International Journal of Pharmacognosy and Pharmaceutical Research
www.pharmacognosyjournals.com
Online ISSN: 2664-7176, Print ISSN: 2664-7168
Received: 27-02-2022, Accepted: 12-03-2022, Published: 28-03-2022
Volume 4, Issue 1, 2022, Page No. 1-9

Effect of *Croton zambescicus* Muell Arg. (Euphorbiaceae) root extracts on aphrodisiac and fertility using Wistar rats and *Drosophila melanogaster* models

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Abstract
Globally, infertility affects approximately 15% of sexually active couples with male factors accounting for 20% to 50% of all cases. Most of the researches on infertility have generally been focused on women and the available chemotherapies exhibit many adverse effects. This study was aimed at investigating the male fertility enhancing activities of *Croton zambescicus* root.
Powdered sample of C. Zambescicus (CZ) root was macerated in methanol at room temperature for 72 hrs, the resulting extract (pilot methanol) was evaluated on Wistar rats for toxicity and male fertility enhancing potentials. The root powder was also successively macerated into n-hexane, dichloromethane, ethyl acetate and methanol, and the extracts were investigated using Wistar rats (sperm indices) and *Drosophila melanogaster* [survival study, mating latency (ML), copulation duration (CD), fly emergence / % fertility] models. Mesterolone (10 mg/kg) and Sildenafil (2 mg/kg) were used as standards for Wistar rats and Drosophila models respectively. All data were analysed using Student T-test and Graph pad prism 5.02, level of significance was placed at $p \leq 0.05$
Results showed that *Croton zambescicus* had LD_{50} of 1523.15 mg/kg bwt., survival study on successive extracts of CZ with drosophila showed that the extracts are safe above 400 mg/kg dose level. 200 mg/kg bwt. of CZ pilot methanol extract produced the most significant sperm count (117.40 ±7.28) when compared to the untreated group (83.27±3.51) and mesterolone (107 ± 5.08). All concentrations of ethyl acetate extract showed significant reduction in ML ($p \leq 0.05$ top ≤ 0.001) and better CD but 400mg/kg diet of ethyl acetate extract was the only significant concentration for copulation duration when compared to untreated group (1 ml of 0.1% Tween 80 per kg diet). Ethyl acetate extract showed highest % fertility increase (58.95± 4.03) in 400 mg/kg ($p \leq 0.001$) relative to negative control (0.00 ± 0.48). Sildenafil (positive control) had significant mating latency and copulation duration but a non-significant % increase (6.10 ± 2.31) in fertility
In this study, *Croton Zambesicus* root was proven to be effective in the regulation of fertility. However, the right choice of extracting solvent has to be made in using *Croton zambesicus* in the management of infertility.

Keywords: *Croton zambesicus,* ethyl acetate extract, *drosophila melanogaster,* mating latency, copulation duration, male fertility booster

Introduction
Croton zambesicus Muell Arg. (Euphorbiaceae) commonly known as *Koriba*or*Icen maser* in Hausa (Keay, 1989; Arbonnier, 2004; Usman *et al*., 2009) [12, 7, 29]. *Ajekobale* in Yoruba; *Mfam* in Ekoi (Agishi *et al* 1975) [4]; and *Moramora* in Kilba, belongs to the Euphorbiaceae family.
It is a shrub of 10-16m high, often branching low down with a spreading crown and characteristic hanging leaves. The leaf decoction is used in Benin for hypertensive, urinary infections fever (Watt and Maria, 1962) and infertility in men. It is also claimed to have reputation of conferring protection to ward off evil influences and commonly called *ajeko bale* (Yoruba) which means "witches do not dare to perch on it" (Adjanohoun *et al.,* 1989) [2].
The leaf extract has been reported to possess antiplasmodial (Okokon *et al.,* 2005a) [15], anti-inflammatory, analgesic and antipyretic activities (Okokon *et al.,* 2005b) [15] while its alkaloidal fraction has been reported for its wide spectrum of antibacterial property (Arbonnier 2004; Okokon *et al.,* 2004; Okokon *et al*., 2005) [7, 18, 15]. The root extract has been reported to possess antimalarial (Okokon *et al.,* 2009a) [19], anticonvulsant and antiulcer activities (Okokon *et al.,* 2009b) [19].
The present study was aimed at evaluating the male fertility boosting potentials of the root extracts of *C. zambesicus (Euphorbiaceae)* on Wistar rats and *Drosophila melanogaster* (Fruit fly) in order to justify or refute the claimed ethnomedicinal use in the treatment of male infertility.

Materials and Methods

Plant collection, authentication and preparation

Fresh roots and voucher specimen of *C. zambesicus* were collected from Moniya, Akinyele local government area, Ibadan, Oyo State, Nigeria in June, 2016 and identified at the Department of Pharmacognosy, University of Ibadan, by Mr Patrick Agu. It was authenticated at the Forestry Herbarium, Ibadan where voucher specimen (FHI 113099) was deposited. The roots were washed with clean tap water and dried at 45˚c in an oven. The dried plant sample was crushed to coarse powder using a 5 KVA electronic miller and kept in an air tight container prior to extraction.

