Pharmacological Profiling of Mangrove Plant in Coastal Area of Sabah Extract, *Rhizophora Mucronata* for its Aphrodisiac Potential

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Abstract: Ethnopharmacological relevance: The potential aphrodisiac activity of mangrove plant in coastal area of Sabah, *Rhizophora Mucronata* (RM) was evaluated for its natural aphrodisiac potential using animal model from the observation of proboscis monkeys at Labuk Bay and Sukau in Sabah with high sexual activity and a high sex ratio towards the female (sex ratio 1:8.4) and their diet in consuming local plant (mangrove) which is believed to contribute to the high sexual activities. Investigation of aphrodisiac potentials on the crude extract was determined by assessing the sexual potency enhancement. The assessment was done on the sexual desire (mounting behaviour test), sexual motivational (partner preference test), sexual performance test and reproductive hormonal assay

Results: Phytochemical screenings showed the presence of flavonoids and saponins believed to be responsible for aphrodisiac properties. Acute oral toxicity of extracts was classified as Category 5 according to the GHS for the classification of chemicals as the LD50 is greater than 2000 mg/kg body weight. Sexual desire (libido) and motivation are improved by the reduction in mount latency. Whereas sexual performance is enhanced by the reduction of intromission latency. Hormonal serum, Testosterone, LH and FSH level in sexually experienced rats was significantly increased.

Keywords: Aphrodisiac, Catechin, Rhizophora Mucronata, Sexual Behaviour.

INTRODUCTION

An aphrodisiac is defined as an agent (food or drug) that arouses sexual desire. In traditional medicine, a variety of plants have been used as sex stimulants. Aphrodisiac is a substance that stimulates/increases sexual desire (libido), motivation and performance. It is also used to modify the impaired sexual functions of human beings. Many synthetic drugs are available to improve sexual function, such as Sildenafil (sold as Viagra) and Tadalafil (sold as Cialis). However, these substances can produce negative side effects such as headache, muscle pain, and blurred vision, and may have dangerous interactions with other medications. Therefore, the demand for a natural aphrodisiac remedy has increased as evidence of its booming business on the internet.

The research was initiated based on the observation of proboscis monkeys of the mangrove areas of Sabah which is seemed to have high sexual activity and a high sex ratio towards the female (sex ratio 1:8.4). Their diet consuming a local plant is believed to contribute to the high sexual activities. Based on the active sexual behaviour and higher sex ratio of adult male proboscis monkeys that fed the mangrove's young leaves, an evaluation on the mangrove leaves for their pharmacological properties as an aphrodisiac has been carried out.

The potential aphrodisiac activity of mangrove plant extracts (aqueous and organics) was evaluated for their aphrodisiac property using an animal model. One mangrove plant in coastal area of Sabah, *Rhizophora mucronata* L. (Rhizophoraceae), was chosen for its aphrodisiac potential. The bark, root, leaves, fruit, and flowers of *Rhizophora mucronata* (RM) have been traditionally used as medicine in the coastal areas of Asian subcontinents for treating health ailments such as diabetes, diarrhoea, hepatitis, inflammation, and cognitive function.

The methanolic fraction of RM extract offers a substantial decrease in diabetes and metabolic impairment in rats [1]. In vitro studies have shown that RM exhibited potent antioxidant, and cholinesterase inhibitory activities [2]. Based on these observations, the present study was carried out to analyse the potential of Catechin-rich methanolic extract of RM in aphrodisiacs. Investigation of aphrodisiac potentials on the RM leave extract was determined by assessing the libido and sexual potency enhancement. The assessment was done on the sexual desire (mounting behaviour test), sexual motivational (partner preference test) and sexual performance test (mating test), sexual hormones and its potential bioactive compound.

MATERIAL AND METHOD

a) Chemical

Estradiol benzoate, progesterone and L-Dopa were purchased from Sigma Aldrich, USA. *Eurycoma longifolia* (*E. Longifolia*) extract was obtained from a research collaborator, the Malaysian Institute of Pharmaceutical and Nutraceutical (IPHARM). The serum testosterone assay kit was purchased from Cayman Chemical Company, USA. Estradiol benzoate, progesterone and L-Dopa were purchased from Sigma Aldrich, USA. The serum testosterone assay kit was purchased from Cayman Chemical Company, USA. Culture Medium: Dulbecco's Modified Eagle Medium (DMEM) (Thermo Scientific, buffered with sodium bicarbonate) supplemented with 10 % (v/v) calf serum (Sigma), 100 IU/mL Penicillin and 100 mg/mL Streptomycin (PAA Lab), Chemical Dilution Medium (CDM): DMEM (Thermo Scientific, buffered with sodium bicarbonate) supplemented with 100 IU/mL Penicillin and 100 mg/mL Streptomycin (PAA Lab), Phosphate Buffered Saline

(PBS), Calcium and Magnesium free (PBS): 8 mg/mL Sodium Chloride, 0.02 mg/mL Potassium Chloride, 0.2 mg/mL Potassium Dihydrogen Orthophosphate and 1.15 mg/mL Disodium Hydrogen Orthophosphate, Trypsin-EDTA (Ethylenediaminetetraacetic acid) : 2 mg/mL trypsin (Sigma) and 0.3 mg/mL EDTA in PBS, Neutral Red (NR) Stock Solution: 2.0 mg/mL NR (Sigma), NR Medium: 50 µg/mL NR in CDM and culture medium, NR Desorb: 1 % Glacial acetic acid and 50 % ethanol, Dimethyl Sulfoxide (DMSO) and Ethanol. Sodium Lauryl Sulphate (SLS) (Calbiochem) was used as Positive Control.

b) Plant materials and preparation of aqueous extract

Young leaves of RM collected around the coastal areas of Sabah were authenticated at the Sabah Forestry Department, Sandakan, Sabah with a voucher specimen, *Rhizophora mucronata* (Bakau Kurap); SAN 149220. The leaves were oven dried at 40 ± 3 ºC overnight, powdered and subjected to 50 % Soxhlet extraction using methanol: water. The extract was filtered, rotary evaporated and freeze-dried to remove excess solvent. The crude extract was kept at 4 ºC in the refrigerator until further use.

c) Animals

Prior approval was taken from SIRIM-IACUC Committee (SIRIM-IACUC/IBRC/B19-137/0023), see Appendices. Male and female Sprague Dawley rats, 8 weeks of age with a weight range of 200 to 300 g were selected for the study. Animals were supplied with feed and water *ad libitum.*

d) Phytochemical screening

Preliminary phytochemical properties of the RM crude extract were studied to detect the presence of some important phytochemical constituents that usually exhibit biological activities using standard procedures as described by Trease and Evans (1989) [3], Harborne (1973) [4], and Sofowora (1993) [5]. These constituents include alkaloids, saponins, flavonoids, tannins and polyphenolic compounds, triterpenes and steroids.

i) Alkaloids testing (Dragendoff's test)

A small amount of both aqueous and lipid extracts was diluted with distilled water and methanol, respectively. Each diluted extract was then mixed with 1 mL of 1 mol/L HCl in a test tube followed by adding 2 to 5 drops of Dragendoff's reagent. The yellow to orange precipitate indicates a positive result.

ii) Steroids testing (Libermann-Burchard test)

A total of 2 mL of each diluted extract was transferred into a spot test plate and allowed to dry before mixing properly with 3 to 5 drops of acetic anhydride. Then, 1 to 2 drops of concentrated Sulphuric Acid were added (from the wall of the spot test plate to let the acid to mix slowly). The colour changes were observed, purple colour indicates positive for steroids.

