be 6.32 ± 0.075 and 5.04 ± 0.23 respectively.

Antioxidant Activity

Extract of NPL exhibit a potent antioxidant property using 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity, nitric oxide scavenging activity assay and reducing power assay (Figures 9, 10 and 11).

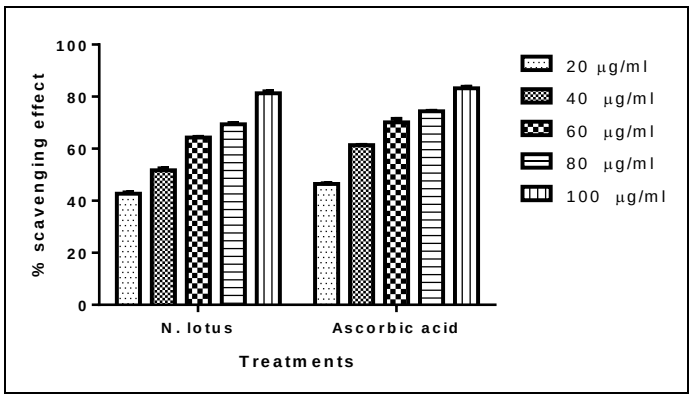

Figure 9: 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

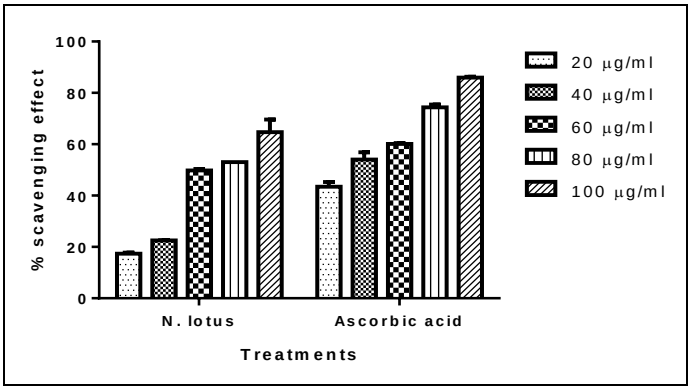

Figure 10: Nitric Oxide Scavenging Activity Assay

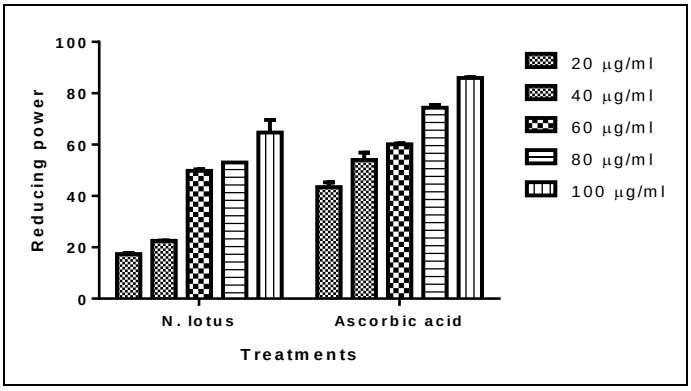

Figure 11: Reducing power activity of *N. lotus*

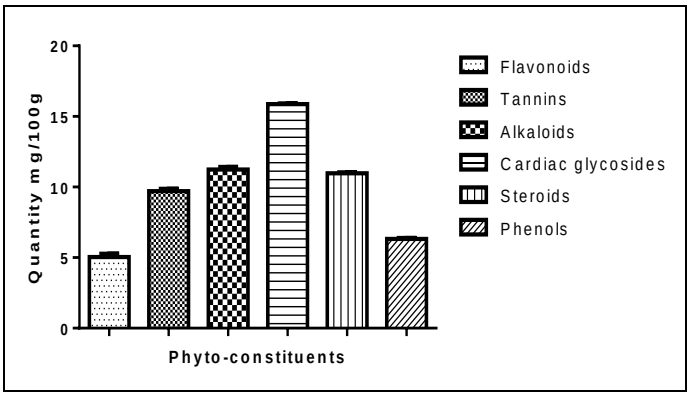

Figure 12: Phytochemical constituents present in NPL

DISCUSSION

The several adverse effects that accompany chronic pain management has necessitated the urgent and continuous search for newer and effective drugs with lower and milder adverse effects [22]. The antinociceptive activity of *N. lotus* was evaluated in the present investigation through the use of different standardized laboratory models. Pain can be altered via manipulation of pain transmission; for example, relief from pain could be achieved through the increase of increase of inhibitory signals from the brain. In elucidating the possible mechanism of anti-nociceptive effect recorded by the extract, a range of studies exploited pharmacological tools involving the use of different receptor antagonists.

Receptors receive information, respond to noxious stimuli, and transmit the information via afferent or sensory fibres to the high centres.