Extraction procedure

One kilogram (1kg) *C. zambesicus* root powder was macerated for 72 h. in methanol. It was filtered and the resulting filtrate was concentrated *in vacou* at 30^0c to obtain 2.71% w/w. The resulting methanol extract (pilot methanol) was used for acute toxicity study and pilot fertility assay. Another batch of the root powder (4.0 kg) was subjected to successive extraction by maceration in n-hexane, dichloromethane, ethylacetate and methanol for 72 hours, respectively. Each filtered extract was separately concentrated *in vacuo* to give n-hexane, dichloromethane, ethyl acetate and methanol extracts which were also used for bioassay.

Toxicity study and fertility study of *C. zambesicus* root methanol extract on male Wistar rats

Thirteen rats weighing 120 - 140g were used for the determination of acute toxicity (1st and 2nd phase of Lorkes, 1983 method (Lorkes, 1983).

Another batch of thirty rats was employed for the evaluation of liver and kidney function, and fertility study. The animals were divided into 5 groups consisting of 6 animals each. Groups 1-3 were administered methanol extract of *C. zambescicus* root orally at 200, 400 and 600 mg/kg. Mesterolone (10mg/kg) (Group IV) and 0.1% Tween 80 (1 mL/kg) (Group V) served as positive and negative control respectively. The administration was carried out 9.00 am daily for 14 days. Tween 80 (0.1%) was used to dissolve the extract to enhance solubility and this necessitated its use as the negative control.

Fertility study of *C. zambesicus* root (CZ) successive extracts on male Wistar rats

The animals were divided into 14 groups of 6 animals each as follows and administered accordingly for 14 days.

Group 1 received 1 ml/kg of 0.1% Tween 80

Groups 2 received 10 mg/kg of mesterolone

Groups 3-5 received 100, 200 and 400mg/kg, *C. zambescicus* root n-hexane extract, respectively.

Groups 6-8 received 100, 200 and 400mg/kg *C.zambescicus* root DCM extract, respectively.

Groups 9-11 received 100, 200 and 400mg/kg *C. zambescicus* root EtOAc extract, respectively

Groups 12-14 received 100, 200 and 400mg/kg *C. zambescicus* root MeOH extract, respectively.

Semen collection

At the end of the treatment period, the rats were dissected to harvest the right epididymis and the caput lancerated on a glass slide using a warm (27ºC) sterile lancet to release the semen (Oyeyemi *et al*., 2000). The semen was examined for sperm motility, count and abnormalities. The testis, kidney and liver were also harvested and weighed.

Sperm motility

Drops of normal saline and eosin stain were added to effect full motility of the spermatozoa. Average gross motility was scored under the microscope x 40 objectives, **%** life-death ratio and caudal epididymis volume were determined (Aweda *et al*., 2010) [9].

Sperm count

The sperm concentration was examined using Neubauer Haemocytometer. (Aweda *et al*., 2010; Osuchukwu *et al*., 2016) [9, 21]

Sperm abnormalities

Different types of abnormalities found in the sperm cells were analysed using Smith *et al*., (1977) [26] method. Two drops of a vital stain eosin-negrosin was added to the semen sample on the slide mixed together and a semen smear prepared on a new clean glass slide. The slide was then scored for such abnormalities as rudimentary tail, bent tail, curved tail, coil tail, tailless head, and headless tail. The scoring was done under x 100 magnification using Olympus microscope.

Hormonal assay

Blood was collected through orbital sinus of the wistar rats using capillary tube. The blood was thereafter centrifuged, the serum was separated and used for hormonal assay (ELISA, England) according to the manufacturer's instructions.

Drosophila collection and preparation

Adult *Drosophila melanogaster* were obtained from Department of Biochemistry, *Drosophila* laboratory, University of Ibadan, Nigeria in 2018. The flies were transported in a controlled environment of 15 to 25^0c to Madonna University where they were bred at the Pharmacognosy laboratory extension, Madonna University, Elele, Rivers State. They were fed with freshly prepared Corn agar media using the procedure of Guruprasad *et al.,* 2010 with some modifications. After 8.5 days of laying eggs on the diet or feed, the eggs metarmephosized into adult fruit flies. Breeding of *Drosophila* continues until the required number of flies for the experiment was obtained

Survival study on successive extracts of *C. zambescicus* using Drosophila

Fourteen groups of thirty five (35) flies each of both sexes from the population were counted into treatment vials and employed for the study. The groups were categorized as follows:

Group 1-3 received 100, 200 and 400 mg/kg *C. zambescicus* root n-hexane extract, respectively in addition to diet.

Groups 4-6 received 100, 200 and 400 mg/kg, *C. zambescicus* root DCM extract, respectively in addition to diet.

Groups 7-9 received 100, 200 and 400mg/kg *C. zambescicus* root EtOAc extract, respectively in addition to diet.

Groups 10-12 received 100, 200 and 400 mg/kg *C. zambescicus* root MeOH extract, respectively in addition to diet.