iii) Flavonoids testing

Two milliliters of each diluted extract were mixed with three pieces of magnesium coils into different test tubes, followed by 0.5 mL of HCl and the solutions were allowed to mix and settle for 10 min. The colour changes were observed, a red colour indicating a positive result.

iv) Saponins testing

Distilled water was added to the diluted samples until about three fourth of the test tube's height. Then, the solutions were vigorously shaken for a few minutes and allowed to stand for 15-20 min. The detection of the saponins content was classified as no froth (negative), froth less than 1.0 cm (weakly positive), froth 1.2 cm high (positive) and froth greater than 2.0 cm (strongly positive).

e) NRU Cytotoxicity Assay

Cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind Neutral Red (NR), a supravital dye, in two cultured cell systems [mouse fibroblast (Balb/C) 3T3 and normal human keratinocytes (NHK)]. The data generated from the *in-vitro* cytotoxicity assays are used to predict the starting doses for rodent acute oral toxicity assays, thus, reducing and refining the use of animals in the toxicological assessment of plant extract studied. The ICCVAM Test Method Evaluation Report: *In-Vitro* Cytotoxicity Test Methods for Estimating Starting Doses for Acute Oral Systemic Toxicity Tests" NIH Publication No. 07-4519 (Published 2006) was followed, which will generate information on the No-Observed-Adverse-Effect level (NOAEL) based on the Inhibition Concentration (IC) obtained.

f) Acute Oral Toxicity Study

This study was conducted on rats to determine the toxicity potential of mangrove extracts from a single dose via oral route according to Organization of Economic Cooperation and Development (OECD) guidelines No: 425 [6]. The acute oral toxicity up-and-down procedure (UDP) – Limit Test is a sequential test which uses a maximum of five animals. Animals are dosed in sequential manner with the next animal receiving the same dose only if the first animal survives the limit dose.

g) Evaluation of aphrodisiac potential

The aphrodisiac potential was evaluated through mounting behaviour study for libido effect [7] partner preference study for sexual motivational [8] and assessment of mating performance for potency effect [9].

i) Selection of female rats

Female rats were used as mating stimulus. The females were bilaterally ovariectomized via lumbar incisions under Ketamine (100 µL/0.1 kg of rat) and Xylazine (10 µL/0.1 kg of rat) anaesthesia at least two weeks before the experiments began. They were allowed to recover after surgery for 10 days. The females were rendered sexually receptive by a single subcutaneous dose of Oestradiol Benzoate (10 µg) 52 h and Progesterone (1 mg) 4 h, before

the copulatory study. Oestrous females displayed a high degree of lordosis responding and proceptivity. A baseline sexual behaviour study was carried out on rats from all groups to render them sexually experienced and was then repeated following the treatment schedule mentioned above [10].

ii) Animal grouping and treatment

The animals were randomized into four groups (I-IV) of six animals each. Animals in Groups I, II, III, and IV were given Control (RO), RM extract (300 mg/kg), *E. Longifolia* extract (500 mg/kg) and L-Dopa (100 mg/kg) respectively.

iii) Male rat sexual behaviour test

a) Mounting Behaviour Test (MBT)

Mounting is the term used to describe the male assuming the copulatory position but failing to achieve intromission. Male rats were placed individually in a clear cage. After 30 min of administration of the extracts, a female rat was introduced into the cage. The animals remain paired for 3 h. The number of mounts was recorded for 15 min observation period at the start of each hour. The analysis of orientation activity was carried out and analyzed in three segments - orientation activities of male rats toward female rats (mounting, licking, anogenital sniffing), toward the environment (exploration, raring, climbing) and toward self (grooming, stretching).

b) Partner Preference Test (PPT)

Male rats were orally treated with the RM crude extract or control groups and caged for 15 min in the separated cage. The animals were then acclimatized in the arena (Figure 1) for five minutes for adaptation. It was then individually caged. Sixty minutes later, one oestrous (receptive) and one dioestrous (nonreceptive) female were introduced to the cage of each of the treated males. The cumulative time spent by a male in physical contact with each of the females was recorded for 15 min. Partner preference index was calculated by subtracting the time spent with dioestrous females from the time spent with oestrous females. A positive score indicates a preference for the sexually receptive female while a negative score indicates its preference for the non-receptive female.

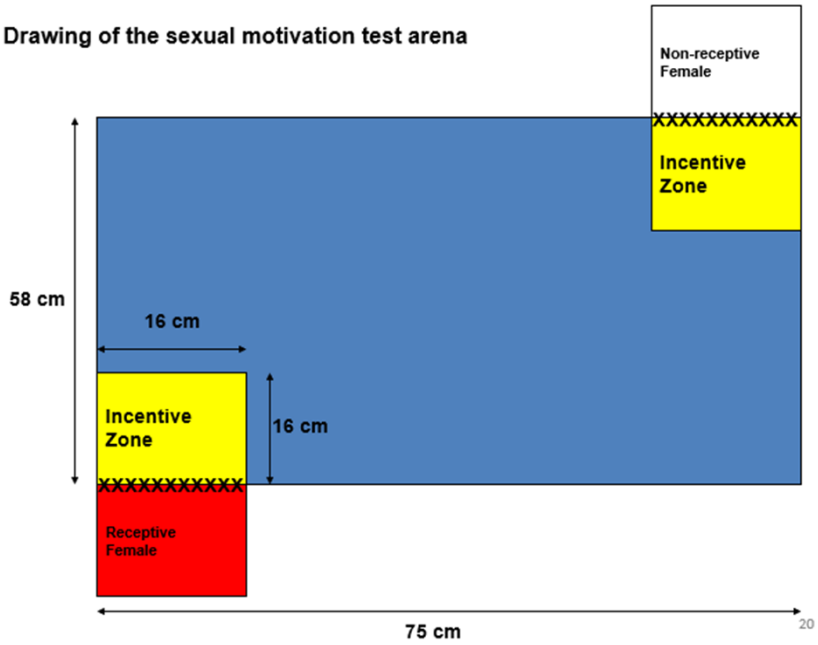


Figure 1: Drawing and measurement of the sexual motivation arena from the top view

c) Mating Performance Test

In this test, female rats were chemically brought into oestrus before their introduction into the cage with male rats. Their receptivity was confirmed by smear analysis according to Ecksterin criteria [11] and by exposing them to the males. Receptive females adopt a copulation stance and beat their ears. The same observation procedure was used as in the mounting behaviour test. All observations were recorded on photos. Many parameters were noted, such as the time from female introduction into the cage to the first mount, i.e. mount latency (ML), the time from female introduction to first intromission, i.e. intromission latency (IL), the number of mounts, i.e. mount frequency (MF), the number of intromissions, i.e. intromission frequency (IF), and finally ejaculation frequency (EF) or libido index.

d) Sperm count and mortality

For the screening of RM crude extract's reproductive toxicity profile, and *in-vitro* sperm count preservation, the method of Thakur and Dixit (2007) [12] was used. A total of six healthy male rats weighing between 250-300 g were taken and sacrificed by cervical decapacitation. Both left and right epididymis were taken into physiological solution (0.9 % NaCl) and squashed thoroughly using needle/forceps until a milky suspension is obtained. A 1 mg/mL solution of each extract group (non-treated, RO Water, RM crude extract and *E. Longifolia*) was prepared and added to the sperm specimens in the extract of each group in a ratio of 0.1:1

(100 µL sperm solution: 1 mL extract solution). The spermatozoa were counted using a hematocytometer at 0 and 30 min after incubation at room temperature (27 ± 1 °C). Manual seminal analysis was used to analyse the semen for motility. A drop of semen (about 10-15 µL) was collected using a dropper and applied on a clean slide. The slide was covered with a cover slip and then examined for motility under x 40 objective lens.

