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Methanol extracts of *Vernonia amygdalina* Del increase the sex ratio of offspring in *Rattus norvegicus* rats

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ABSTRACT

Several sex selection methods are available but with varying success rates, availability, and affordability. *Vernonia amygdalina* has been used by some Kenyan communities for the male child pre-selection at conception but these claims have not been scientifically ascertained. This study aims at evaluating the ability of this herbal plant to pre-select for the male child at conception. The roots of the plant were collected and processed before extraction using methanol. The obtained crude plant extracts were orally administered to *Rattus norvegicus* rats before and during mating for one week. Crude extracts at doses of 50, 87, and 150 mg/kg body weight were used to evaluate the effects of the plant on the sex ratio of pups and factors related to sex ratio. A P value of $P<0.05$ was set as the limit of significance. The extracts were also subjected to quantitative phytochemical and mineral analysis. The results indicated that treating female rats with the plant extract significantly increased the ratio of male to female pups in all the treatment groups. There was a significant decrease in serum cortisol levels in both male and female rats and an increase in vaginal pH in female rats. The 50 and 87 mg/kg doses caused a significant increase in estrogenic levels compared to the untreated group. These results validated the use of the plant by herbalists for the preselection of a male child at conception.

Keywords: Sex Selection, Sex Ratio, *Vernonia Amygdalina*, Vaginal pH, Estrogen, Cortisol.

INTRODUCTION

Vernonia amygdalina belonging to the daisy family is a shrub that grows to a height of 2-5 m and is commonly known as the bitter leaf. It has elliptical leaves that have a maximum length of about 20 cm [1]. It is among the most popular medicinal plants belonging to the genus *Vernonia*. Information obtained from traditional herbalists shows that the plant has also been used to select for male offspring in different communities in Kenya such as the Maasai and the Samburu but no scientific evidence has been provided for these claims. Traditionally, the roots of the plant have been used by Kenyans and South Africans to treat infertility [2]. Scientific studies by Kamatenesi-Mugisha *et al* [3] have shown that the roots of *V. amygdalina* increased uterine motility and milk production while Steen-Kamp [2] described potential infertility remedies. In Nigeria, females from the Hausa tribe consume leaves of the plant claiming that it makes them more sexually attractive. It has also been used in horses as a diet supplement to induce fattening and increase strength by the same community. Besides this, many herbalists have recommended it as a laxative for the treatment of infertility as well as sexually transmitted diseases [4]. A study conducted in rats showed that administration of saponins from *V. amygdalina* resulted in the elevation of sperm cell motility as well as sperm count but the percentage of abnormal spermatozoa was not significantly reduced [5] and therefore, are recommended for treatment of male infertility. Dietary supplements of *V. amygdalina* were observed to significantly increase fertility in African catfish (*H. dorsalis*) where the egg size, fertilization percentage, milt volume, motility, and count were significantly elevated in the bloodstock group that received 100 g/kg bitter leaf supplement [6].

Sex selection can be viewed as any effort that tries to determine the gender of the offspring to be born [7]. Once the sperm cells are mature, ejaculated, and deposited into the female reproductive tract, they need to move across a long-distance via the uterus to the oviduct where fertilization takes place. Various factors are involved in maintaining sperm motility during this journey. These include sperm morphology, molecular signaling, glucose metabolic switch between glycolysis and oxidative phosphorylation, protein phosphorylation, enzymatic and ionic factors among others [8-10]. Differences in DNA content of the X and Y sperm result in differential gene expression which results in differential response to factors affecting sperm motility [11]. These differences in the X and Y sperm eventually lead to a shift in the ratio of X: Y sperm cells that reach the ovulated oocyte.

Once the sperm cell reaches the oocyte in the oviduct, there is a need to successfully bind and penetrate the oocyte membrane for successful fertilization. Functional differences between the Y-chromosome-bearing sperm and X-chromosome-bearing sperm in the ability to recognize the oocyte or undergo the acrosomal reaction with the zona pellucida of the oocyte would result in a skewed sex ratio. On the other

hand, selective susceptibility of the oocyte to interaction and penetration by X- versus Y-chromosome-bearing sperm would also result in a change in sex ratio [12,13]. Each of these step's functions to determine the primary sex ratio of offspring.

Once fertilization has successfully occurred, the rate at which male and female embryos undergo cleavage events can vary due to differences in gene expression during spermatogenesis [14]. Differential survival of the male and female embryos and implantation in the uterine lining may also lead to significant changes in sex ratios of offspring [15-17]. Finally, successful survival through embryonic and fetal development represents the final stage during which secondary sex ratios may be altered. Sex-specific mortality and miscarriages would skew the numbers of males and females at parturition [18].

This study is therefore aimed at evaluating the potential of methanol extracts of *V. amygdalina* to pre-select for the male child at conception by determining its effects on maternally and paternally driven factors of sex selection including vaginal pH, sperm count, sperm morphology, serum estrogen, testosterone, and cortisol levels as well as determining its phytochemical and mineral composition.