Groups 13 – untreated group; received diet only (negative control).

Groups 14 received Sildenafil citrate (positive control).

Each group was carried out in five replicates. The flies were carefully transferred into a freshly prepared diet containing the corresponding extracts at intervals of four days. The same was applicable to the untreated group only that it was not fed with the extracts. The number of dead flies were counted and recorded daily for 28 days. Any death occurrence at day zero was regarded as death due to handling while deaths from day one to the end of the study were referred to death due to tested extract(s). The percentage surviving flies was determined based on initial number (35).

Effect of C. *zambesicus* root extracts (CZ) on mating latency, copulation duration and fly emergence of *D. melanogaster*

The procedure of Suchira and Shakunthala (2014) [27] was followed with some modifications. Both sexes were aged for 3 days during which the following procedures were undertaken. The flies were separated into bachelor males and virgin females before the neurons for mating were activated, which is usually 8-10 hours after emergence. The males were starved for 8 hours then divided into groups of 5 each. They were fed for 64 hours with different concentrations of *C. zambesicus* root extracts (100, 200 and 400mg/kg diet) of n- hexane, dichloromethane, ethyl acetate and methanol extracts. The females were also divided into groups of five each but placed on fresh diet (without extracts). After treatment of the male flies for 64 hours with the extracts and both sexes were 3 days old, a pair of both sexes was kept in a mating chamber for maximum of 1 hour to observe their courtship behaviour, mating latency and copulation duration. After copulation, male and female fruit flies were removed from the mating chamber and kept on fresh feeds (without extract) for them to lay eggs for 24 hours. At 24 hours interval, they were transferred into new vials containing freshly prepared diet, this was done for a period of five days. All the vials in which the flies have been transferred within the period of the five days were not discarded but left for observation so as to enable the eggs laid metarmorphosized in to adult flies. The newly emerged flies were counted and compared to untreated group to determine percentage fertility. Sildenafil citrate (2 mg/kg) was used as positive control

Data analysis

Data were analysed using one-way analysis of variance (ANOVA) employing GraphPad Prism version 5.02 for Windows (Graph pad software, San Diego California, USA, www.graphpad.com) at $P < 0.05$. Dunnet multiple comparison test at 95% Confidence Interval of difference was considered as significant.

Results and Discussions

The LD_{50} of CZ root was found to be 1523.15 mg/kg. The various extracts of CZ showed no significant effect on the body (Table 1 and 2) and organ (Fig 1) weights of the animals at the tested doses when compared to the negative control. This implies that the extracts might not be hazardous to the animals at the concentration used (Oyeyipo *et al*., 2010) [23] unlike Mesterolone which elicited a reduction in organ weights as shown in Fig 1. Also, all the biochemical indices were normal, the concentrations of Aspartate aminotransferase, Alanine aminotransferase, Alkaline phosphate, Bilirubin urea nitrogen and Creatinine were not significantly different from the control group indicating that the extracts at the tested doses did not have any adverse effect on the liver and the kidney (Table 3). These biochemical indices, if altered, will impair the normal functioning of the organs (Afolayan and Yakubu, 2009; Appidi *et al*., 2019) [3].

The pilot methanol extract showed a significant increase in the percentage sperm motility at 200 mg/kg (Fig 2) but did not affect live / dead ratio whereas there was no significant difference in sperm motility of the successive extracts (Fig 3). A significant increase in sperm motility is an indication of beneficial effect to the spermatozoa and will enable easy transport of sperm cells into the ovule (Oladeinde *et al*., 2007) [20]. A significant 41%

increase in sperm count was elicited by pilot methanol extract (200 mg/kg) compared to the negative control (0.1 % Tween 80) and this was higher than that obtained for the positive (29%) control (10 mg/kg Mesterolone). A significant increase in testosterone level was also observed at all concentrations of the pilot methanol extract with the highest percentage increase of 254% in 600 mg/kg which was higher than 10 mg/kg Mesterolone (190%) as represented in Table 4. The extract showed no abnormalities to spermatozoa (Fig 4). In the successive extracts, significant increase in sperm count was recorded for DCM 200 mg/kg, ethyl acetate 100 mg/kg, 100 and 200 mg/kg of methanol extracts. Methanol extract (100 mg/kg) showed a 24.1% increase followed by 18.8% increase in ethyl acetate (100 mg/kg) while there was high level of subacute lethality in the groups treated with 400 mg/kg of ethyl acetate extract. The fertility boosting activity was seen to be largely retained in the polar extracts as indicated in Table 5.

