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Rodent-based aphrodisiac and toxicological evaluations of Waltheria indica ethanol root extract

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Abstract

Background: The root of Waltheria indica is used in Nigeria to enhance libido. This study evaluated the aphrodisiac potential and toxicological profile of ethanol root extract (EEWI) in male Wistar rats.

Methods: The aphrodisiac potential was evaluated on the 7th and 14th days of oral administration of graded doses (100, 200, and 400 mg/kg/day) of EEWI. Anxiolytic properties were evaluated on the 7th day, while memory retention properties were evaluated on the 14th, 21st, and 28th days. Sperm parameters, toxicological profile, and antioxidant potential were evaluated on the 28th day. The in vitro antioxidant property was also evaluated.

Results: In a dose-dependent manner, the extract increased mounting frequency and intromission frequency but significantly decreased mount and intromission latencies ($p<0.001$) on the 7th and 14th day of treatment. It prolonged ejaculatory latency. Anxiolytic and memory studies showed that the extract compared agreed with standards. Toxicologically, it was observed that all the doses used neither caused death nor any gross toxicological symptom. A significant increase ($p<0.05$) was observed in the concentrations of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), as well as hematological parameters such as red blood cells and hemoglobin. A significant reduction in total sperm and motile sperm cell counts was observed in rats given the highest dose.

Conclusion: The extract possesses aphrodisiac action and is relatively safe. The antioxidant property may accentuate the aphrodisiac and anti-anemic use in ethnomedicine.

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Highlights

What is current knowledge?

- Folklore practitioners use the extract for many diseases
- As enhancement of sexual performance (aphrodisiac)
- No known likely mechanism of action
- No report on its safety

What is new here?

- Extract is safe on the vital organs of the liver and kidney
- It indeed possesses aphrodisiac potential, as previously reported
- It has no fertility-enhancing effect in males.
- Extract has antianxiety property
- Extract enhances memory, likely to enhance testosterone secretion
- Extract has an anti-anemic effect

Introduction

Nearly 500 plant species have been recognized as stimulants of sexual activity most of which are used as food, spices, and herbal medicine worldwide. In many resource-poor nations, including Nigeria, the ability, facilities, and cost of appropriately diagnosing sexual dysfunction with long-term consequences are often high. Accordingly, many of their citizens prefer to employ alternative or traditional herbal treatments. Accessibility, cost-effectiveness, reduced side effects, and favorable therapeutic outcomes contribute to the growing demand for plants with aphrodisiac and reproductive qualities.

The roots of Waltheria indica L. (syn. Waltheria americana) Sterculiaceae, have been indicated as a potential aphrodisiac (1, 2). The plant is also known as velvet leaf, marshmallow, monkey bush, boater bush, leather coat, and buff coat. In Africa, all plant parts are employed for medicinal purposes, such as antidiarrhea (3) and as an analgesic (4). The roots and aerial parts have been found to exhibit moderate antiplasmodial activity (5). In Nigeria, different parts of the plant are used traditionally against hemorrhoids, syphilis, anemia, and cough (6). Its aphrodisiac potential in rats (1) and fruit flies (2) has already been reported. Some compounds have been identified in the whole plant, such as flavonoids, epicatechin, kaempferol derivatives, tiliroside, and quercetin (6).
Erectile dysfunction (ED) is a condition in which a man cannot maintain adequate penile erection for normal sexual intercourse or sexual satisfaction. The currently available treatment for ED has several unpleasant side effects and limited efficacy (7). Consequently, alternative treatments considered safer and more effective are needed, which might be examined through herbal/medicinal plants with intrinsic aphrodisiac effects. An aphrodisiac refers to any food, medicine, perfume, or equipment that has the potential to stimulate or enhance sexual desire or libido (8).

Although W. indica has been used in the folklore management of ED among locals, this needs to be sufficiently evaluated. In addition, there needs to be more information on the likely mechanism of its action, as well as safety. This study aimed to evaluate the effects of an extract of the plant on sexual behavior, sperm indices, and safety in rats.

However, reports on the safety evaluation of the plant have shown some histopathological changes in organs (9). Aphrodisiac substances aimed at managing a condition as recurring as erectile dysfunction may require constant and consistent use. Hence the need for toxicological evaluation.

Methods

Plant material

The roots of *W. indica* were collected from around Lekan Salami stadium (Longitude 3° 54' 59.99″ E; latitude 7° 23' 28.19″ N), University of Ibadan, Ibadan, Nigeria, in April 2018. Mr. Donatus O. Esimekhuai of the Department of Botany, University of Ibadan, Oyo State, Nigeria, identified and authenticated the plant. A specimen with voucher number UIH-22850 was deposited at the University of Ibadan Herbarium.

Animals

Sexually experienced male Sprague-Dawley rats weighing between 140-220 g were sourced from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria. The animals were given free access to food and maintained under natural lighting conditions and a temperature range of 28-30 °C. The experimental animals were allowed two weeks of acclimatization before the experiments. They were fed growers feed (Ewu feeds growers with big feed cereals, Ewu, Edo state). All the animals were treated humanely, as specified in the National Academy of Science's 'Guide for the Care and Use of Laboratory Animals (10). Institutional ethical approval (EC/FP/022/13) was obtained from the Animal Ethics Committee of the Faculty of Pharmacy, University of Benin.

Preliminary phytochemical screening

Standard procedures reported by (11) and (12) were used for the preliminary phytochemical screening of the root extract of *W. indica*.

