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Antioxidant, anti-inflammatory and antinociceptive activities of methanolic extract of *Justicia secunda* Vahl leaf

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Abstract *Background of study:* Plants used for traditional medicine contain a wide range of substances which can be used to treat various infectious diseases.
Aim: The study evaluated the *in vitro* antioxidant, antinociceptive, and anti-inflammatory activities of the methanolic extract of *Justicia secunda* Vahl leaf.
Methods: The acute toxicity was performed with up and down method and the highest dose used was 2 g/kg. The anti-inflammatory activity was evaluated using the carrageenan and formalin-induced paw edema models, and antinociceptive activity was evaluated using acetic acid-induced writhing reflex and tail flick test models while the antioxidant activity was evaluated using 2,2-diphenyl-2-picryl hydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) photometric assay.
Results: The extract was well tolerated as no signs of toxicity or death were noticed during the period of observation. The extract produced a concentration dependent increase in antioxidant activities in both DPPH and FRAP models. The extract produced its optimum activity at 400 μg/ml in both DPPH (54.07%) assay and FRAP (1.58 μM) assay. The extract produced significant ($P < 0.05$) dose-dependent increase in both anti-inflammatory and antinociceptive activities. The antinociceptive and anti-inflammatory activities of the extract (0.4 g/kg) were comparable with the reference drugs (aspirin and pentazocine) used in the study.
Conclusion: This study suggests that *J. secunda* possesses anti-inflammatory, antinociceptive and antioxidant activities and also provide the pharmacological basis for its uses in traditional medicine for these purposes.

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1. Introduction

Medicinal plants are the most common sources of drugs used in traditional medicine.[1] Despite improvement in health care delivery system, medicinal plants play a vital role in both human and animal health care systems and about 60% of world population depend on herbs for their primary health care.[2,3] The reason for this may be due to high cost of orthodox drugs, side effects and unavailability of orthodox medicine and personnel in remote areas. Poverty and cultural inclination may have contributed to the trend.[3,4] One of the diseases that are usually treated with herbal medicine is inflammatory conditions.[5,6] Inflammation is a vascular tissue response to injury which involves chemical and cellular infiltration of the affected areas.[7] The cardinal signs of inflammation are pain, edema, loss of function, redness and heat.[8] Some herbs used in ethnomedicine in the treatment of inflammation are *Taraxacum officinale* Linn., *Magnifera indica* Linn., *Crocus sativus* Linn., *Ocimum sanctum* Linn.[9–12]

Justicia secunda Vahl belonging to the family *Acanthaceae* is known as "Blood root" and "Sanguinaria" in Barbados and Venezuela respectively.[13] The folkloric uses of the plant include wound healing, anemia and abdominal pain.[14,15] In South-Eastern Nigeria, Congo and South Côte-d'Ivoire the leaf decoction is consumed by Jehovah's Witness believers in the management of anemia.[16] The anti-sickling, haematinic, antimicrobial and anti-hypertensive activities of *J. secunda* have been reported.[13,16–18] Phytochemical screening of the plant has shown the presences of tannins, flavonoids, alkaloids, quinines and anthocyanins.[19] Luteolin, aurantamide acetate, auranamide, quindoline and pyrrolidone derivatives; secundarellone A, B and C have been isolated from *J. secunda*.[19–21] Notwithstanding the popular uses of *J. secunda* in folkloric medicine only few pharmacological studies have been done on the plant.[22] This study investigated the antioxidant, anti-inflammatory, and antinociceptive effects of *J. secunda* leaf methanolic extract (*J. secunda*).

2. Materials and methods

2.1. Plant collection and identification

The leaves of *J. secunda* were collected from uncultivated farmlands located at Eastern parts of Nigeria in June 2015 and its botanical identification was authenticated by Dr. Garuba Omosun of the Department of Plant Science and Biotechnology of Micheal Okpara University of Agriculture, Umudike. A voucher specimen catalogued MOUAU/VPP/2015/14 was deposited in the departmental herbarium for reference purposes.