The life span of a fruit fly is about 50 days and the survival test is a means of investigating the level of toxicity of the test substance as indicated by Taylor *et al.,* 2013. However, the survival study showed no significant difference between the control (untreated group) and the groups treated with different concentrations of the extract of *C. zambesicus* root (Fig 5a and 5b). This means that the extracts are not toxic to the flies at the concentration used (Mathew and Krishnamurthy, 2018) [14]. In Drosophila species, successful mating depends on male activity and female receptivity, and courtship/mating latency is one of the parameter which indicates vigor of male. It represents the time between introduction of male and female flies into observation chamber and initiation of mating. A male with high vigor reacts quickly in the presence of female while a male with less vigor, reacts slowly (Taylor *et al.,* 2013) [28]. The mating latency and copulation duration here indicate the aphrodisiac property of *Croton zambesicus* (Fig 6), it was observed that Sidenafil citrate, all concentration of ethyl acetate extract and 400mg/kg diet of methanol extract showed significant reduction in mating latency ($p \leq 0.05$ top≤ 0.001) and prolonged copulation duration but 400mg/kg diet of ethyl acetate extract was the only significant concentration for copulation duration when compared to untreated group (0 mg/kg diet). Decrease in mating latency means increase in the vigor of male (Singh *et al.,* 2014), the present study showed that ethyl acetate extract had increased copulation duration in Drosophila. Extended duration of copulation allows enough time for the male to introduce more sperms into the female flies (Pankaj *et al*., 2011) [24].

In Drosophila, successful mating depends on male activity and female receptivity. Courtship latency is one of the parameter, which indicates vigor of male. It represents the time between introduction of male and female flies into observation chamber and initiation of courtship. A male with high vigor reacts quickly in the presence of female while a male with less In Drosophila, successful mating depends on male activity and female receptivity. Courtship latency is one of the parameter, which indicates vigor of male. It represents the time between introduction of male and female flies into observation chamber and initiation of courtship. A male with high vigor reacts quickly in the presence of female while a male with less Eastwood, L. and Burnet, B. 1977. Courtship latency in male D. malenogaster. Behav Genet., 7: 359–372. The fly emergence study was used to evaluate the fertility enhancing property of successive extracts of *Croton zambesicus* root. The results obtained revealed that all concentration of ethyl acetate produced significant percentage increase in fertility having highest in 200 mg/kg of ethyl acetate extract (55.4% ± 8.3) at $p \leq 0.001$ as compared to (control) untreated group (0.0 ± 4.8). Sildenafil citrate (2 mg/kg) had a significant aphrodisiac effect but a non significant % fertility increase (6.1 ± 8.4) as represented in Fig 7. This study indicates that in test groups, increased fly emergence account for the fact that the flies are under the influence of the extracts.

C. Zambesicus root ethyl acetate extract was proven to be effective in boosting fertility. This study justifies its ethnomedicinal claim in the treatment of male infertility by traditional healers.

Conclusion

The use of *Croton zambesicus* root in the management of infertility was justified and since it is relatively nontoxic, it can serve as a lead to discovery of new drug in the treatment of male fertility although further studies will be required to isolate the chemical constituents responsible for this activity.

Table 1: Effect of pilot methanol extracts (PME) of, *C. zambesicus* root, on body weight of wistar rats per week

WEEKS	Distilled water (0.9ml/kg))	10mg/kg mesterolone	Extract (200mg/kg)	Extract (400mg/kg)	Extract (600mg/kg)
0	141.2±5.46	145.6 ± 3.2	151.45 ± 4.67	148.83± 6.51	137.41± 2.34
1	170.8±3.82	160.4 ± 3.6	173.71 ± 3.12	186.23± 3.71	169.48± 3.27
2	185.4±5.1	156.4±1.76	191.24± 4.72	189.62±6.08	180.82± 4.43

Data are expressed as Mean ± SEM (n=6)

Table 2: Effect of successive extracts of *C. zambescicus* on body weight of treated rats

Grps/ wks	0	1	2
Dist water	128.4±4.5	146.5±6.3	167.8±5.1
Mest 10 mg/kg	126.6±6.5	141.3±8.3	159.2±4.7
N-H 100	134.1±2.9	156.3±3.8	162.9±2.6
N-H 200	127.0±3.7	139.2±4.9	151.7±6.2

N-H 400	135.0±6.7	139.6±3.7	146.2±9.4
DCM 100	127.5±3.9	136.1±9.2	158.8±4.9
DCM 200	126.0±8.5	144.9±3.6	149.2±5.3
DCM 400	121.3±9.3	142.8±3.4	155.2±8.8
EtOAc 100	122.5±7.2	136.0±4.4	152.1±4.9
EtOAc 200	127.5±2.7	139.5±5.8	151.4±4.9
EtOAc 400	139.0±6.2	146.5±8.5	156.9±5.8
MeOH 100	130.0±7.1	142.1±4.8	154.3±3.9
MeOH 200	129.5±2.9	143.8±7.7	155.0±4.3
MeOH 400	129.5±8.1	148.9±6.2	154.0±9.6

Data expressed as Mean ± SEM (n=6)