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High-performance liquid chromatography analysis of the extract

This analysis was done using the method described by (13). Chromatographic analysis was performed using a Shimadzu liquid chromatograph (Kyoto, Japan) equipped with a vacuum degasser (DGU-14A), quaternary pump LC-10AT, UV-vis detector (SPD-10AV), and an injector (Rheodyne) with a 20 µL loop. A C18 reverse-phase column (4.6 mm × 250 mm, 5 µm particle size) (Hichrom, Europe) was used. Preceding the analytical column was a C18 guard column (4.6 mm × 12.5 mm, 5 µm particle size) (Hichrom, Europe), used to prevent any non-soluble residues from the samples from contaminating the column. Peak areas were determined at 280 nm for all phenolic compounds except quercetin and naringenin, tested only at 330 nm. The ambient temperature was controlled and maintained at 20±1°C. Retention time and peak area were checked for repeatability by injecting the mixture of standards at 0.01 mg/ml concentration into the HPLC system over six runs. Phenolic solutions present were identified by comparing chromatographic peaks with the retention time of individual standards and further confirmed by co-injection with isolated standards and summarized in a table.

Evaluation of extract on the mating performance of male rats

Preparation of chemicals and drugs

EEWI, sildenafil citrate, and estradiol were prepared for oral administration by reconstituting each in 10% Tween 80. Meanwhile, progesterone was dissolved in olive oil and administered through a subcutaneous injection.

Preparation of animals for mating

Each male rat was paired with a female in which estrus had been induced, and mating behavior was recorded on days 7 and 14. Estrus was induced in the rats by the oral administration of ethinylestradiol at a dose of 100 g/animal 48 h and subcutaneous progesterone at a dose of 1 mg/animal 6 h before the pairing. The study only included the most responsive female rats (14).

Experimental design

Twenty male rats were randomly allotted to five groups (n=4). Group I was the control group, to which 0.5 ml of 10% Tween 80 was given. Group II was administered 5 mg/kg of sildenafil citrate (15, 16). Groups III, IV, and V received 100, 200, and 400 mg/kg of EEWI. All treatments were by the oral route. The mating experiments occurred on the 7th and 14th days after the male rats began receiving the treatments. The experiment was carried out at 19:00 h in the same laboratory and with the same degree of luminosity. Receptive female rats were placed into male rat cages at a ratio of one female to one male (17). Daily administration continued for 28 days before the rats were humanely sacrificed using chloroform anesthesia. Blood and tissues were harvested for sub-acute toxicity testing.

Determination of mating performance in rats

This was carried out according to the method described by Singh et al. (2013). Each male rat was placed in a glass cage measuring 60 cm x 30 cm x 30 cm (L, B, and H) and given 10 min to acclimatize. Following that, an experimentally-induced estrus female rat was placed in the cage and allowed to cohabit for 60 min. A digital camera (HD Digital Video Camera Recorder Camcorder) was used to record the sequence of events. The video was then played again to assess the parameters of the mating behavior of interest (mount, intromission, and ejaculation). The male assuming the copulation posture without inserting the penis into the female vagina is occurred the "mount." Intromission is the entry of the male penis into the female vaginal canal, characterized by pelvic thrusting and jumping dismount. Ejaculation is the release of semen into the female vaginal canal by the male. Deeper pelvic drive and a sluggish dismount, followed by a period of inactivity, are the hallmarks of this condition. The latencies and frequencies of the mount, intromission, and ejaculation were recorded.

Effect of extract on anxiety

The anxiety test used Pellow and File's method (19). Twenty-four rats were randomly allotted to six groups (n=4). Group A received 0.5 ml of 10% Tween 80; group B received 5 mg/kg/day of sildenafil; groups C-E received 100, 200, and 400 mg/kg/day, respectively; group F received 1 mg/kg/day diazepam. The rats were treated for seven days and subjected to an anxiety test on the 7th day using an elevated plus maze (EPM). The apparatus consists of a "+"-shaped maze elevated above the floor with two oppositely positioned closed arms, two open arms, and a center area. As animals freely explore the maze, their behavior is recorded. The preference for closed arms over open arms (expressed either as a percentage of entries and/or a percentage of time spent in the open arms) is a measure of anxiety-like behavior (20). Every rat was positioned on the EPM and monitored for 5 minutes. Subsequently, the apparatus was sterilized with ethanol to eliminate any residual cues before the next rat was introduced for observation.

Effect of extract on learning and memory/ transfer latency

The method described by Singh and Parle (21) was slightly modified and used. The control group (A) received 10% tween 80 (10 ml/kg/day), and group B received (sildenafil 5 mg/kg/d. Different doses of EEWI (100, 200, and 400 mg/kg) were administered to groups C-E, and the standard drug piracetam (400 mg/kg/i.p) was administered once daily to group F. On the 14th, 21st, and 28th days, the rats were placed on the elevated plus-maze test (EPM) for 90 s, and the time spent in the open arm was recorded. Transfer Latency (TL) was recorded as the time the rat took to move from an open arm to any one of the closed arms with all four legs crossing the middle line. If the animal did not enter the closed arm within 90 seconds, it was gently pushed into the closed arm, and a transfer latency of 90 seconds was assigned to it. The reduction in the TL retention value indicated memory improvement (22).