Note is made of the number of spermatozoa that are motile and the nature of such motility out of a population of 20 spermatozoa (motility type is categorized into 'a'- rapid, progressive motility, 'b'- slow or sluggish, progressive motility, and 'c'- non-progressive motility/immotile [13] [14]. Counts obtained of the motile spermatozoa are multiplied by 5 to obtain the percentage motility in each category.

e) Quantification of reproductive hormones

The animals were grouped into four groups and administered orally daily for six days at various doses based on body weight with group I - Control (RO), group II - RM crude extract (300 mg/kg), group III - *E. Longifolia* extract (500 mg/kg) and group IV - L-Dopa (100 mg/kg). Animals were sacrificed 24 h after the last dose on the sixth day. Trunk blood was collected into centrifuge tubes. The tubes were centrifuged (3000 rpm, 20 min and 4 °C) to give serum and stored frozen until time for analysis. The serum hormone concentrations of Luteinizing hormone (LH), Follicle-Stimulating Hormone (FSH) and Testosterone (T) were quantified according to the manufacturer's instruction, using a microplate immunoenzymometric assay (EIA/EMA/ELISA) kit. The serum hormone concentrations were then interpolated from their respective calibration curves.

RESULTS AND DISCUSSION

a) Preliminary phytochemical properties

The phytochemical screening can help to reveal the chemical constituent of the plant extract and the one that predominates over the others. It may also be used to search for bioactive agents as starting products used in the partial synthesis of some useful drugs [15]. Table 1 shows the relative concentration of the secondary metabolites in the samples which were determined by considering the colour intensity of the positive results of the tests. An intense colour was taken as an indication of a high concentration of the metabolite. The same was done for moderate and low intensity coloration of the positive results. It was observed that the RM crude extract had a moderate concentration of flavonoids, saponins and steroids. A low concentration of tannins was also detected. There were no alkaloids in the RM leaf extract detected. This result corresponded to Rohini et al., (1999), where RM leaf is a natural source of tannins and flavonoid, mainly Catechin.

The presence of flavonoids in plant extract has been implicated to have a role in altering androgen levels and may also be responsible for the enhanced male sexual behaviour in this study [16]. The potential for application of natural flavonoids in delaying the decline in testosterone is supported by the animal studies on the flavonoid-enhanced testosterone production and reproductive function. Po-Ling Yu, et al., 2009 [17], claims Catechins increased plasma testosterone in vivo in male rats. Meanwhile, *in-vitro* study, low-dose concentration of Catechins increased gonadotropin releasing hormone (GnRH)-stimulated luteinizing hormone (LH) release by anterior pituitary gland and hCG-stimulated testosterone release by LCs of male rats. Therefore, studies to identify the potential active constitutes (Catechin) in RM crude extract that is responsible for the sexual function improvement activities and the mechanism whereby these activities implanted has been carried out.

Table 1: Phytochemical screening of RM leave extract

Alkaloids	Saponins	Flavonoids	Tannins & Polyphenolic compounds	Triterpenes & Steroids
-	++	++	+	++

*Absent (-), low concentration (+), moderate concentration (++), high concentration (+++)

b) NRU Cytotoxicity

NRU assay is more sensitive and reliable in determining cytotoxicity in plant extracts. The possibility of plant extracts to react chemically with the indicator in NRU assay is eliminated as the protocol for this assay involves the washing step whereby the treated BALB/c 3T3 cells were washed with PBS before adding the NR solution. The neutral red dye does not accumulate in the lysosomes of dead or damaged cells [18] thus, the compounds that could have contributed to the chemical reaction were removed although neutral red reacted chemically with the plant extracts in the interaction test.

The RM crude extract was extracted in Chemical Dilution Medium (CDM) for 24 h (37 ºC) at the highest stock concentration of 200,000 µg/mL. Dimethyl sulfoxide (DMSO) was used as the vehicle control. Range Finder Test was carried out at the highest treatment concentration of 100,000 µg/mL. The concentration-response curves are presented in Figure 2. Positive control was carried out at the highest treatment concentration of 250 µg/mL. The concentration-response curves are presented in Figure 3. Mean IC50 from two main tests was calculated as the result and applied to the following regression formula for estimation of a median lethal dose or LD50 in mg/kg: log LD50 (mg/kg) = 0.372 log IC50 (µg/mL) + 2.024

Conclusion: The median inhibition concentration (IC50) level of RM crude extract predicted an LD50 value of more than 2,000 mg/kg body weight. Therefore, the proposed starting dose for acute oral toxicity test according to the Up-and-Down Procedure is 2,000 mg/kg body weight.