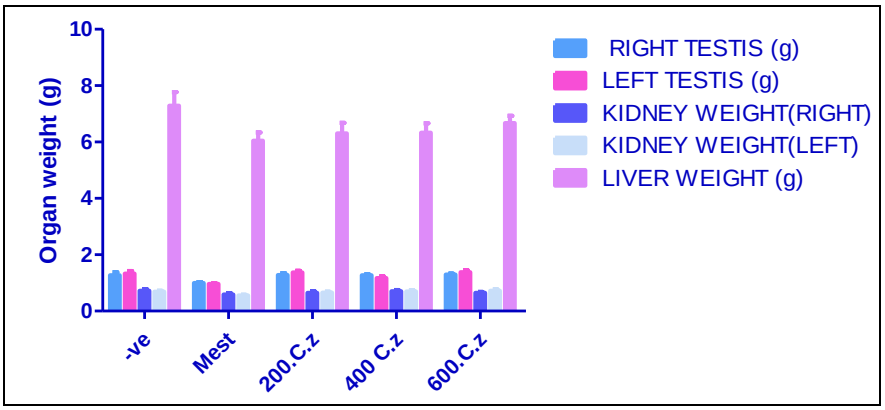

Fig 1: Effects of C. zambesicus pilot methanol root extract on organ weight of animals after 14 days of administration.

Table 3: Effects of pilot methanol extracts of C. *zambesicus* on liver and kidney parameters of the treated animals

Liver/kidney function test (IU/L)	**Distilled water**	**Mesterolone 10 mg/kg**	**MeOH 200 mg/kg**	**MeOH 400 mg/kg**	**MeOH 600 mg/kg**
AST	37.7±0.7	40.7±0.3	40.0±1.2	37.7±2.2	38.0±1.5
ALT	28.7±0.3	30.0±0.0	30.3±0.7	29.3±1.5	29.0±1.0
ALP	116.6±1.0	119.3±0.7	110.3±5.5	120.7±1.8	115.0±0.6
BUN	11.1±0.1	14.0±0.1	11.9±0.5	11.2±0.7	11.7±0.2
CREATININE	0.8±0.0	1.0 ±0.0	0.8±0.1	0.7±0.2	0.6±0.1

Data expressed as Mean ± SEM (n=6)

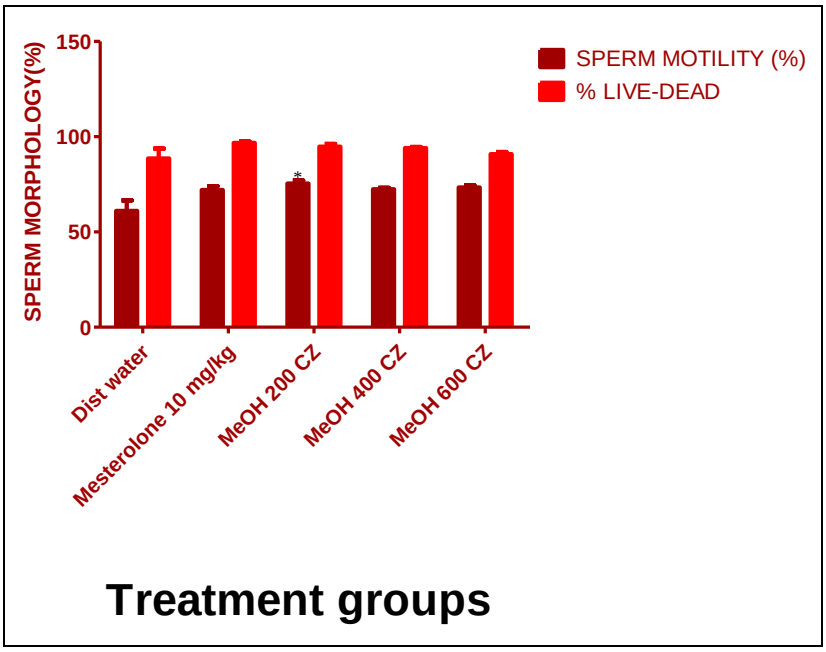

Fig 2: Effect of *C. zambescicus* pilot methanol root extracts on Sperm morphology of animals after 14 days administration. Data expressed as Mean ± SEM (n=6), * = $p \leq 0.05$