Determination of acute toxicity (LD50) in rats

Twenty healthy male albino rats were deprived of feeds for 3-4 hours before being exposed to acute toxicity tests using a procedure specified in the Organization for Economic Cooperation and Development Guidelines No: 423 of Europe (23). During the trial, the rats were randomly allotted to five groups (n=4) and housed in separate cages. The control group was given 10 ml/kg of distilled water orally. The ethanol extract of *W. indica* (EEWI) was given orally to groups 1 to 5 at doses of 10, 1000, 2000, 3500, and 5000 mg/kg, respectively. For 24 h, the rats were monitored for behavioral, neurological, autonomic characteristics, and death.

Sperm collection and sperm analyses

These analyses were done following the guidelines of the World Health Organization (24). Computer Assisted Semen Analysis (CASA) equipment and software (SCA® version 6.0, Microptic S.L, Barcelona, Spain) were used to determine sperm concentration and motility. All CASA were performed with a Basler A312FC digital camera mounted on a Nikon Eclipse 50i microscope (IMP, Cape Town, South Africa).

Oral sub-acute toxicity evaluation in rats

Oral sub-acute toxicity was evaluated according to OECD Guidelines 407 (25). Rats (16) were randomly allotted to four groups (n=4). Group 1 was given 1 ml/100 g/day of distilled water. Groups 2, 3, and 4 were given 100, 200, and 400 mg/kg/day of EEWI, respectively. All treatments lasted for 28 days. On the 29th day, under excessive chloroform inhalation, blood samples were obtained from the abdominal aorta and divided into two portions: ethylene diamine tetra-acetic acid (EDTA) sample bottles for hematological assays and lithium heparinized bottles for biochemical assays. The blood samples for biochemical assays were centrifuged at 3000 rpm for 15 min, and the sera obtained were stored at -20 °C. The animals were dissected, and the heart, liver, and kidneys were obtained and weighed as described by (26).

(a) Relative organ weights of liver and kidney

This was estimated using the formula:

$$\text{ROW} = \frac{AOW}{BW} \times 100$$

Where ROW is relative organ (liver, kidney, penis, and testes) weight (g), AOW is absolute organ weight (g), and BW is the body weight (g) of the rat on the final day.

(b) Effect of extract on hematological parameters

The hematological parameters include white blood cell (WBC) count, red blood cell (RBC) count, percentages of neutrophils (NEUT), eosinophils (EOS), lymphocytes (LYM), monocytes (MON), hematocrit (HCT), hemoglobin concentration (HGB), and platelet (PLT) count were determined by an automated blood analyzer (Mindray BC5300, China).

(c) Histology of the liver and kidney

As done by (27), these organs were washed in Phosphate-Buffered Saline (PBS), fixed in Bouin's solution, and dehydrated in graded ethanol concentrations. The tissues were further cleared in xylene, infiltrated in molten paraffin wax in the oven at 58°C, embedded in wax, and blocked out. Blocks were cut at 5 µm thickness in a microtome (Leica Microsystems Inc., Germany). The fixed tissues were stained using hematoxylin and eosin (H&E), cleared in xylene, and mounted in DPX (a mixture of distyrene, a plasticizer, and xylene). The tissues were examined and analyzed using photomicrographs taken with the Accu-scope Digital Photomicrographic system (3001-LED-10, New York, USA).

The antioxidant potential of the extract

(a) Effect of the extract on antioxidant defense enzymes

Glutathione peroxidase (GPX) activity in erythrocyte hemolysis was measured using a technique in which the activity of GPX was linked to NAD oxidation by glutathione reductase (28). At 37°C, the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was measured spectrophotometrically at 340 nm and was finally reported as U/g of Hb.

Sun *et al*. method (29) determined total superoxide dismutase (SOD) activity. The basis of this method is the resuscitation inhibition of nitroblue tetrazolium (NBT) with the xanthine-xanthine oxidase system. After adding 1 ml of ethanol/chloroform combination (v:v = 5:3) to the same volume of the centrifuged blood sample, SOD activity in the ethanol phase was measured, and the amount of SOD activity was represented as U/g of Hb. Catalase (CAT) was determined using the Aebi technique. The premise of this method is that the decomposition of hydrogen peroxide is determined by measuring the velocity constant K. The enzyme velocity constant was estimated by monitoring absorbance changes per minute, as described by (30). The amount of measured CAT activity was represented as U/g of Hb.

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(b) Free radical scavenging ability

The ability of the aqueous extract to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radicals was determined using the method described by Rathi et al. (2021) as given by the formula:

$$\%\ DPPH\ Radical\ Scavenging\ Activity = \frac{A_0 - A_1}{A_0} \times 100$$

A_0 is the absorbance of the control, and A_1 is the absorbance of the extract or standard. The percentage inhibition was plotted against concentration, and the IC_{50} was derived from the graph. The experiment was performed three times for each concentration of the extract.

Statistical analysis

Results are represented as mean ± standard error of the mean (SEM). Data were subjected to a one-way analysis of variance (ANOVA) with Dunnett's post hoc test using GraphPad Prism software (version 5.01). P< 0.05 was considered a statistically significant difference between the compared data.

Results

Table 1 shows the results of qualitative phytochemical tests on the ethanol root extract of *W. indica* (EEWI). While alkaloids, flavonoids, sterols, tannins, saponins, anthraquinones, and carbohydrates were detected, cardiac glycosides were absent.