MATERIALS AND METHODS

Study site

This study was carried out at the laboratory of the Department of Biochemistry, Microbiology, and Biotechnology, School of Pure and Applied Science, Kenyatta University.

Plant Materials

Collection and preparation of plant extract

The fresh plant roots of *Vernonia amygdalina* were obtained from their natural environments in the Molo sub-county, Kenya. This was based on ethnobotanical information provided by the local herbalists. The plants were then identified by a qualified Taxonomist in the Department of Pharmacognosy and Pharmaceutical Chemistry Herbarium of Kenyatta University where voucher specimens were stored for future reference. The identified plant material was then left to dry in a dark room with an average temperature of 25 degrees Celsius for 28 days before grinding them into a fine powder using an electric grinding miller.

Extraction

Extraction was done repeatedly by soaking 4.5 kg of *V. amygdalina* roots powder in analytical grade methanol (Sigma-Aldrich) for 2 hr., 6 hrs., and 24 hours in a clean conical flask while shaking was done constantly by using an orbital shaker KS130 IKA™ at 120 rpm at room temperature. After each time interval, the extracts were decanted and filtered using a Whatman No. 1 paper before concentration using a rotary evaporator at 56°C. The resulting gum-like crude extract was stored in a -20°C freezer until the day of use.

Experimental animals

Adult *Rattus norvegicus* rats weighing 200-300 g obtained from Kenyatta University, Department of Biochemistry, Microbiology & Biotechnology`s animals breeding facility were used for the study. The animal handling and experimental procedures were according to The Guide for the Care and Use of Laboratory Animals by the National Research Council. The animals were kept under standard conditions and fed appropriately according to the guidelines established for the care and use of laboratory animals [19].

Experimental design

Determination of the effects of V. amygdalina on sex ratio assay

One hundred and twenty rats were randomly allocated into four different experimental groups each having 10 female and 5 male rats for the study. The animal house's environmental conditions were maintained at 22-26 Degrees Celsius, 12 hours of light/dark cycle, and a Relative Humidity of 60 ± 10%. Each standard polypropylene cage contained three rats (two females and one male) to allow mating for four days before the separation of the male rats from the females. The rats in the control group received 0.2 ml of physiological (0.9% w/v) saline while the female rats in the other three groups were treated with different doses of the extracts (50, 87, and 150 mg/kg body weight) for eight days (four days before mating and four days during mating) in 0.2 ml physiological saline. The estrous stage of the estrous cycle was confirmed microscopically before allowing the animals to mate. After mating, the male rats were immediately disposed according to the laboratory SOP. Upon parturition (after 18-21 days), the sex of the pups was determined macroscopically by inspection of the distance between the anus and external genitalia. The number of male and female pups was recorded and the ratio of males to females was established.

Determination of effects of V. amygdalina on vaginal pH

A vaginal lavage was performed using distilled water on female rats before and after the treatment period (day one and day five) with various extracts and the pH of the reinstate was determined using a pH meter (Hanna, pHep®5). The changes in vaginal pH before and after treatment were calculated and recorded.

Evaluation of sperm count and sperm morphology

Sperm cells were obtained from cauda epididymis; in this process, the head of the epididymis was obtained and immersed in 5ml of Dulbecco's phosphate buffer at 37.5°C and a sharp incision was done across it. Sperm were allowed to diffuse into the buffer for 10 minutes [20]. Sperm count was done using an improved Neubauer's counting chamber in three replications. 1% of eosin yellow stain was used for the determination of morphological integrity of the sperm head and tail and the percentage of morphologically intact sperm cells was determined and recorded.

Determination of effects on cortisol, estrogen, and testosterone hormone levels

After four days of drug administration, 0.5 ml of whole blood was obtained from the animals by the pricking of the jugular vein with a sterile gauge 21 needles (BD Medical Systems). The blood was allowed to clot, and serum was obtained by centrifugation at 2300 rpm (500 g) at 4 Degrees Celsius. Serum levels of cortisol and testosterone in males while cortisol and estrogen hormones in females were determined using a well-calibrated, quality-controlled Olympus AU-640 (Olympus Optical Co., Ltd) Clinical Chemistry Autoanalyzer. All the tests were conducted according to the standard operating procedures (SOPs) used in the laboratory of the School of Medicine, University of Nairobi. The hormone levels recorded in the extract treatment groups were compared against the levels in the control group.

Qualitative phytochemical screening and minerals analysis

The methanol extracts of V. amygdalina were subjected to qualitative phytochemical screening with the help of gas chromatography coupled with mass spectrometry (GCMS 7890/5975 Agilent Technologies) to identify the present chemical constituents as described by Harborne [21]. Quantitative analysis of mineral components was done on the methanol extracts using Atomic Absorption (AAS) Model: 210 VGP (Scientific equipment) and Flame Photometry using Cole-Parmer® Dual-Channel Flame Photometer as described by Maruga *et al* [22]. Among the chemicals and reagents used included, Na_2SO_4 NaF, H_2SO_4, Calcium carbonate, 1N HCL, KCL, Spec-pure magnesium rods, NaCl, lanthanum chloride

($LaCl.7H_2O$, 0.15%), phenolphthalein indicator from Sigma Aldrich and Fujairah chemicals.