2.2. Preparation of J. secunda extract

The plant material *J. secunda* were air dried under shed in the Veterinary Physiology, Pharmacology, Biochemistry, Animal and production Laboratory of Michael University of Agriculture. It was later ground with manual milling machine (Corona, China). Two hundred grams (200 g) of the fine powder of *J. secunda* was weighed, using weighing balance (Mettler, Germany). The plant material was soaked with 80% of methanol in a winchester bottle. This was shaken every 3 h interval and allowed to stand for 48 h (2 days) at room temperature and then filtered using Whatman No. 1 filter paper into a beaker. The filtrate was concentrated at temperature of 40 °C with the use of an electric oven and the extract was stored in a refrigerator at 4 °C as J. secunda until when needed.[4]

The percentage yield was calculated using the formula below:

$$\%\ \text{yield} = (\text{weight of extracted material} \div \text{weight of plant material}) \times 100/1$$

2.3. Antioxidant study

2.3.1. Assessment of 2,2-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging activities of J. secunda

The antioxidant activity of *J. secunda* was evaluated using DPPH photometric assay.[23] The test extract (2 ml) at different concentrations (25, 50, 1, 200 and 400 μg/ml) was mixed with 0.5 mM DPPH (in 1 ml of methanol) in a cuvette. The absorbance at 517 nm was taken after 30 min of incubation in the dark at room temperature. The concentrations were prepared in triplicates and the percentage antioxidant activity was calculated as follows:

$$\%\ \text{antioxidant activity (AA)} = 100 - (\{[\text{absorbance of sample} - \text{absorbance of blank}] \times 100\}/\text{absorbance of control}$$

A volume of 1 ml of methanol plus 2.0 ml of the extract was used as the blank while 1.0 ml of the 0.5 mM DPPH solution plus 2.0 ml of methanol was used as the negative control. Ascorbic acid (vitamin C) was used as reference standard.[24]

2.3.2. Ferric reducing antioxidant power (FRAP) of test

The ferric reducing antioxidant power was carried out as described by Benzie and Strain.[25]

2.3.2.1. Reagent composition.

1. Acetate buffer (300 mM), pH 3.6 (3.1 g sodium acetate. $3H_2O$ and 16 ml glacial acetic acid in 1000 ml buffer solution).
2. 2,4,6-Triphridyl-s-triazine (TPTZ) (10 mM) in 40 mM HCl.
3. $FeCl_3$ś$6H_2O$ (20 mM) in distilled water.

The working solution was prepared by mixing solution 1, 2, and 3 in the ratio of 10:1:1 respectively. The working solution was freshly prepared in test. The aqueous solution of known amount of ascorbic acid was used for calibration.

FRAP reagent (3 ml) and 100 μl sample solution at concentrations of 25, 50, 100, 200 and 400 μg/ml were mixed and allowed to stand for 4 min. Colorimetric reading was recorded at 593 nm, at 37 °C. The ascorbic acid standard was tested in parallel process. Calculations were made by a calibration curve.

$$\text{FRAP value of sample}(\mu\text{mol/L}) = \{[\text{Changes in absorbance of sample 4–0 min} / \text{Changes in absorbance of standard 4–0 min}] \times \text{FRAP Value of standard } (2\ \mu\text{mol/L})\}$$

2.4. Experimental animals

One hundred and twenty-six (126) Wistar albino rats of both sexes weighing 90–105 g, obtained from the laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka, Enugu State, were used for the study. The animals were housed in aluminum cages at room temperature and under natural light/darkness cycles. They were supplied with clean drinking water and fed *ad libitum* with standard commercial pelleted feed (Vital feed® Nigeria). They were maintained in accordance with the recommendations of the Guide for the care and use of laboratory animals.[26] The rats were acclimatized for two weeks prior to the study. The experimental protocol was approved by the University Animal Ethics Committee with reference MOUAU/CVM/EAEC/2013/210.

2.5. Acute toxicity test

This study was carried out using the up and down method of acute toxicity.[27] Six rats were randomly selected, weighed and placed in a cage. Three rats were treated with 2 g/kg of plant extract while three other rats were given equal volume of distilled water, orally by gastric gavage. The rats were observed for 48 h for signs of toxicity and mortality.

2.6. Anti-inflammatory study

2.6.1. Carrageenan – induced paw edema

This was done using the method of Owoyele *et al.*[6] The rats were fasted overnight and had free access to water prior to the day of the experiment but were denied access to feed and water during the experiment. Thirty albino rats were weighed and randomly divided into five groups (A–E) of 6 rats per each. Group A was given 10 ml/kg of distilled water (negative control), group B was treated with 0.2 g/kg of acetylsalicylic acid (aspirin) (positive control) and groups C, D and E were treated with 0.1, 0.2 and 0.4 g/kg of the plant extract respectively. One hour after treatment, paw edema was induced by injecting 0.1 ml of 0.6% solution of carrageenan into the sub-plantar surface of the hind right paw. Their left paw volumes were measured using the volume displacement method, as control. Thereafter, the right paw volume was determined at 1, 2, 3, and 24 h post treatment.