Table 1. Results of the preliminary qualitative screening of ethanol root extract *W. indica*

Parameters	Inference
Alkaloids	+
Flavonoids	+
Sterols	+
Tannins	+
Cardiac glycosides	-
Saponins	+
Anthraquinones	+
Carbohydrates	+

+ Presence
- Absence

Table 2 shows the composition of the flavonoids detected by HPLC. Observingly, rutin was the most abundant of the flavonoids detected in the extract, with a concentration of 20 mg/100 g.

Table 2. The flavonoids identified by HPLC in the ethanol root extract of *W. indica*

Name of compound	Amount (mg/100 g)	Retention time (Min)
Catechin	11.15	9.692
Protocathechuic acid	0.000508	10.657
P-coumaric acid	6.97	11.022
Epicatechin	35.8	11.368
Vanillic acid	4.5	11.696
O-Coumaric acid	0.00022	12.254
Eugenol	0.000076	12.445
Isoeugenol	0.000354	12.834
P-hydroxybenzoic acid	0.059	13.401
Gallic acid	3.50	13.834
Caffeic acid	4.60	14.160
Ferrulic acid	2.30	14.950
Synrigic acid	5.50	15.389
Piperic acid	0.000090	15.805
Sinapinic acid	0.000026	16.247
Diadzein	0.000047	16.543
Coumestrol	0.000014	17.086
Apigenin	0.045	17.696
Naringenin	2.9	18.131
Naringenin Chalcone	0.000507	18.400
Genistein	0.00012	18.775
Shagaol	0.000304	18.957
Kaempferol	1.9	19.483
Luteolin	0.35	20.208
Capsaicin	0.000123	21.800
Epigallocatechin	0.0042	22.672
Ellagic acid	13.6	23.341
Gingerol	0.000025	24.341
Quercetrin	3.43	24.145
Isorhamnetin	4.05	24.275
Myricetin	4.05	24.783
3-o-caffeoylquinine	0.0000018	25.254
Chlorogenic acid	26.8	25.549
Quercitrin	0.0000501	26.029
Isoquercitrin	0.000052	26.737
Rutin	20	27.748

Acute toxicological profile in rats

No death was observed at all doses of the extract (Table 3). In addition, no changes were found in the eyes, fur, skin, paw, and behavior, and no signs of convulsion and constipation in the animals. The oral D50 of the extract was estimated to be greater than 5000 mg/kg.

Effect on mating behavior in male rats

On the 7th day of administering the extract, a significant (P<0.05) decrease was found in mounting latency in the treated groups compared to the control group (Table 4). The shortest mount latency was observed in the group that received 400 mg/kg/day of the extract (P<0.01). Mount frequency, an index for potency, was also higher in extract-treated groups (P<0.01). Like sildenafil citrate, doses of the extract produced a significant (P<0.05) decrease in intromission latency,

Table 3. Acute toxicity test (LD_{50}) of *W. indica* ethanol root extract in rats

Dose administered (mg/kg)	Mortality (Number of death/Number of rats used)
10	0/4
1000	0/4
2000	0/4
3500	0/4
5000	0/4

No death was observed in all the doses. Oral LD_{50} > 5000 mg/kg

Table 4. Effect of ethanol root extract of W. indica (EEWI) on sexual behavior on the 7th day

	Mount latency (seconds)	Mount frequency	Intromission latency (seconds)	Intromission frequency	Ejaculatory latency (seconds)	Post ejaculatory Interval (seconds)
Control (10% Tween 80)	308.50 ± 128.12	3.500 ± 0.64	700.00 ± 400.00	1.33 ± 0.33	74.66 ± 18.67	300.00 ± 0.00
Sildenafil (5 mg/kg)	62.00 ± 10.49**	21.50 ± 1.04**	254.00 ± 100.54***	5.75 ± 1.79*	239.25 ± 12.36**	116.00 ± 37.30*
EEWI (100 mg/kg)	69.00 ± 7.29**	16.00 ± 1.87*	421.50 ± 47.70**	3.25 ± 1.31	161.75 ± 20.91*	99.50 ± 12.50**
EEWI (200 mg/kg)	39.00 ± 7.49***	19.00 ± 3.03**	310.75 ± 41.45**	5.50 ± 41.45*	220.75 ± 2.29**	74.50 ± 13.72***
EEWI (400 mg/kg)	26.25 ± 4.17***	20.00 ± 3.34**	272.00 ± 28.81***	5.75 ± 1.11*	273.25 ± 33.63**	63.75 ± 12.6***

*P< 0.05; **P< 0.01; ***P< 0.001 when compared with control group. The values are mean ± SEM, n = 4.

increase in intromission frequency, decrease in ejaculatory latency, and post-ejaculatory interval.

Table 5 shows the same pattern of results seen on the 7th day of treating the rats with EEWI. The effects at the end of the 14th day were dose-dependent, as the higher doses tended to reduce the mounting latency more. The highest mount frequencies (MF) were seen in the rats administered with 400 mg/kg/day body weight or sildenafil, followed by those administered with 200 mg/kg/day, 100 mg/kg/day, and control, in that order. The mean ejaculation latency (EL) was higher in rats treated with sildenafil and 200 mg/kg/day and 400 mg/kg/day of the extract. The 400 mg/kg/day dose had lower post-ejaculatory intervals (PEI) than the standard drug (sildenafil).

Effect on anxiety

The extract-treated groups showed a significant (P<0.05) increase in time spent in the open arm of the EPM at 200 mg/kg/day and 400 mg/kg/day doses. There was a corresponding significant decrease in time spent in the closed arm of the maze in rats treated with the extract at doses of 200 and 400 mg/kg/day compared to the control rats. Similarly, the sildenafil-treated rats showed a significant (P<0.05) increase in the time spent in the open arm. The extract at 100 mg/kg/day did not significantly affect the time spent in the maze's open or closed arms (Figure 1).