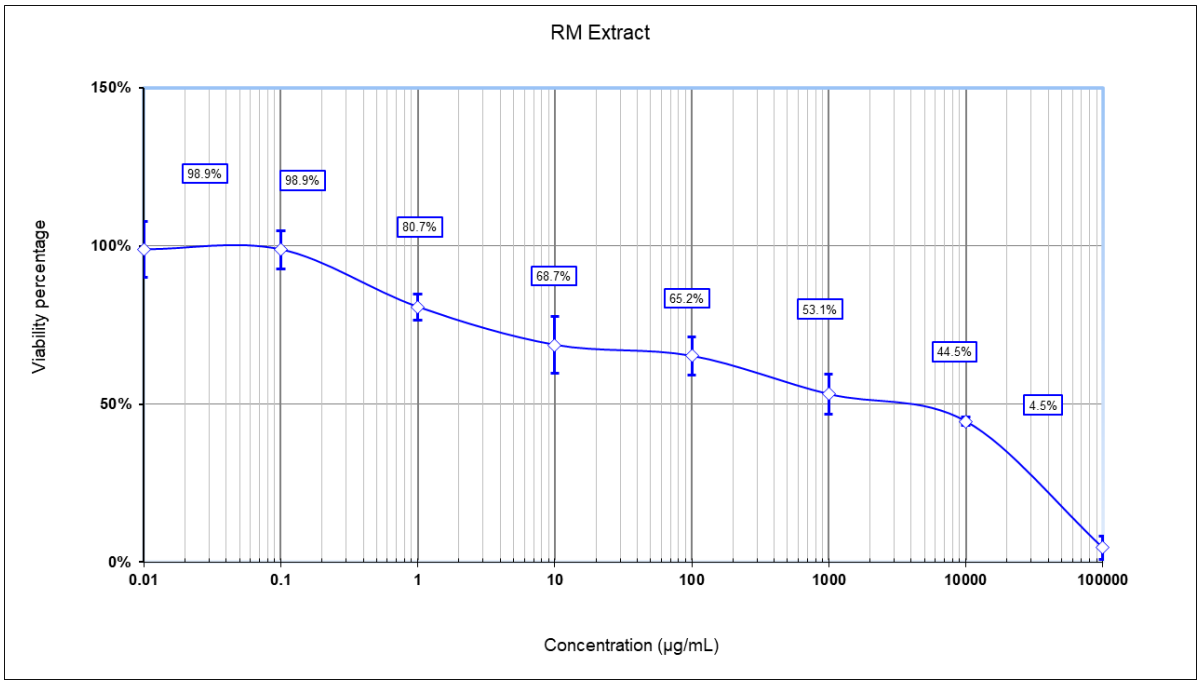


Figure 2: Range Finder Test: Concentration-response curve of RM crude extract

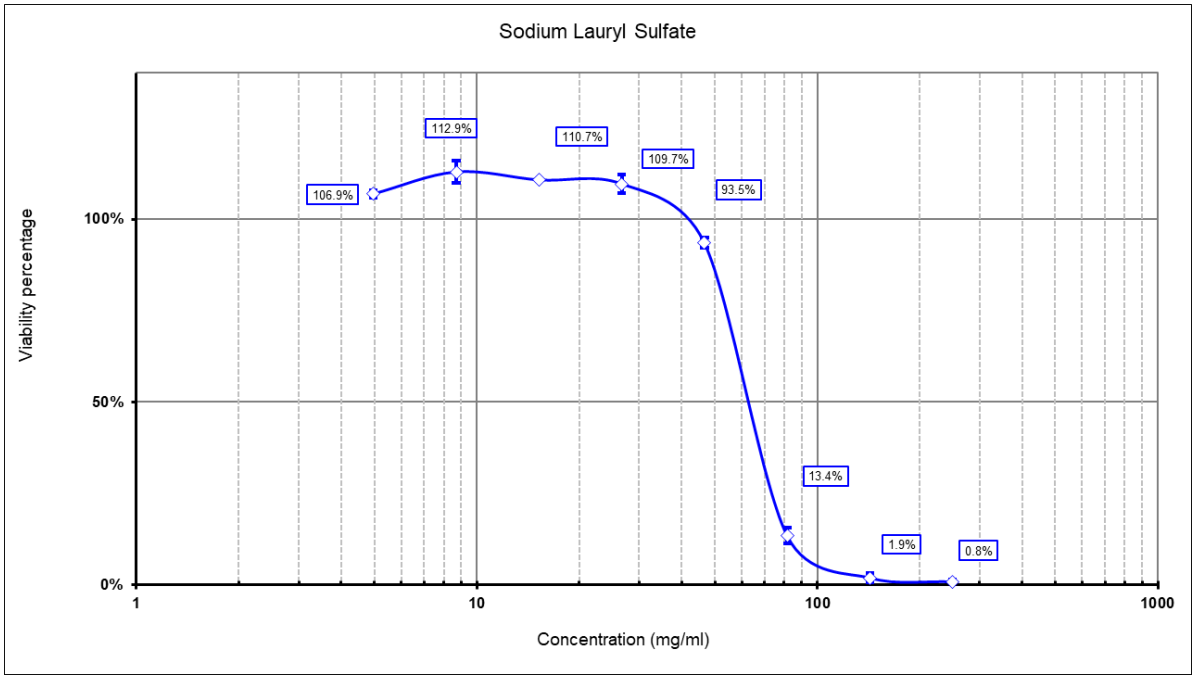


Figure 3: Positive Control: Concentration-response curve of Sodium Lauryl Sulphate

c) Acute Oral Toxicity

The toxicity studies are useful parameters to investigate the therapeutic index of chemicals, drugs and xenobiotics (Rang, 2001). In this study, all animals were observed individually for mortality, signs of gross toxicity and behavioural changes once during the first 30 minutes after dosing. No mortality was observed within the 14 days procedure. All animals gained body weight, appeared normal and did not demonstrate any abnormal behaviour during the observation period. This data is shown in Table 2. Under the conditions of this study, the acute oral toxicity of RM crude extract studied was classified as Category 5 which is nontoxic according to the Globally Harmonised System (GHS) for the classification of chemicals (LD50 is greater than 2000 mg/kg body weight).

Earlier reports have shown that if the median lethal dose of a test substance is three times more than the minimum effective dose, the substance is considered as a good aspirant for further studies [19]. Therefore, RM crude extract indicates that it does not cause any toxicity and is safe for oral use. Nevertheless, such acute toxicity data are of limited clinical application since cumulative toxic effects do occur even at very low doses.

Table 2: Data of animal body weight (day 0, day 7 and day 14), daily observation and necropsy observation of tissue and organ.

Dose (mg/kg)	Body Weight (g)			Daily Observation	Necropsy Observation of Tissue/Organ	Findings
	Day 0	Day 7	Day 14			
175	199	218	232	Active and healthy the 14-day observation period	All tissues/organs	No gross abnormalities
550	201	222	239		All tissues/organs	No gross abnormalities
2000	210	231	239		All tissues/organs	No gross abnormalities
2000	213	227	235		All tissues/organs	No gross abnormalities
2000	217	229	237		All tissues/organs	No gross abnormalities

d) Evaluation of aphrodisiac potential

Assessment of aphrodisiac potential was done on the sexual desire (mounting behaviour study), sexual motivational (partner preference study) and mating performance study after administration of RM crude extract (300 mg/kg, p.o). All activities were recorded using CCTV video camera.

(i) **Mounting Behaviour**

The mounting behaviourial study results are summarized in Table 3. Results showed that rats treated with RM crude extract has increase the number of mounts throughout the observation period compared to the Control (RO) group. RM crude extract at the dose level of 300 mg/kg body weight markedly influenced the orientation behavior, which showed more attraction towards female rats, and also indicated sexual motivation. The studies on the licking and anogenital smelling revealed that a significant increase in the number of licking and a moderate increase in anogenital smelling of treated male rats towards receptive females was observed in animals treated with RM crude extract.

The behavioural assessment of rats towards the environment (exploration, raring and climbing) was significantly decreased in experimental animals and moderately in the standard group. The studies on genital grooming revealed that there was a significant increase in genital grooming in RM crude extract group, while a moderate decrease in non-genital grooming was observed as compared with the control group.

L-Dopa (100 mg/kg) and *E. Longifolia* (500 mg/kg) also show a significant decrease in genital and non-genital grooming as compared to the control group. L-DOPA has been shown to be involved only in energetic aspects of appetitive sexual behaviour in men, but not genital or subjective sexual arousal [20]. As shown in other studies, L-Dopa is proven not to show that the animals are more attractive or receptive as mounting objects.

Finding in this study also tallied with another separate study which revealed that mice treated with 800 mg/kg of *E. Longifolia* increased orientation activities towards the receptive females (anogenital, licking, and mounting), increased genital grooming towards themselves and restricted movements to a particular area of the cage, but decreased interest in the external environment (raring, climbing, exploration) [21].

(ii) **Partner Preference**

The goal of this experiment was to assess the effect of the administration of RM crude extract to male rats on sexual motivation. The results in Table 4 showed that the male rats treated with RM crude extract (300 mg/kg) increased in preference for the sexually receptive female rats when compared to the Control (RO) group by the high percentage cumulative time spent in the female area compared to male area. Therefore, sexual desire is altered or observed to increase in rats treated with RM crude extract and L-Dopa when subjected to partner preference study. However, low percentage is showed in *E. Longifolia* (500 mg/kg).

Table 3: Effect of Orientation Behaviour of RM crude extract. Figures are mean score ± SEM of the six rats in each group. *P<0.05 indicates a statistically significant difference from the Control (RO) group.