The increase in paw volume = *right paw volume* − *left paw volume*

2.6.2. Formalin-induced paw edema

The method described by Ezeja *et al.*[28] was used for this experiment. The rats were fasted overnight and were given free access to water and feed prior to the experiment. Thirty albino rats were weighed and randomly divided into five groups (A–E) of 6 rats per group. Group A was given 10 ml/kg of distilled water (negative control), group B was treated with 0.2 g/kg of acetylsalicylic acid (aspirin) (positive control) and groups C, D and E were treated with 0.1, 0.2 and 0.4 g/kg of the plan extract respectively. One hour after treatment, paw edema was induced by injecting 0.1 ml of 1.0% solution of formalin into the sub-plantar surface of the hind right paw. Their left paw volumes were measured using the volume displacement method, as control. Therefore, the right paw volume was determined at 1, 2, 3 and 24 h post treatment. The increase in paw volume = *right paw volume* − *left paw volume*.

2.7. Antinociceptive study

2.7.1. Acetic acid-induced abdominal writhing in rats

The method described by Hajhashemi *et al.*[5] was used. Thirty rats were assigned to five groups (A–E) of 6 rats each and were fasted for 12 h but free access to tap water was allowed. Group A served as negative control and received distilled water (10 ml/kg). Group B served as positive control and received aspirin (0.2 g/kg), while Groups C–E received 0.1, 0.2 and 0.4 g/kg of *J. secunda*, respectively. The drug and extract were administered orally. 45 min post treatment, the rats received 10 ml/kg of 0.7% acetic acid intraperitoneally. The number of writhing or abdominal stretches produced in each rat was counted for the next 30 min.

2.7.2. Tail flick test in rats

The experiment was carried out as described by Adzu *et al.*[29] Thirty rats were assigned to 5 Groups (A–E) of 6 rats each and fasted for 12 h with free access to drinking water. Group A received distilled water 10 ml/kg orally (negative control), Group B received pentazocine 0.003 g/kg intraperitoneally (positive control), while Group C–E received *J. secunda* 0.1, 0.2 and 0.4 g/kg orally, respectively. One hour post drug treatment about 3 cm of the tail of each rat was dipped into a water bath containing warm water maintained at a temperature of 55 ± 1 °C. The time taken for the mouse to flick the tail known as the pain reaction time (PRT) was recorded for all the rats.

2.8. Data analysis

The results were presented as mean ± standard error of mean (SEM). Data obtained were analyzed using one way analysis of variance (ANOVA) and the variant means were separated by Least Significant Difference (LSD) of the different groups. Significance was accepted at the level of $p < 0.05$.

3. Results

3.1. Plant extraction

The yield of *J. secunda* extract was 10.7% w/w dry matter.

3.2. Antioxidant study

3.2.1. DPPH free radical scavenging activities of J. secunda

The result of the DPPH photometric assay of *J. secunda* is presented in Fig. 1. *J. secunda* extract caused a concentration dependent increase in percentage antioxidant activity increasing the antioxidant activity from 7.22% at 25 μg/ml concentrations to 54.07% at 400 μg/ml concentrations while ascorbic acid had 94.97% at 400 μg/ml concentrations.

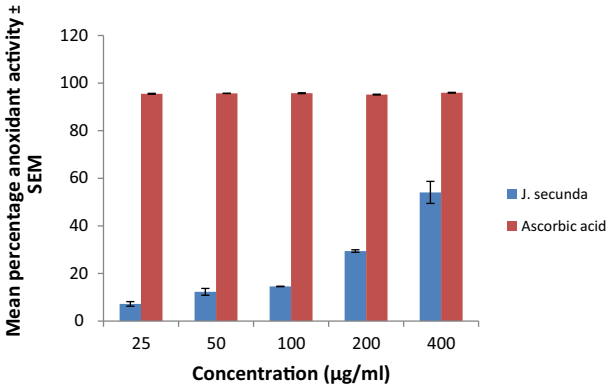

Figure 1 DPPH free radical scavenging activities of *J. secunda*.

3.2.2. Ferric reducing antioxidant power (FRAP) of J. secunda

The result of the FRAP photometric assay of *J. secunda* is presented in Fig. 2. *J. secunda* extract caused a concentration dependent increase in FRAP value from 0.35 µM at the concentration of 25 µg/ml to 1.58 µM at the concentration of 400 µg/ml.

3.3. Acute toxicity test

Oral administration of 2 g/kg of *J. secunda* and an equal volume of distilled water produced no death or any sign of toxicity after 48 h.