Effect on cognition /transfer latency

The treated rats with EEWI compared well with those treated with the standard agent, piracetam. The rats treated with the highest dose of extract did not show any difference from that of the standard (piracetam). Although, on days 14 and 21, rats treated with 100 mg/kg/day and 200 mg/kg/day showed a significant (P<0.001 and P<0.05). Increase in the time it took to move into the closed arm. By day 28, the latency of transfer of all treated rats was comparable to that of the standard. In the time taken to move into the close arm (P<0.05) (Figure 2).

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Table 5. Effect of ethanol root extract of W. indica (EEWI) on sexual behavior on the 14th day

	Mountlatency (second)	Mount frequency	Intromission latency (seconds)	Intromission frequency	Ejaculatory latency (second)	Post ejaculatory interval (seconds)
Control (10% Tween 80)	335.50 ± 153.06	3.75 ± 0.47	658.75 ± 410.73	1.75 ± 0.85	84.33 ± 14.19	351.67 ± 116.00
Sildenafil (5 mg/kg)	43.50 ± 15.56***	33.25 ± 4.31***	234.75 ± 84.66**	6.75 ± 2.78**	256.25 ± 22.26**	146.50 ± 47.33**
EEWI (100 mg/kg)	85.25 ± 6.53***	18.50 ± 2.39**	441.75 ± 65.91*	4.00 ± 0.91*	251.25 ± 51.63**	247.75 ± 24.79*
EEWI (200 mg/kg)	58.25 ± 9.26***	19.00 ± 2.83**	329.25 ± 46.40**	5.75 ± 1.03*	231.50 ± 5.97**	187.750 ± 42.31*
EEWI (400 mg/kg)	32.50 ± 4.52***	36.50 ± 7.96***	99.25 ± 5.12***	7.75 ± 2.28***	388.50 ± 24.56**	63.75 ± 12.61***

*P< 0.05; **P< 0.01; ***P< 0.001 when compared with control group. Values are mean ± SEM, n = 4.

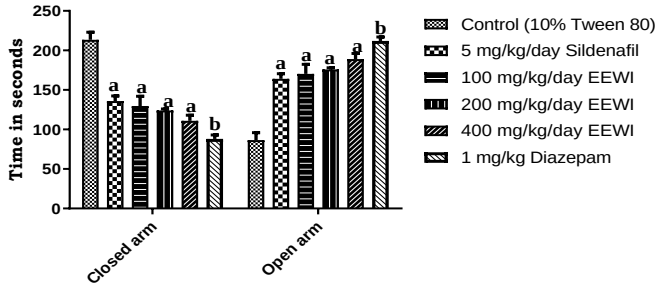

Figure 1. Effect of 7-day treatment of rats with the ethanol extract of W. indica (EEWI) on time spent in the open and closed arms of the elevated plus-maze. aP<

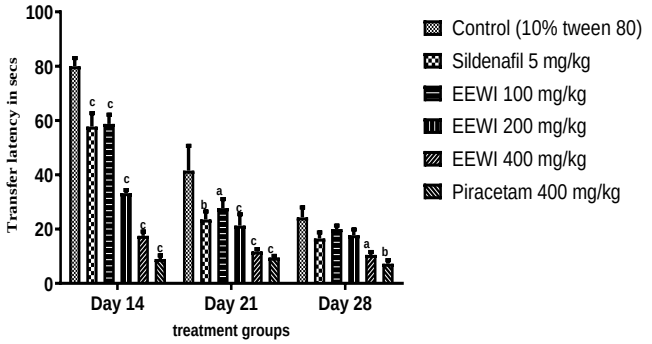

Figure 2. Effect of the ethanol root extract of W. indica (EEWI) on memory/cognition in male rats after a 28-day treatment. aP< 0.05; bP< 0.01; cP< 0.001 when compared with control group. The values are mean ± SEM, n = 4.

Effect on sperm parameters

Table 6 shows that sperm count decreased but was not statistically significant in 100 and 200 mg/kg/day treated rats compared to untreated (control) rats. However, 400 mg/kg/day of EEWI significantly (P<0.05) decreased sperm count compared to the control. The percentage of progressively motile sperms in the treated rats reduced as the dose increased, even though no significant difference was found between the groups treated with 100, 200 mg/kg/day of the extract, 5 mg/kg/day of sildenafil, and control (10% Tween 80). However, the rats treated with 400 mg/kg/day of the extract had a significant (P<0.001) decline in progressively motile sperm cells.