Table 4: Effect on partner preference study (sexual motivational)

(iii) **Mating Performance**

This study examined the effect of RM crude extract on male sexual competence in rats, with L-Dopa and *E. Longifolia*

Treatment group	Time (min)	Mounting		Mean activity score towards Female		Mean activity score towards Environment			Mean activity score towards self	
		No of Mounting	Mount Latency	Anogenital Sniffing	Licking	Climbing	Raring	Exploration	Non-genital grooming	Genital grooming
Control (RO)	15	0.17 ± 0.2	00.00 ± 0.0	5.32 ± 3.3	5.17 ± 1.2	0.83 ± 0.5	0.83 ± 0.4	10.50 ± 4.2	12.50 ± 2.7	2.33 ± 1.2
	75	0.17 ± 0.2	00.00 ± 0.0	4.41 ± 2.1	4.32 ± 2.4	1.17 ± 0.4	0.17 ± 0.2	8.00 ± 3.0	12.67 ± 2.2	1.83 ± 0.6
	180	0.33 ± 0.3	00.00 ± 0.0	2.03 ± 1.7	2.13 ± 1.6	2.30 ± 0.8	0.00 ± 0.0	6.33 ± 1.4	10.67 ± 2.2	2.83 ± 1.8
RM (300 mg/kg)	15	2.00 ± 1.0	102.11 ± 43.2	3.33 ± 1.3	4.33 ± 1.5	0.88 ± 0.4	0.33 ± 0.3	4.33 ± 2.7	6.90 ± 2.3	1.45 ± 0.4
	75	3.17 ± 0.7	35.50 ± 10.1	1.88 ± 1.2	3.31 ± 1.3	0.00 ± 0.0	1.00 ± 0.5	5.00 ± 0.2	9.45 ± 3.7	5.90 ± 1.5
	180	3.67 ± 1.7	15.50 ± 4.3	7.33 ± 1.3	4.40 ± 1.4	0.00 ± 0.0	3.75 ± 0.5	4.89 ± 1.3	8.88 ± 2.8	8.00 ± 1.5
E. Longifolia (500 mg/kg)	15	1.67 ± 1.4	13.17 ± 2.2	3.40 ± 2.0	2.00 ± 1.2	1.88 ± 1.0	1.00 ± 0.8	4.33 ± 1.7	4.00 ± 1.4	6.17 ± 0.2
	75	2.17 ± 1.9	11.00 ± 2.3	4.22 ± 1.6	3.00 ± 1.0	1.15 ± 0.7	0.00 ± 0.0	2.17 ± 0.8	4.83 ± 1.8	5.17 ± 0.2
	180	2.50 ± 1.9	10.12 ± 2.1	4.83 ± 1.9	4.17 ± 1.7	1.83 ± 0.8	0.17 ± 0.2	3.33 ± 1.4	3.00 ± 1.8	7.67 ± 1.0
L-Dopa (100 mg/kg)	15	0.83 ± 0.7	129.00 ± 44.4	10.67 ± 3.7	11.00 ± 6.3	0.33 ± 0.2	2.47 ± 1.0	12.33 ± 5.5	7.33 ± 2.6	8.83 ± 0.5
	75	1.50 ± 0.7	64.33 ± 15.1	3.67 ± 1.4	10.17 ± 7.4	1.17 ± 0.6	1.87 ± 1.4	7.00 ± 0.2	10.50 ± 4.1	3.33 ± 1.2
	180	2.67 ± 1.2	42.00 ± 13.4	3.00 ± 1.6	8.33 ± 5.4	0.83 ± 0.5	0.83 ± 0.4	10.50 ± 4.2	12.50 ± 2.7	2.35 ± 1.2

Treatment	Receptive Female		Non-Receptive Female	
	Cumulative Time Spent (s)	Percentage (%)	Cumulative Time Spent (s)	Percentage (%)
Control (RO)	296.67	32.96	118.50	13.17
RM (300 mg/kg)	414.83	46.09	202.50	22.50
L-Dopa (100 mg/kg)	498.00	55.33	287.17	31.91
E. Longifolia (500 mg/kg)	36.17	8.56	377.83	33.89

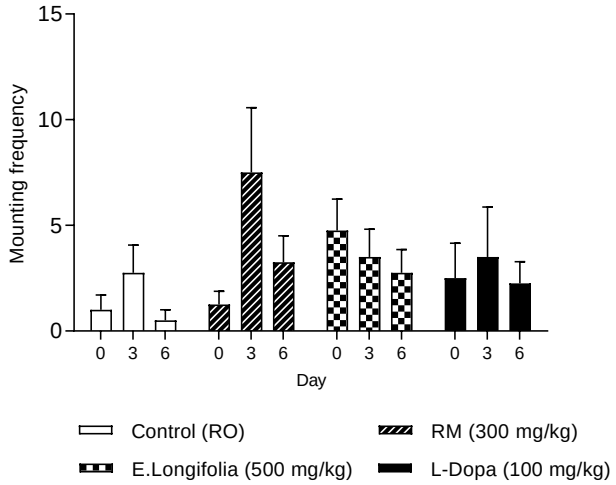
as a positive reference. The present study provides special evidence that the RM crude extract is a potent stimulator of sexual behaviour, particularly on sexual arousal in male rats.

Mount Frequency and Intromission Frequency are useful indices of vigour, libido and potency. While the number of mount (MF) reflects sexual motivation, an increase in the number of intromissions (IF) shows the efficiency of erection, penile orientation and the ease by which ejaculatory reflexes are activated [9]. Therefore, the increase in MF (Figure 4) and IF (Figure 5) following the administration of aqueous extract of RM crude extract at 300 mg/kg body weight on 0, 3rd and 6th day of observation suggests enhanced libido [22]. It is postulated that such enhancement of libido might have arisen from an increase in the number of concentrations of several anterior pituitary hormones and serum testosterone, which in turn stimulated dopamine receptor synthesis and sexual behaviour [23]. The result may therefore be logical to attribute these behaviours to flavonoid and or saponin constituents of the plant since they have been reported to alter androgen levels [24]. The alteration in the androgen level was supported by the increase in testosterone content of the animals in the present study. Furthermore, since intromission is not possible without adequate erection and coordinated activity of penile muscles [25]. the increase in IF by the extract in this study suggests that the mechanism of penile erection was activated. Therefore, aqueous extract of RM crude extract may increase potency by allowing or sustaining

erection. The increase in ejaculation frequency (EF) by RM crude extract on 0, 3rd and 6th day is an indication of the enhanced aphrodisiac effect of the plant (Figure 6). The presence of a plug in the vagina of the female rats indicated that ejaculation occurred. This was further complemented by the genital toileting observed in the male rats.

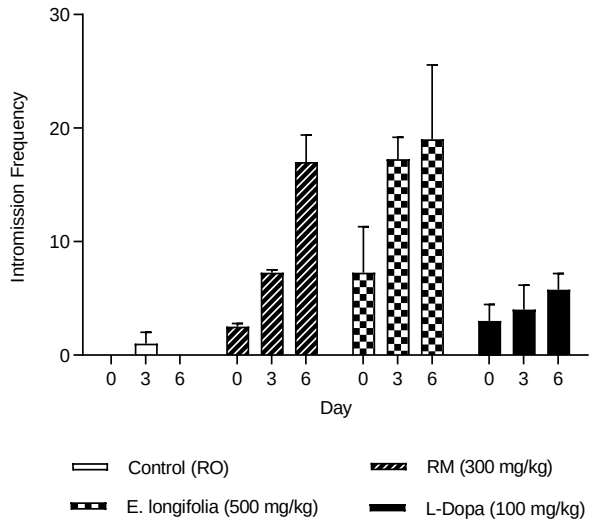
Mount latency (ML) and intromission latency (IL) are indicators of sexual motivation. ML and IL are inversely proportional to sexual motivation. Therefore, the decrease in the ML (Figure 7) and IL (Figure 8) observed in RM crude extract might imply stimulation of sexual motivation and arousability. It may also be an indication of enhanced sexual appetitive behaviour in male rats. RM crude extract treated rats showed a decrease in ejaculation latency (EL) on 0, 3rd and 6th day (Figure 9) but not significant as compared to standard groups (L-Dopa and *E. Longifolia*). This suggests that RM crude extract does not give an effect on increasing ejaculation latency, thus may not give effect in enhancing copulatory performance. The post-ejaculatory interval (PEI) is considered an index of potency, libido and the rate of recovery from exhaustion after the first series of mating and is important in measuring the rats' copulatory [22]. PEI of more than 5400 sec indicates that the male is sexually exhausted, and the intensity of sexual behaviour will be reduced in subsequent mating [9] Therefore, the significantly decreased PEI of RM crude extract on 0, 3rd and 6th day (Figure 10) may be attributed to enhanced potency and libido or less exhaustion in the first series of mating since the values of PEI obtained in this study are not up to the cut-off. The longest time taken for PEI was Control (RO) group which is up to cut-off time at 1800.0 ± 0.0 (s). Meanwhile, the shortest time taken for PEI was RM crude extract at day 6, which is 3 ± 9.6 (s).