3.4. Anti-inflammatory study

3.4.1. Formalin-induced paw edema

The results of *J. secunda* on the paw volume of formalin-induced paw edema in rats are presented in Table 1. The extract (0.1, 0.2 and 0.4 g/kg) and aspirin (0.2 g/kg) produced a significant ($p < 0.05$) dose-dependent decrease in the mean paw volume of the treated rats when compared to the negative control rats. The extract (0.4 g/kg) produced a significant ($p < 0.05$) time dependent decrease in the paw volume when compared to the negative control group. At the 3rd hour post treatment the Aspirin (0.2 g/kg), and extract 0.1, 0.2 and 0.4 g/kg produced 18%, 9%, 12% and 44% decrease in paw volume respectively, when compared to negative control.

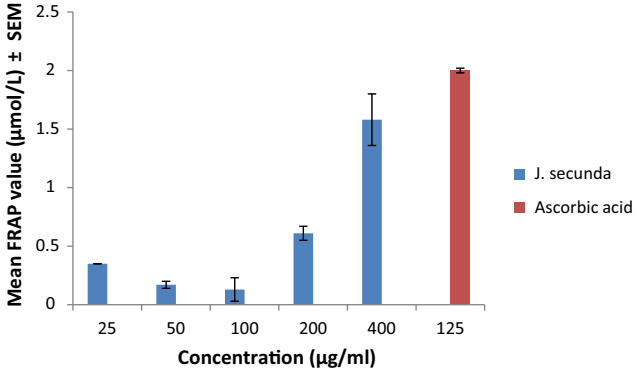

Figure 2 Ferric reducing antioxidant power of *J. secunda*.

3.4.2. Carrageenan-induced paw edema

The results of the *J. secunda* on the mean increase in paw volume of carrageenan induced paw edema in rats are presented in Table 2. The extract (0.1, 0.2 and 0.4 g/kg) and Aspirin (0.2 g/kg) produced a significant ($P < 0.05$) decrease in the mean paw volume of the treated rats when compared to the negative control rats. The extract (0.1 g/kg) produced a significant ($P < 0.05$) time dependent decrease in the paw volume when compared to the negative control group. At 24th hour post treatment the Aspirin (0.2 g/kg), and extract 0.1, 0.2 and 0.4 g/kg produced 43%, 45%, 57% and 60% decrease in paw volume respectively when compared to negative control.

3.5. Antinociceptive study

3.5.1. Tail flick test

The results of the effects of *J. secunda* on tail flick test in rat are presented in Table 3. The extract produced dose-dependent increase in PRT of the treated rats. Pentazocine 0.003 g/kg and *J. secunda* 0.4 g/kg significantly ($P < 0.05$) increased the PRT of the treated rats when compared to negative control. Pentazocine 0.003 g/kg and *J. secunda* 0.4 g/kg caused 44.98% and 25.76% increase in PRT in treated rats respectively, when compared to the negative control group.

3.5.2. Acetic acid-induced writhing reflex

The results of the effects of *J. secunda* on acetic acid-induced writhing reflex in rats are presented in Table 4. The extract produced a significant ($P < 0.05$) dose-dependent reduction in the number of writhing reflex in the treated rats, when compared to the negative control group. Distilled water 10 ml/kg, aspirin and *J. secunda* 0.1, 0.2 and 0.4 g/kg caused 38.00, 22.00, 36.50, 29.00 and 13.75 mean number of writhing reflex in treated rats respectively.

4. Discussion

The anti-inflammatory, antinociceptive and antioxidant capacity of methanolic extract of *J. secunda* were evaluated in this study. The extract was well tolerated by the rats as neither death nor signs of toxicity were observed in the rats dosed with 2 g/kg of *J. secunda* during the period of observation.[27] The LD_{50} of the extract is greater than 2 g/kg.

The extract demonstrated a potent anti-inflammatory, antinociceptive and antioxidant properties which may have been mediated by the phytochemical constituents.[19] Tannins have been documented to possess antinociceptive, anti-inflammatory and antioxidant activities.[9] The antinociceptive, anti-inflammatory activities and antioxidant capacity of flavonoids (luteolin) have been reported.[30–32]

The mechanism of the *in vitro* antioxidant effects of the extract may be through the scavenging of the reactive oxygen species (ROS) and *in vivo* may be via the inhibition of the ROS generating oxidases, enhancement of the endogenous antioxidant and/or direct inhibition of enzyme that catalyzes oxidation of cellular components.[32–35] The antioxidant effects of the extract may be responsible for its anti-inflammatory and antinociceptive activities.[32]

Table 1 Effects of *J. secunda* on formalin induced paw edema.