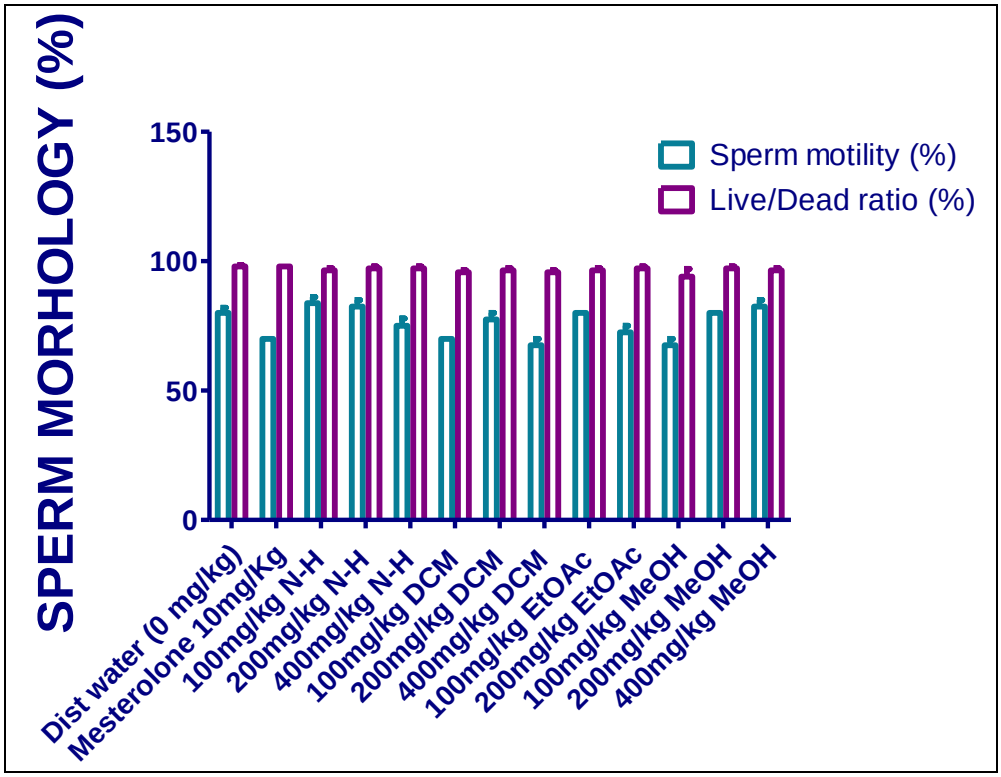

Fig 3: Effect of *C. zambesicus* root extracts on sperm morphology, * = p ≤ 0.05, N-H: Normal hexane, DCM: Dichloromethane, EtOAc: Ethyl acetate, MeOH: methanol. Data expressed as Mean ± SEM (n=6)

Table 4: Effect of *C. zambesicus* root pilot methanol extract on sperm indices and testosterone level of treated animals.

Treatment/Parameters	Sperm Count (10^6/ml)	Sperm volume (ml)	Testosterone(IU/ml)
Dist water + 0.1ml	83.27±3.51	0.18±0.02	0.39 ± 0.03
Mest 10mg/kg	107.00 ± 5.08* (29%)	0.16±0.02	1.13 ± 0.12*** (190%)
200 mg/kg CZ	117.40 ±7.28** (41%)	0.20±0.00	0.67 ± 0.23*** (72%)
400 mg/kg CZ	104.40 ± 8.61 (25%)	0.18±0.02	0.61 ± 0.71*** (56%)
600 mg/kg CZ	106.42 ± 8.47* (28%)	0.16±0.02	1.38 ± 0.83*** (254%)

Data expressed as Mean ± SEM (n=6), *P≤0.05, **P≤0.01, *** P≤ 0.001, Mest: mesterolone

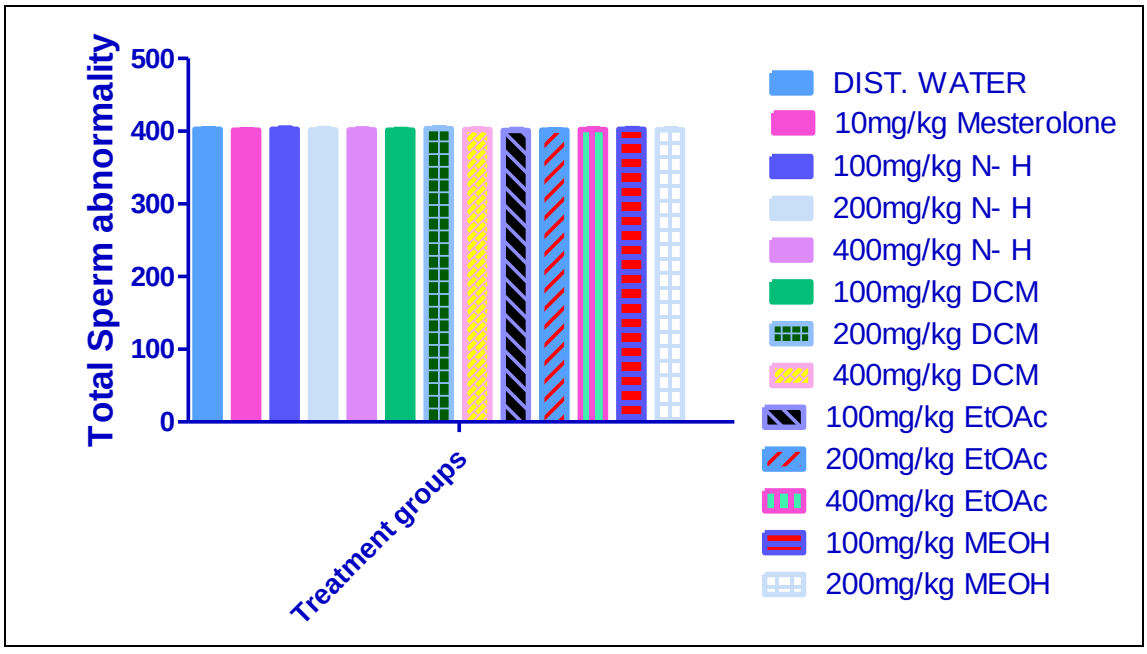

Fig 4: Effects of *C. zambesicus* root successive extracts on Total sperm abnormalities. N-H: normal hexane, DCM: dichloromethane, EtOAc: ethyl acetate, MeOH: methanol. Data expressed as Mean ± SEM (n=6)