In addition, the higher values of the computed male rat sexual behaviour parameters following treatment with the RM crude extract when compared with the control (RO) group are indications of significant and sustained increase in sexual activity.



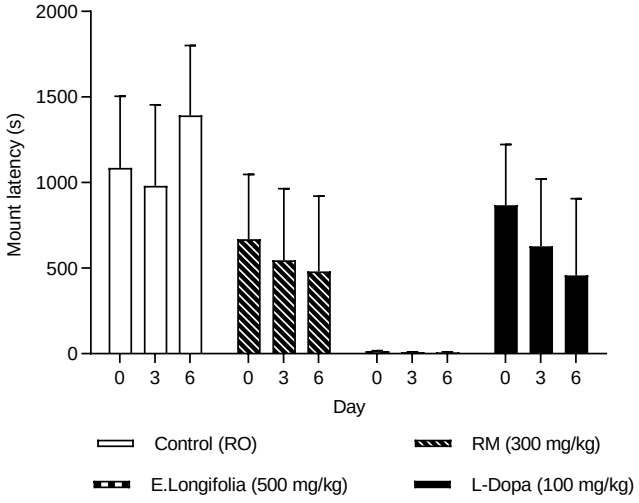
Group	Day		
	0	3	6
Control (RO)	1.0 ± 0.7	2.8 ± 1.3	0.5 ± 0.5
RM (300 mg/kg)	1.3 ± 0.6	7.5 ± 3.1	3.3 ± 1.3
E. Longifolia (500 mg/kg)	4.8 ± 1.5	3.5 ± 1.3	3.8 ± 1.1
L-Dopa (100 mg/kg)	2.5 ± 1.7	3.5 ± 2.4	4.3 ± 1.0*

Figure 4: Figure show frequency of mounting by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



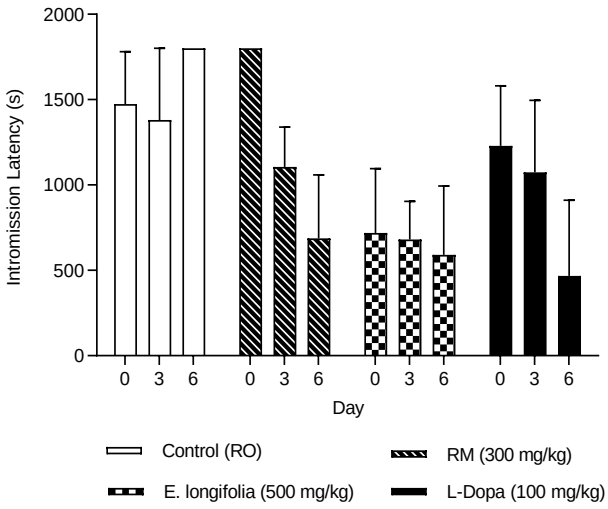
Group	Day		
	0	3	6
Control (RO)	0.81 ± 0.5	1.00 ± 1.0	0.00 ± 0.02
RM (300 mg/kg)	2.50 ± 0.6	7.30 ± 3.3	17.52 ± 8.5
E. Longifolia (500 mg/kg)	7.25 ± 4.1	17.25 ± 1.9	19.00 ± 6.6
L-Dopa (100 mg/kg)	3.00 ± 1.5	4.0 ± 2.2	5.75 ± 1.4

Figure 5: Figure shows frequency of intromissions by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



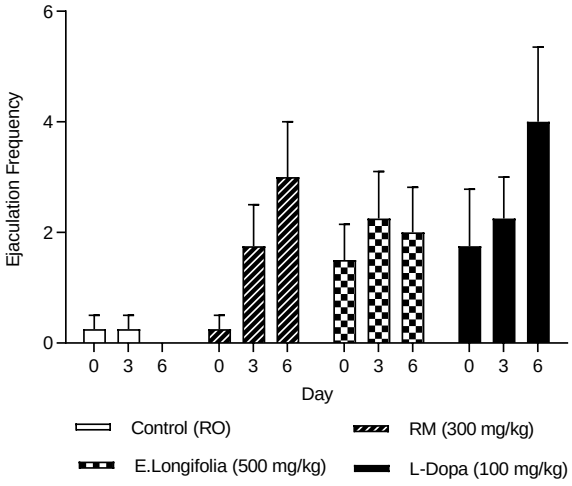
Group	Day		
	0	3	6
Control (RO)	1086.5 ± 417.5	980.0 ± 473.4	1392.5 ± 407.5
RM (300 mg/kg)	669.5 ± 378.5	545.3 ± 417.5	480.5 ± 440.4
E. Longifolia (500 mg/kg)	16.0 ± 1.7	8.5 ± 1.7	8.0 ± 2.2*
L-Dopa (100 mg/kg)	867.0 ± 355.7	627.0 ± 393.7	458.0 ± 447.3

Figure 6: Figure shows mount latency by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



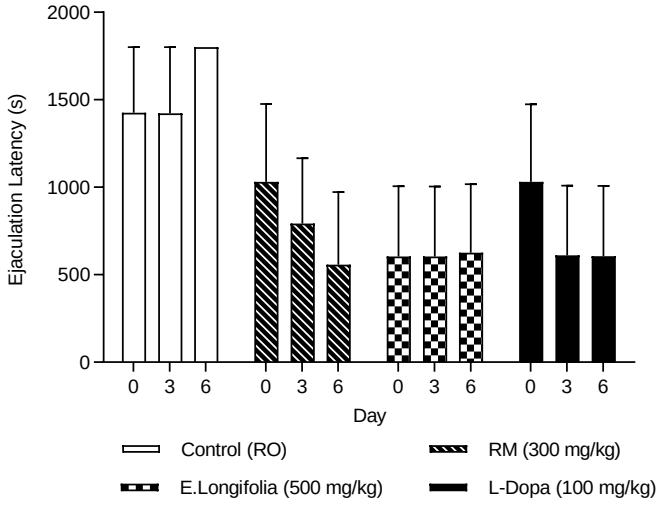
Group	Day		
	0	3	6
Control (RO)	1474.5 ± 305.8	1380.5 ± 419.5	1800.0 ± 0.0
RM (300 mg/kg)	1464.8 ± 335.3	699.3 ± 368.2	568.3 ± 414.2
E. Longifolia (500 mg/kg)	719.0 ± 377.3	680.5 ± 223.8	590.0 ± 403.5
L-Dopa (100 mg/kg)	1229.5 ± 351.5	1074.5 ± 421.4	467.0 ± 444.4*