Table 6. Effect of ethanol root extract of W. indica (EEWI) on sperm parameters after 28 days of treatment

	Sperm count (x106/m)	Progressive motility (%)	Non-progressive motility (%)	Non-motile (%)	Normal sperm (%)
Control (10% tween80)	46.88 ± 4.67	62.64 ± 1.68	20.79 ± 0.62	22.33 ± 4.31	82.86 ± 1.62
Sildenafil (5 mg/kg)	39.52 ± 7.45	56.12 ± 2.07	26.78 ± 3.41	23.65 ± 1.03	82.85 ± 2.03
EEWI (100 mg/kg)	39.15 ± 6.17	53.02 ± 6.35	29.08 ± 2.38	26.02 ± 2.90	81.71 ± 5.08
EEWI (200 mg/kg)	30.65 ± 3.11	50.08 ± 5.93	27.20 ± 2.31	27.95 ± 5.60	77.19 ± 7.34
EEWI (400 mg/kg)	29.97 ± 5.13*	40.55 ± 4.93*	33.98 ± 3.09	32.58 ± 4.15	75.51 ± 4.04

*P< 0.05 when compared with the control group. n = 4. Values are mean ± SEM

Sub-acute toxicological effects in rats

Effect on hematological parameters

The hematological parameters following the daily oral administration of ethanol root extract of W. indica (EEWI) for 28 days are shown in (Table 7). A significant (P<0.05) increase was observed in total RBC count at 400 mg/kg/day doses. Besides, hemoglobin concentrations increased significantly (P<0.05) with 400 mg/kg/day. White blood cell count (WBC) also increased significantly (P<0.05) in the 400 mg/kg/day group. The values of the other parameters were similar in the control and other groups.

Table 7. Effect of 28-day oral administration of ethanol root extract of W. indica on hematological parameters in rats

Dose (mg/kg/da)	WBC (x 103/µ)	NEU (%)	EOS (%)	LYM (%)	MON (%)	RBC (x 106/µl)	HCT (%)	HGBX (g/dl)	PLT (x 106/µ)
Control	15.14 ± 0.71	42.03 ± 0.07	0.32 ± 0.22	8.76 ± 0.33	1.30 ± 0.44	6.63 ± 0.21	44.18 ± 0.76	13.66 ± 0.45	568 ± 3.23
100	15.32 ± 0.22	38.33 ± 0.66	0.22 ± 0.55	7.56 ± 0.33	1.25 ± 0.45	7.45 ± 0.22	45.33 ± 0.34	13.45 ± 0.33	567 ± 0.33
200	16.47 ± 0.12*	44.35 ± 0.55	0.24 ± 0.24	8.59 ± 0.42	1.32 ± 0.33	7.55 ± 0.12*	43.23 ± 0.43	16.00 ± 0.22	591 ± 0.21
400	16.53 ± 0.21*	47.66 ± 0.44	0.28 ± 0.26	8.45 ± 0.33	1.29 ± 0.21	7.64 ± 0.44*	45.33 ± 0.33	18.77 ± 0.44*	597 ± 0.13

*P < 0.05. Values represent mean ± standard error of the mean (SEM), n = 4 for each group. WBC: white blood cell; RBC: red blood cells; NEU: neutrophils EOS: eosinophils; LYM: Lymphocytes; MON: monocytes; HGB: hemoglobin; HCT: hematocrit; PLT: platelets.

Effect of extract on the histology of the kidney

The extract had no deleterious effect on kidney histology, as the treated groups were comparable to the control group. Just slight obliterations in bowman's capsules and tubules in the kidneys of rats treated with 200 and 400 mg/kg (Figure 3).

Effect of extract on the histology of the liver

The extract did not have any deleterious effect on the liver histology as treated groups were comparable with the control group (Figure 4).

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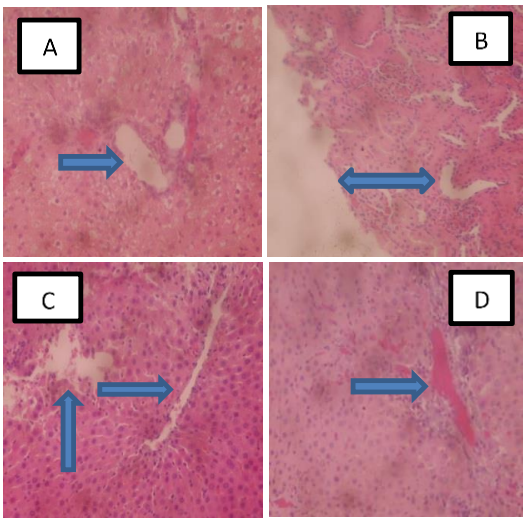

Figure 3. Histological sections of the liver samples of rats. A: Control shows normal liver architecture with the central vein (v), normal hepatocytes, and blood sinusoids. B: 100 mg/kg/day, shows normal hepatocytes and blood sinusoids. C: 200 mg/kg/day shows normal hepatocytes and blood sinusoids. D: 400 mg/kg/day congested blood vessels with perivascular leucocytic infiltration. X100, H&E

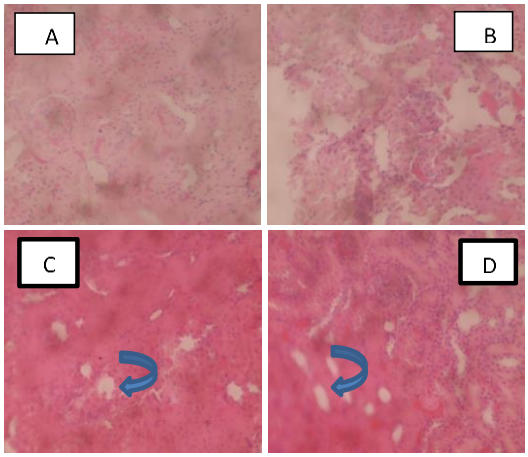

Figure 4. Histological sections of the kidney samples of rats. **A**: Control, shows normal Bowman's capsule and tubules. **B**: 100 mg/kg/day of extract shows distal convoluted tubule, glomerulus, urinary space, and squamous cells. **C**: 200 mg/kg/day of extract shows obliterated Bowman's capsule. **D**: 400 mg/kg/day of extract shows obliterated Bowman's capsule. x 100, H&E.