Table 5: Effect of *C. zambesicus* root successive extracts on Sperm count of wistar rats. N-H: Normal hexane, DCM: Dichloromethane, EtOAc: Ethyl acetate, MeOH: methanol, Mest: mesterolone, CZ: *Croton zambescicus.* Data expressed as Mean ± SEM (n=6), * = $p \leq 0.05$

Treatment Groups	Sperm count (10^6/mL)	% fertility increase relative to control
Distilled water (control) 5ml/kg	97.5 ± 3.3	-
10mg/kg Mest.	108.0 ± 3.6	10.8
100 N-H CZ	111.5±1.7	14.4
200 N-H CZ	105.8±3.0	8.5
400 N-H CZ	111.3±3.4	14.2
100 DCM CZ	108.3±4.0	11.1
200 DCM CZ	115.0±4.1*	17.6
400 DCM CZ	104.5±4.6	7.2
100 EtOAc CZ	115.8±3.7*	18.8
200 EtOAc CZ	112.5±4.4	15.4
100 MeOH CZ	121.0±2.7*	24.1
200 MeOH CZ	115.3±2.5*	18.3
400 MeOH CZ	114.0±3.1	16.9

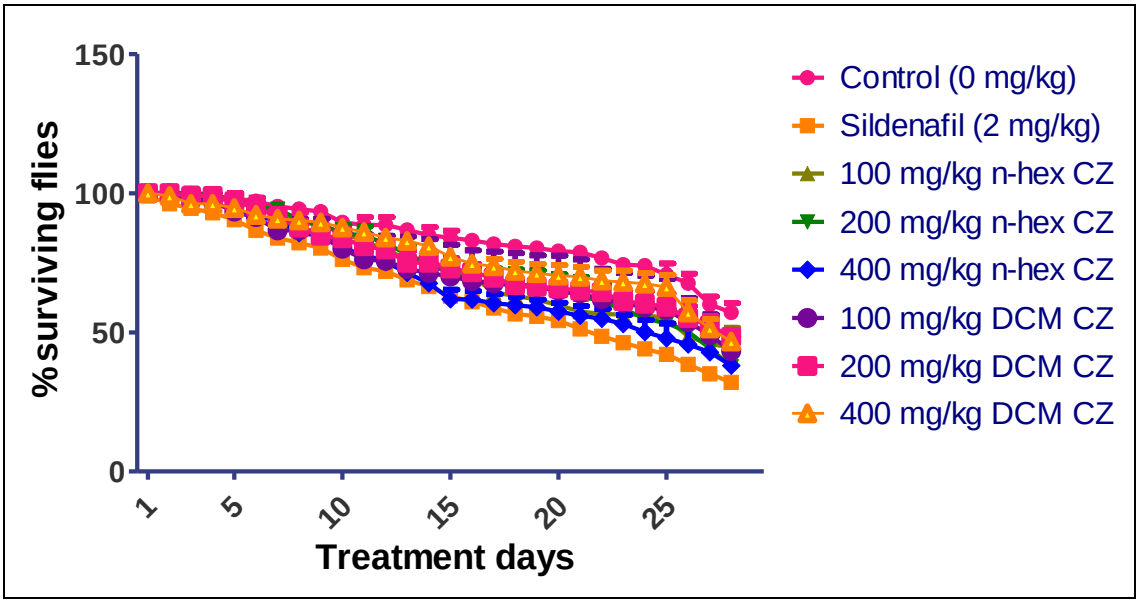

Fig 5a: Effect of *C.zambescicus* root extracts on 28 days survival study of *D. melanogaster*. N-hex: normal hexane, DCM: dichloromethane, EtOAc: ethyl acetate