The receptors are usually classified as opioid and non-opioid receptors, when discussing pain. In the study, acetic acid-induced writhing, formalin, tail clip and hot plate tests were employed for scoring the anti-nociceptive potential of the plant. Observations recorded that it possesses anti-nociceptive activity, evident in the reduced number of writhes in acetic acid, reduction in the duration of biting and licking in formalin test, increased in latency in tail clip test and increased in the time spent on the hot plate-induced pain.

When administered after naloxone pre-treatment, the antinociceptive effect, of NPL was significantly reversed by naloxone (a non-specific opioid receptor antagonist), suggesting that the extract produces anti-nociception via interaction with opioid receptors.

Non-opioid analgesics have been reported to be among the most widely used treatment for pain [23]. Other than the inhibition of COX and prostaglandin synthesis, accumulating evidence has demonstrated the multiple actions of analgesics with other systems during pain. These systems, classified as non-opioid receptor systems, include interaction with the monoaminergic pathways, such as noradrenergic, dopaminergic and serotonergic systems [23, 24]. The search for new novel non-opioid analgesics quest to find new/novel non-opioid analgesics is paramount, since it may possibly alleviate and consequently overcome the side effects associated with the use of opioids analgesics, especially the psychological addiction, abuse, diversion of uses, dependence and tolerance [22, 24]. Involvement of noradrenergic system in nociception at spinal and supraspinal levels has been proven to be mediated through α-adrenoceptors and descending inhibitory pathways [25]. Yohimbine, the α_2 adrenoceptor antagonist, has been observed to significantly antagonize the anti-nociceptive effect of NPL, suggesting the involvement of α_2–adrenergic systems in its antinociceptive activity.

The serotonergic system has gained much attention as a therapeutic target for treating migraine pain [26, 27]. Therapeutics targeting 5-HT receptors and 5-HT reuptake are being clinically tested for their in their treatment of migraine and fibromyalgia-induced pain [28].

Cyproheptadine insignificantly ($p>0.05$) curtailed the anti-nociceptive effect of NPL.

Modulation of pain perception via the dopaminergic pathway has been established [29]. Dopamine is pronociceptive at peripheral D_1- and antinociceptive effect at D_2-like receptors, located centrally in the trigeminocervical complex [30]. In the study, treatment with metoclopramide produced a non-significant effect on the extract effect.

Ion channels are essential for controlling neuronal excitability, which is one of the steps in generation of most pain signals in human nervous system. Electrical excitation, which is controlled by an intricate set of ion channels that are coordinated to produce a degree of excitation that is proportional to the strength of the external stimulation, which starts in the peripheral somasensory nerves and is controlled by an intricate set of ion channels [31]. Upon activation, K^+ which are most common and more widely distributed, facilitates an extremely rapid transmembrane K^+ efflux that can influence action potential threshold, waveform and frequency. K^+ channel opening repolarizes, or even hyperpolarizes the neuronal membrane, and can limit action potential generation and firing rate. A variety of antinociceptive drugs mediate their action by directly opening spinal K^+ channels [32]. In the present study, glibenclamide (an inhibitor of ATP-sensitive K^+ produced a non-significant inhibition of the antinociceptive activity of NPL.

Phytochemical analysis of NPL showed the following constituents - flavonoids, alkaloids, glycosides, tannins, steroids, and phenols, some of which have been reported to elicit anti-nociception [33]. Flavonoids have been shown to interact directly with the cyclooxygenase pathway by inhibiting prostaglandins [34]. They have also been also reported to suppress the increased level of intracellular Ca^{2+} ion and the release of pro-inflammatory mediators in a dose-dependent manner. Tannins and glycosides also elicit analgesic and anti-inflammatory activities through cyclooxygenase inhibition [35, 36]. The presence of these phyto-constituents in the extract of NPL may be responsible for the recorded antinociceptive activity.

Extract of NPL showed potent antioxidant property, which compared effectively with ascorbic acid as shown from the present study. Oxidative stress, an imbalance between pro-oxidants and antioxidants, generate free radical, which could be overwhelm the efficiency of endogenous antioxidant system, when there is increased exposure to pro-oxidant, or reduced antioxidant capacity [37]. Oxidative stress is a generate free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are prone to oxidizing a wide range of biomolecules. Apart from the latter, they can turn-on many stress-activated pathways.[38]

The proposed mechanisms of injury from free radicals include direct attack, lipid peroxidation, DNA modification, and enzyme degradation/inactivation [38]. Potent antioxidant effect exhibited by NPL might have contributed to its antinociceptive property and this might also be one of the mechanisms of its antinociceptive activity.

The extract of NPL was found to be relatively safe up to 4000 mg/kg, since no mortality was recorded.

CONCLUSION

The study recorded the anti-nociceptive property of NPL aqueous leaf extract, as well as its antioxidant activity, and its ability to interact opioid and alpha2-adrenergic systems, which could possibly be the pathways for producing the recorded effect. The results justify the use of NPL for treating pain in traditional African medicine.

Conflict of interest

The authors declare no conflict of interests

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