Antioxidant properties of ethanol root extract of *W. indica*
Effect of 28-day oral administration on antioxidant biomarkers

In Table 8, the administration of doses of 200 and 400 mg/kg/day of the extract resulted in a significant ($P<0.05$) increase in the concentrations of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT). The concentrations of these biomarkers did not significantly increase with the dose of 100 mg/kg/day.

Table 8. Effects of 28-day oral administration of ethanol root extract of *W. indica* on antioxidant biomarkers of rats

Dose	GPX (U/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control	7.03 ± 0.21	7.52±025	9.32 ± 0.22
100 mg/kg/day	7.65 ± 0.22	7.46 ± 0.55	10.22 ± 0.55
200 mg/kg/day	9.55 ± 0.12*	8.85 ± 0.54*	11.44 ± 0.24*
400 mg/kg/day	9.64 ± 0.44*	8.28 ± 0.21*	12.48 ± 0.26*

*P<0.05 versus control. Values are mean ± SEM, n = 4. SOD: superoxide dismutase; CAT: catalase; GPX: glutathione peroxidase.

DPPH radical scavenging activity of the extract

The DPPH radical scavenging activity of the ethanol root extract of W. indica is presented in (Figure 5). The concentrations causing 50% inhibition of the production of the radical (IC50) of the extract and of ascorbic acid were 210 µg/l and 800 µg/l, respectively.

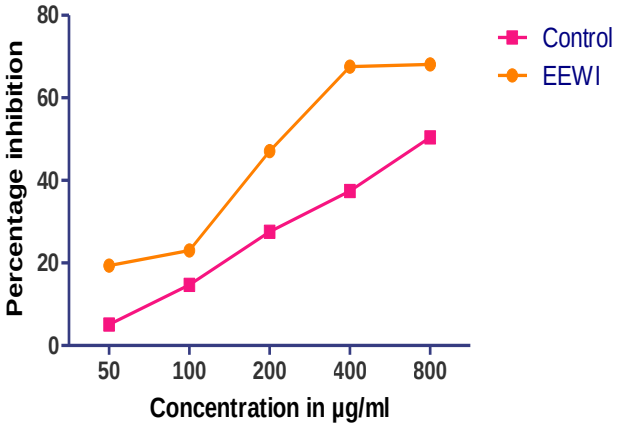

Figure 5. DPPH radical scavenging activity of ethanol root extract of W. indica (EEWI) compared to control (vitamin C). n = 3

Discussion

The aphrodisiac potential and mechanism of aphrodisiac activity in the ethanol root extract of W. indica were investigated in this study. Preliminary phytochemical screening showed that the extract contained flavonoids, alkaloids, sterols, tannins, saponins, anthraquinones, and carbohydrates, corroborated by previous studies (31, 6). Interestingly, some of these classes of compounds have been shown to have aphrodisiac properties. For example, some saponins have been linked to aphrodisiac properties (32). Saponins can cause corpus cavernosum muscle relaxation through the nitric oxide-cyclic GMP pathway and increase testosterone levels, improving libido and sexual performance (33). Similarly, phenolic compounds such as flavonoids have been reported to exhibit an aphrodisiac effect through a mechanism that is similar to that of phosphodiesterase inhibitors such as sildenafil (34, 35) and by interacting with central pathways that participate in libido or sexual arousal (36, 37). Some flavonoids have been shown to raise androgen levels, which results in greater libido and sexual performance (33, 38). In addition to altering androgen levels (thus enhancing sexual stimulation), flavonoids have been reported to increase superoxide dismutase and catalase activities, thereby imparting an indirect potentiating effect on the parameters of sexual behavior (39). Furthermore, flavonoids have been associated with increased testosterone levels by inhibiting aromatase, an enzyme responsible for converting testosterone to estrogen (40). Therefore, using HPLC, carrying out the quantitative and qualitative assay of flavonoids in EEWI was essential.

The assay for flavonoids in the ethanol root extract of W. indica using HPLC revealed the presence of rutin, isoquercitrin, luteolin, apigenin, kaempferol, quercetin, syringic acid, and gingerol, to name a few. Rutin was observed to be the most abundant constituent (20 mg/100 g of extract). Interestingly, rutin possesses a variety of reported pharmacological properties, including antioxidant, cytoprotective, vasoprotective, anticarcinogenic, neuroprotective, and cardioprotective effects (41)

The aphrodisiac potential of the plant was studied in rats to corroborate the observations in *D. melanogaster* (1, 2). Some sexual behavioral indices were used to appraise the aphrodisiac property of the extract. Mount frequency (MF) and intromission frequency (IF) are valuable indices of vigor, libido, and potency. This is because the number of mounts (MF) is thought to reflect sexual motivation, and an increase in the number of intromissions (IF) shows the efficiency of erection, penile orientation, and the ease with which ejaculatory reflexes are activated (39). As a result, the increased MF and IF at the end of weeks 1 and 2 of daily dosing with 100, 200, and 400 mg/kg of EEWI suggest increased libido. The fact that the extract increased IF in this study shows that penile erection mechanisms were involved. Indicators of the sexual drive include mount latency (ML) and intromission latency (IL), and sexual motivation is inversely proportional to ML and IL (42, 43) Azizi *et al*., 2021). Accordingly, the decrease in mount and intromission latencies reported in this study at all doses of the extract could indicate that sexual motivation and arousability were stimulated in the rat. Taken together, the extract improved sexual function at these doses, and the results obtained were increasingly comparable to those obtained in sildenafil-treated rats.