Figure 7: Figure shows intromissions latency by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



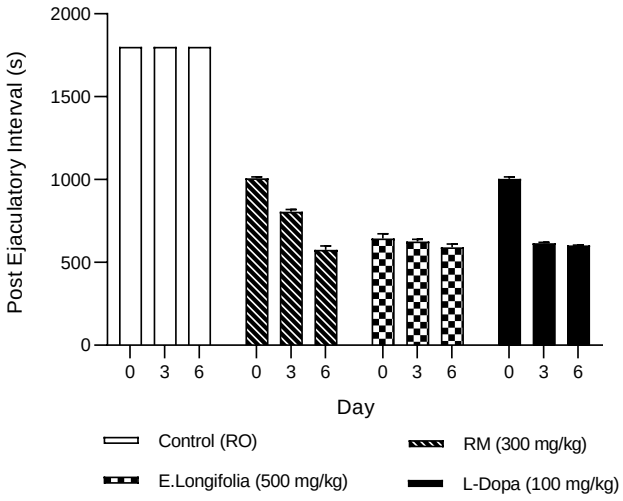
Group	Day		
	0	3	6
Control (RO)	0.25 ± 0.25	0.25 ± 0.25	0.00 ± 0.00
RM (300 mg/kg)	0.25 ± 0.25	1.75 ± 0.75	3.00 ± 1.00
E. Longifolia (500 mg/kg)	1.50 ± 0.65	2.25 ± 0.85	2.00 ± 0.82
L-Dopa (100 mg/kg)	1.75 ± 0.25	2.25 ± 0.75	4.00 ± 1.35

Figure 8: Figure shows the frequency of ejaculations by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



Group	Day		
	0	3	6
Control (RO)	1425.0 ± 375.0	1422.0 ± 378.0	1800.0 ± 0.0
RM (300 mg/kg)	1031.0 ± 443.9	792.5 ± 374.1	557.5 ± 414.4
E. Longifolia (500 mg/kg)	604.0 ± 401.2	603.5 ± 400.0	626.3 ± 391.3
L-Dopa (100 mg/kg)	1030.5 ± 444.7	610.5 ± 398.2	605.0 ± 402.7

Figure 9: Figure shows ejaculation latency by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**



Group	Day		
	0	3	6
Control (RO)	1800.0 ± 0.0	1800.0 ± 0.0	1800.0 ± 0.0
RM (300 mg/kg)	1008.0 ± 3.0	806.0 ± 5.4	576.3 ± 9.6
E. Longifolia (500 mg/kg)	644.1 ± 11.7	625.5 ± 5.5	591.6 ± 8.0
L-Dopa (100 mg/kg)	1003.6 ± 4.8	615.4 ± 2.0	602.0 ± 0.9

Figure 10: Figure shows post ejaculation latency by group, with n=6.
Figures are mean score ± SEM of the six rats in each group.
***P<0.05 indicates a statistically significant difference from the Control (RO) group.**

(iv) **Sperm count and mortality**

Further investigation was done to screen the RM crude extract's reproductive toxicity profile with *in-vitro* sperm count preservation method. Result shown in Table 5, RM crude extract has a reduction only by 9.7 % after 30 min. The sperm motility is observed to reduce from category 'a' at 0 min to category 'b' after 30 min. The non-treated group (sham) showed sperm reduction by 36.7 % after 30 min. The sperm motility showed a reduction from the category 'a' at 0 min to category 'b' after 30 min. The control group (RO) showed the highest reduction in sperm count by 43.6 % after 30 min. The sperm motility showed category 'c' at time 0 until after 30 min. Meanwhile, *E. Longifolia* showed the magnificent result of sperm motility 'a' even after 30 mins and a reduction of sperm

count by only 21.3 %. This finding tallied with previous findings, the water-soluble extract was able to increase the concentration of sperm, mobility rate and percentage of progressive sperm. The results showed that the water-soluble extract could increase the quantity and quality of the sperm and increase the fertility rate [26].

Preservation of *in-vitro* sperm count is an indicative that the RM crude extract contains water soluble nutritive substances that help sperms in avoiding death due to lack of energy. One the other hand the fructose content is also regulated by androgens and fructose content establishes a narrow relationship among the levels of testosterone and fructose production by the seminal vesicle [27].

(v) **Quantification of reproductive hormones**

On the last day of sexual behavioural studies, serum Testosterone (T), Luteinizing hormone (LH) and Follicle-Stimulating Hormone (FSH) level were determined for all groups (Table 6). Groups receiving RM crude extract produced a significant increase in testosterone concentration compared with the Control (RO) group ($p<0.001$). A significant increase ($p<0.001$) in serum testosterone level confirmed that the RM crude extract being studied is able to improve sexual function [8].

Administration of RM crude extract increased serum LH, FSH and T suggesting the stimulation of hypothalamic–pituitary–gonadal axis. The fundamental regulator of reproduction is controlled by GnRH which informs the hypothalamus and its release is influenced by different neurotransmitters. The components of the extract maintain the pulse episodes of GnRH, hence an elevated level of FSH and LH is observed. LH and FSH are called gonadotrophins because they stimulate the gonads, the testes in males. In the testes, LH binds to receptors on Leydig cells (LCs), stimulating the synthesis and secretion of testosterone. The increase in the concentration of LH significantly stimulates the synthesis and release of high levels of testosterone in the blood. This leads to assuming that some phytoconstituent present in the ethanolic extract may possibly mimic the function of LH to stimulate interstitial cells.

Testosterone is used to stimulate the cell of epididymis and seminal vesicles to activate and nourish spermatozoa in corresponding organs. The combination of FSH and T are qualitatively and quantitatively responsible for fully normal spermatogenesis [28]. These effects are likely to be mediated via the hypothalamic GnRH system. The synergistic effect of FSH and testosterone accelerates spermatogenesis so that a large number of spermatozoa are produced in the lumen of seminiferous tubule. The excessive number of Sertoli cells causes high production of nutrients in the cells to meet the requirements of nourishment to spermatozoa. Some constituents of the extract are instrumental in producing Sertoli cells which may be responsible for low production of inhibin resulting a in continuous inflow of FSH. As this situation remains consistent for a longer time, high levels of testosterone levels are observed. Increased testosterone level does not account for the persistent long sexual behaviour through activation of brain receptors. The possibility of any components activating the parasympathetic pathway in the brain cannot be ruled out.

Saponins and flavonoids in the aqueous extract of this plant might have assisted in stimulating increase in natural endogenous testosterone levels probably by raising the level of LH, which is translated into the male sexual competence observed. The steroidal nature of saponins may facilitate its role as an intermediary in the steroidal pathway of androgen production [29]. Saponins may bind to hormone receptors which may result in conformational change that will enhance the physiological function of the hormone or bind to enzymes that are involved in the synthesis of such hormones and thus enhance its production as observed.