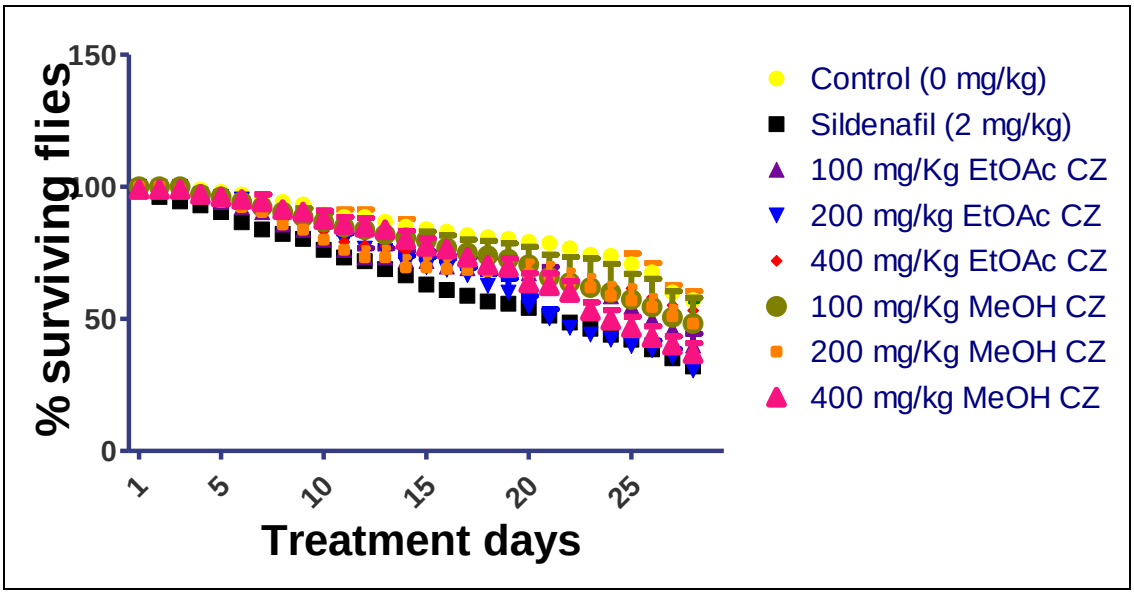

Fig 5b: Effect of *C.zambescicus* root extracts on 28 days survival study of *D. melanogaster*. N-hex: normal hexane, DCM: dichloromethane, EtOAc: ethyl acetate

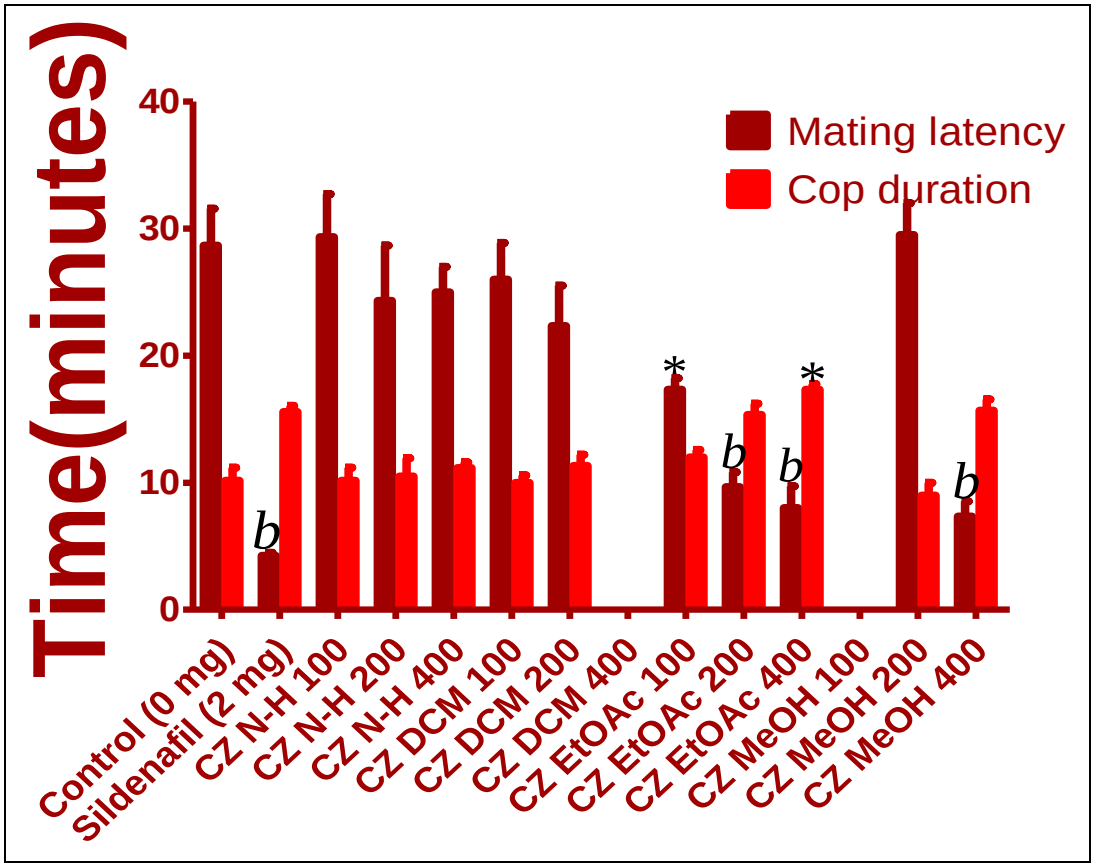

Fig 6: Effect of *C. zambescicus* root successive extracts on mating latency and copulation duration of *D melanogaster* (fruit flies), N-H = n-hexane, DCM = dichloromethane, EtOAc = ethyl acetate, MeOH = methanol. * = $p \leq 0.05$, b = $p \leq 0.001$

Fig 7: Effect of *C. zambescicus* root extracts on fertility using *D. melanogaster* (fruit flies) emergence. N-H= n-hexane, DCM = dichloromethane, EtOAc = ethyl acetate, MeOH = methanol, a = $P \leq 0.01$, b = $p \leq 0.001$ malenogaster. Behav Genet., 7: 359–372.

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