The longer ejaculation latency caused by the 200 and 400 mg/kg/day of the extract indicated that the copulatory performance of the rats was improved. The

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post-ejaculatory interval (PEI) measures potency, libido, and the pace of recovery from exhaustion (44). Consequently, the much shorter PEI observed in treated rats could explain the increased potency and libido, less tiredness during the first mating session, or both.

The behavioral indicators of sexual performance and facilitation are erection and ejaculation (45). Compared to the control group, the much higher mounting frequency in the extract-treated rats suggests arousal and, thus, aphrodisiac activity. Some plants with aphrodisiac characteristics function through one or more of the following mechanisms: vasodilation (46), nitric oxide production (47), increased concentration of androgens (48), and gonadotropin (49). Sexual behavior and erection are also influenced by androgens, which may act centrally and peripherally (50). More specifically, drugs that change circulating hormone levels, such as testosterone and prolactin, could influence sexual behavior (17).

In anxiety trials, the extract-treated rats spent more time in the open arm of the Elevated Plus Maze (EPM) than the control group rats, indicating that the extract had an anxiolytic effect (51). A definite link exists between erectile dysfunction and thoughts and emotions. Any event that impairs message transmission between the brain and the penis can significantly impact erectile performance (52). According to some previous studies, the prevalence of anxiety problems in men with ED ranges from 2.5 % to 37 % (53). Neurobiological manifestations of anxiety include the release of adrenaline and noradrenaline (54). Increased release of these monoamines may negatively affect arousal and orgasmic phases, interfering with sexual arousal (55). Various features of anxiety, particularly the vicious circle of anxiety/dysfunction/performance anxiety, have long been explored in arousal disorders (53, 56). This condition is exemplified by honeymoon impotence (57). Drugs that stimulate open-arm exploration of the EPM have previously been shown to have anxiolytic effects (20). Since the treated rats spent significantly more time in the open arm of the EPM, it may be assumed that the extract has anxiolytic properties and may be effective in sexual dysfunction caused by anxiety. A transfer latency study was carried out to observe the effect of the extract on learning, as the underlying mechanism of sildenafil appears to be related to increased nitric oxide (NO) production in the brain, leading to cerebral vasodilatation which may result in enhanced acquisition and retention of memory (21). Alternatively, the enhanced NO production leading to facilitated cortical cholinergic transmission (58) may be responsible for memory improvement.

The report has shown that cognitive impairment negatively affects sexual activity (59). At the same time, the production of sex hormones has been linked to improved memory since lower levels of cognitive impairment have been linked to higher levels of sexual activity (60). In older men, low estradiol and high testosterone levels have shown better performance on several cognitive function tests (61), indicating that cognitive memory may be a function of testosterone concentration. The memory studies in this research revealed that concomitant exposure of rats to extract EEWI improved the memory of treated rats, such that results of days 21 and 28 were improvements on day 14. The study underscored the dose-dependent effect of the extract, as rats that were given 400 mg/kg/d compared very well with the standard agent piracetam. The results showed that the extract has a memory enhancement activity effect correlated with sexual enhancement.

Previous evaluations of fecundity-enhancing characteristics revealed that the extract did not affect fertility, as there was no increase in the quantity of progeny or eggs deposited by D. melanogaster (2). This is corroborated by the drop in sperm count and an increase in defective sperm cells in the extract-treated rats. Proposedly, the extract has no fertility-enhancing properties, agreeing with previous reports (62).

Toxicological tests are used to determine whether or not a plant material is safe. The toxicological profile of the ethanol root extract of W. indica was investigated using several methods. Reports by (62) and (63) are agreeable with the present study that acute doses of the extract did not cause mortality. Sub-acute toxicity evaluation of the extract in this study showed a considerable rise in red and white blood cells at the dosages of 200 and 400 mg/kg/day, which aligns with the report by Oladiji et al. (6), who attributed the increase in RBC to the presence of iron and proteins in the aqueous extract of W. indica plant. The increase in RBC in this study supports the view that erectile dysfunction (ED) is inversely correlated with RBC, expected to favor the oxygenation of blood flow to the penis during erection (64). More importantly, histology revealed that the extract did not appear to alter the liver or kidney architecture, except for minor vascular congestion in the kidney. Hence, the present study differs slightly from that of Adedokun et al. (9), who reported mild interstitial congestions in the kidney and mild vascular congestions in the liver after 21 days of administration of doses (1000 mg/kg) of methanol leaf extract of the plant. Treatment with varied doses of EEWI did not affect the weights of organs such as the heart, kidney, and liver.

Conclusion

The ethnomedicinal use of the ethanol root extract of W. indica (EEWI) for aphrodisiac action has been validated by this study. The extract lacks fertility-enhancing effects but appears safe. Although the extract contains flavonoids and saponins, which have been associated with aphrodisiac actions, further studies will identify the pharmacologically active compounds.

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Ethical statement

Institutional ethical approval (EC/FP/022/13) was obtained from the Animal Ethics Committee of the Faculty of Pharmacy, University of Benin.

Conflicts of interest

The authors declared no competing interest in this research.

Author contributions

R.I.O. designed the work, B.E.E. made the first draft, and B.E.E., along with E.E.O., did the laboratory experiments.

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