Table 5: Effect of *in-vitro* sperm count preservation and motility in rats treated with mangrove extracts of RM. Figures are mean score ± SEM of the six rats in each group

Treatment (1 mg/mL)	Sperm Count (millions/mL)		Reduction (%)	Sperm Motility (a/b/c)	
	0 min	30 min		0 min	30 min
Non-treated	109.5 ± 12.93	69.3 ± 5.49	36.7	a	b
Control (RO)	66.5 ± 1.44	37.5 ± 3.22	43.6	c	c
RM	79.8 ± 1.37	72.4 ± 1.78	9.7	a	b
E. Longifolia	97.5 ± 4.21	76.7 ± 5.58	21.3	a	a

Motility category according to WHO, 1999 and Nwafia *et al.*, 2005:
'a' - rapid, progressive motility
'b' - slow or sluggish, progressive motility
'c' - non-progressive motility /immortally

Table 6: Serum reproductive hormones level in sexually experienced rats treated with RM crude extract. Each column represents the mean ± S.E.M for 6 animals; *p<0.05 significantly different from control, **p<0.01 significantly different from control, ***p<0.001 significantly different from control.

Treatment	Testosterone (ng/mL)	FSH (ng/mL)	LH (ng/mL)
Control (RO)	9.98 ± 0.55	1.20 ± 0.11	3.30 ± 0.96
RM (300 mg/kg)	19.58 ± 2.04***	2.32 ± 0.55	5.54± 0.42
L-Dopa (100 mg/kg)	9.28 ± 0.88	1.66 ± 0.73	5.62 ± 0.30
E. Longifolia (500 mg/kg)	9.55 ± 1.06	1.40 ± 0.15	3.38 ± 0.97

CONCLUSION

From the present investigation, we conclude that RM extract possesses potent aphrodisiac activity in normal male albino rats with adverse effects on sperm quality. This result is the scientific evidence it is clinically useful as a sexual invigorator in males. More studies are proposed to find out the exact mechanism of saponins and/or flavonoids and/or other phytoconstituents to validate and confirm the aphrodisiac activity.

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REFERENCES

1. A. Adhikari, M. Ray, T.K. Sur, S. Biswas, R.K. Roy, A.K. Hazra, A.K. Das, 2018. Anti-diabetic activity of *Rhizophora mucronata* leaves in streptozotocin-nicotinamide induced animal model. J Middle East North Afr Sci 4 (8): 1-7.
2. N. Suganthy, K. Pandima Devi, (2015). In vitro antioxidant and anti-cholinesterase activity of *Rhizophora mucronata*. Pharm. Biol. 1–12, http://dx.doi.org/10.3109/13880209.2015. 1017886.
3. G.E. Trease, & M.C. Evans (1989). Textbook of Pharmacognosy 13th Edition Bailiere Tindall, London, Toronto. Tokyo. Pgs, 200-201.Y.
4. J.B. Harborne (1973). Phytochemical methods Chapman and Hall. Ltd. London, 4, 49-188.
5. A. Sofowora (1993). Phytochemical Screening of Medicinal plants and traditional medicine in Africa, pp: 150–156.
6. E.D. OECD, (2010). Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests. OECD Series Test Assess, 20, 1-54.
7. S. Zamblee, V. Norain, & Y.K. Gupta, (2008). Evaluation of the aphrodisiac activity of *Tribulus terrestris* Linn. in sexually sluggish male albino rats. Journal of Pharmacology and Pharmacotherapeutics, 3(1), 43-47.
8. P. Zanoli, A. Benelli, M. Rivasi, C. Baraldi, F. Vezzalini, & M. Baraldi, (2003). Opposite effect of acute and subchronic treatments with *Ferula hermonis* on copulatory behaviour of male rats. International Journal of Impotence Research 15, 450– 455.
9. A. Ågmo, (1997). Male rat sexual behaviour. Brain Research Protocols, 1(2), 203-209.
10. A. Abedi, M. Parviz, S.M. Karimian, & H.R.S. Rodsari, (2013). Aphrodisiac activity of aqueous extract of *Phoenix dactylifera* pollen in male rats.
11. P. Eckstein, S. Zuckerman, A.S. Parkes, (1960). Marshall's physiology of reproduction. Part I Volume I, 127-9.
12. M. Thakur, & V.K. Dixit, (2007). Effect of some vajikaran herbs on pandiculation activities and in vitro sperm count in male. Sexuality and Disability, 25(4), 203-207.
13. World Health Organization (1999). Laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 4th, NY; Cambridge University Press.
14. W.C. Nwafia, J.C. Igweh, I.N. Udebuani, (2006): Semen analysis of infertile Igbo males in Enugu, Eastern Nigeria. Niger. J. Physiol. Sci. 21(1-2): 67-70
15. A.J. Harborne, (1998). Phytochemical methods a guide to modern techniques of plant analysis. springer science & business media.
16. S.A. Padashetty, & S.H. Mishra, (2007). Aphrodisiac studies of *Tricholepis glaberrima*. With supportive action from antioxidant enzymes. Pharmaceutical Biology, 45(7), 580-586.
17. P.L. Yu, H.L. Chao, S.W. Wang, P.S. Wang, 2009. Effects of evodiamine and rutaecarpine on the secretion of corticosterone by Zona *fasciculata–reticularis* cells in male rats. J Cell Biochem 108:469–475.
18. G. Repetto, A. Del Peso, & J.L Zurita, (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature protocols, 3(7), 1125-1131.
19. O.A. Salawu, B.A. Chindo, A.Y. Tijani, I.C. Obidike, T.A. Salawu, & A.J. Akingbasote, (2009). Acute and sub-acute toxicological evaluation of the methanolic stem bark extract of *Crossopteryx febrifuga* in rats. Afr. J. Pharm. and Pharmacol, 3(12), 621-626.
20. S. Both, W. Everaerd, E. Laan, & L. Gooren, (2005). Effect of a single dose of levodopa on sexual response in men and women. Neuropsychopharmacology, 30(1), 173-183.
21. H.H. Ang, & K.L. Lee, (2002). Effect of *Eurycoma longifolia* Jack on orientation activities in middle-aged male rats. Fundamental & clinical pharmacology, 16(6), 479-483.
22. A, Tajuddin. (2008). Aphrodisiac activity of 50% ethanolic extracts of *Myristica fragrans* Houtt.(nutmeg) and *Syzygium aromaticum* (L) Merr. & Perry.(clove) in male mice: a comparative study. BMC Complementary and alternative Medicine, 3(1), 1-5.
23. S.A. Taha, M.W. Islam, & A.M. Ageel, (1995). Effect of ambrein, a major constituent of ambergris, on masculine sexual behaviour in rats. Archives internationales de pharmacodynamie et de thérapie, 329(2), 283-294.
24. B.L. Hart & C.M. Haugen, (1968). Activation of sexual reflexes in male rats by spinal implantation of testosterone. Physiology & Behaviour, 3(5), 735-738.
25. J.M. Davidson, (1982). Sexology: Sexual biology, behaviour and therapy. In selected papers of Fifth World Congress of Sexology: 1981; Jerusalem Edited by: Zewi H. Excerpta Medica, Amesterdam-Princeton–Oxford, 42-47.
26. N.A. Wahab, N.M. Mokhtar, W.N. Halim, S. Das, (2010). The effect of Eurycoma Longifolia jack on spermatogenesis in estrogen-treated rats. Clinics, São Paulo 65(1), 93-98.

27. G.F. Gonzales, (1989). Functional structure and ultra-structure of seminal vesicles. Archives of andrology, 22(1), 1-13
28. J.B. Kerr, S. Maddocks, & R.M. Sharpe, (1992). Testosterone and FSH have independent, synergistic and stage-dependent effects upon spermatogenesis in the rat testis. Cell and tissue research, 268(1), 179-189.
29. S.A. Padashetty, & S.H. Mishra, (2007). Aphrodisiac studies of *Tricholepis glaberrima* with supportive action from antioxidant enzymes. Pharmaceutical Biology, 45(7), 580-586.