Treatment (n = 6)	Mean increase in paw volume in ml ± SEM (% inhibition)			
	1 h	2 h	3 h	24 h
Distilled water 10 ml/kg	0.57 ± 0.02 (–)	0.77 ± 0.069 (–)	0.77 ± 0.04 (–)	0.37 ± 0.029 (–)
Aspirin 0.2 g/kg	0.5 ± 0.05 (12)	0.67 ± 0.03* (12)	0.63 ± 0.04* (18)	0.37 ± 0.06 (0)
J. secunda 0.1 g/kg	0.67 ± 0.02 (−18)	0.67 ± 0.02* (12)	0.70 ± 0.00 (9)	0.30 ± 0.03 (18)
J. secunda 0.2 g/kg	0.67 ± 0.06 (18)	0.63 ± 0.04* (18)	0.67 ± 0.06 (12)	0.23 ± 0.04* (37)
J. secunda 0.4 g/kg	0.43 ± 0.04* (25)	0.50 ± 0.00* (35)	0.43 ± 0.03* (44)	0.27 ± 0.03 (27)

* $P < 0.05$ when compared to negative control.

Table 2 Effect of *J. secunda* on carrageenan-induced paw edema.

Treatment (n = 6)	Mean increase in paw volume in ml ± SEM (% inhibition)			
	1 h	2 h	3 h	24 h
Distilled water 10 ml/kg	0.50 ± 0.04 (–)	0.91 ± 0.04 (–)	1.03 ± 0.019 (–)	0.9 ± 0.01 (–)
Aspirin 0.2 g/kg	0.46 ± 0.03 (8)	0.70 ± 0.04* (23)	0.72 ± 0.02* (30)	0.51 ± 0.01* (43)
J. secunda 0.1 g/kg	0.42 ± 0.01 (16)	0.67 ± 0.06* (26)	0.68 ± 0.05* (33)	0.49 ± 0.02* (45)
J. secunda 0.2 g/kg	0.57 ± 0.02 (−14)	0.78 ± 0.02* (14)	0.86 ± 0.04* (17)	0.38 ± 0.03* (57)
J. secunda 0.4 g/kg	0.43 ± 0.04 (14)	0.85 ± 0.05 (7)	0.87 ± 0.04* (16)	0.35 ± 0.02* (61)

* $P < 0.05$ when compared to negative control.

Table 3 Effects of *J. secunda* on tail flick test.

Treatment (n = 6)	Pain reaction time (s)	% increase
Distilled water 10 ml/kg	2.29 ± 0.17	–
Pentazocine 0.003 g/kg	3.32 ± 0.28*	44.98
J. secunda 0.1 g/kg	2.54 ± 0.10	10.92
J. secunda 0.2 g/kg	2.50 ± 0.21	9.19
J. secunda 0.4 g/kg	2.88 ± 0.17*	25.76

* $P < 0.05$ when compared to negative control.

Table 4 Effect of *J. secunda* on acetic acid-induced writhing reflex.

Treatment (n = 6)	Number of writhing reflex	% inhibition
Distilled water 10 ml/kg	38.00 ± 0.60	–
Aspirin 0.2 g/kg	22.00 ± 0.27*	42.11
J. secunda 0.1 g/kg	36.50 ± 0.42*	3.95
J. secunda 0.2 g/kg	29.00 ± 0.71*	23.68
J. secunda 0.4 g/kg	13.75 ± 0.31*	63.81

* $P < 0.05$ when compared to negative control.

Carrageenan and formalin induce paw edema through the release of inflammatory mediators such as histamine, serotonin, protease, cytokine, lysosome and prostaglandins.[36,37] The anti-inflammatory effect of the extract may be due to the inhibition of production and/or migration of these inflammatory mediators.[38–40] Another possible mechanism of the anti-inflammatory and antinociceptive effects may be the inhibition of cyclooxygenase activities, just as the aspirin.[41] Cyclooxygenase catalyzes the biosynthesis of prostaglandins from arachidonic acid.[42] Again the mechanism of antinociceptive effects may be through the inhibition of pain perception, impulse transmission and/or elevation of pain threshold in the hypothalamus.[42] The antinociceptive activity of *J. secunda* is effective against both peripheral and deep pain sensation.

In conclusion, this study suggests that *J. secunda* possesses anti-inflammatory, antinociceptive and antioxidant activities and also provide the pharmacological basis for its uses in traditional medicine for these purposes. We recommend further studies to isolate and characterize the active compound(s) responsible for the activities.

Conflict of interest

The authors have to conflict of interest to declare.

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