Data analysis

All the numerical data obtained from the assays were recorded in a laboratory notebook and entered into the excel spreadsheet, cleaned, and exported to SPSS statistical software by which descriptive statistical analysis was carried out. Results were expressed as mean ± standard deviations. The difference of results for the efficacy and the related parameters explaining efficacy were statistically compared using independent one-way ANOVA followed by *Schefft's* post hoc test. Data on the number of female rats that conceived among the groups was compared using the Pearson Chi-square test. The level of statistical significance was set to $P < .05$.

RESULTS

Effects of methanol extracts of *Vernonia amygdalina* to male and female rats on the rate of conception, number of litters born and the male: female litter count ratio

Mating male rats treated with the different doses of *V. amygdalina* methanol extracts with untreated females did not result in any statistically significant changes ($P < .05$) in the number of females that conceived, the number of pups born as well as the male: female litter count ratio compared to the control group as shown in Table 1 and Figure 1A, B & C. However, when treated female rats were mated with untreated males, significant changes in the ratio of males to females in the litter were observed in all the extract-treated groups count compared to the control group (Table 2 & Fig. 1C). Specifically, female rats treated with the 50, 87, and 150mg/kg body weight doses of *V. amygdalina* had a significantly elevated ratio of males to female pups (2.52±0.45, 2.07±0.83, and 1.74±0.52, respectively) compared to the untreated females (1.10±0.12) ($P < .05$) as shown in Table 1 and Figure 1 A. Interestingly, the effect of treatment on the male: female litter count ratio was decreasing with an increase in dose levels. Additionally, although the female rats that received the 150mg/kg extract dose showed a significant reduction in the number of females that delivered (6/10) in comparison to the control (10/10), the average number of pups born by each rat was significantly elevated (14.2±3.9) compared to the control group (10.4±1.6) at a *P*-value of $P < .05$ (Table 1; Figure 1 A & B).

Effects of methanol extracts of Vernonia amygdalina to male and female rats on factors that may contribute to the sex selection, epididymal sperm count, the percentage of normal spermatozoa, serum testosterone, and cortisol levels

Treating male rats with therapeutic doses of *V. amygdalina* root methanol extracts did not cause any significant change in the epididymal sperm count and the percentage of normal spermatozoa as shown in Fig. 2 A & B. Treatment of male rats with methanol extracts of *V. amygdalina* did not cause any significant changes in the serum levels of testosterone compared to the untreated control group (Figure 2 C). However, all the groups treated with the 50, 87 and 150 mg/kg doses of extracts of *V. amygdalina* demonstrated a significant decrease in serum cortisol levels (129.00±16.45, 102.80±8.44, and 84.96±51.97 nmol/L, respectively) in comparison with the untreated group with a mean value of 186.86±22.03 nmol/L ($P < .05$, Figure 2 D).

Effects of methanol extracts of V. amygdalina to female rats on the changes in vaginal pH, serum estrogen, and cortisol levels

Significant increases in vaginal pH by 1.18±0.22, 1.12±0.08, and 0.87±0.17 were recorded when the 50, 87, and 150mg/kg doses of *V. amygdalina* respectively were administered compared to the untreated females which had a pH change of 0.06±0.11 ($P < .05$, Fig. 2 A). Estrogen levels in female rats were significantly elevated upon administration of the 50 mg/kg and 87mg/kg doses of *V. amygdalina* (87.96±13.85 and 80.39±17.73 pg./mL respectively) compared to the untreated group (43.74±3.39 pg./mL) (Figure 2 B; $P < .05$). A significant decrease in serum cortisol levels was observed in all the female rats treated with methanol extracts at 50, 87, and 150mg/kg body weight of *V. amygdalina* (364.3±128.6, 422.1±34.1, and 256.8±112) nmol/L, respectively, compared to that of the untreated group with a mean of 955.3±94.3 nmol/L ($P > .05$, Figure 2 C).

Phytochemical composition of methanol root extract of V. amygdalina

A total of 60 phytochemical compounds were detected in the methanol root extract of *V. amygdalina* by Gas Chromatography coupled with Mass Spectrometry (GC-MS) method (Supplementally Table 1). These phytochemical compounds included phenols and polyphenols, alkaloids, fatty acids, and terpenes, among others. The quantity of each phytochemical was expressed as a percentage abundance. Resorcinol had the highest concentration with a percentage abundance of 9.69% followed by eugenol at 8.74%, thymol at 7.37%, and vanillin with 6.32%. The rest had a percentage abundance of less than 5%. Phenols were the most abundant class of phytochemical compounds with a total percentage abundance of 34.57%, fatty acids and fatty alcohols had a total abundance of 14.76% while phthalic acid was 2.97%. The retention time, name, chemical class, molecular formula, molecular mass, percentage abundance, and known uses of all the identified phytochemical compounds are listed in Supplementally Table 1.

Mineral composition of methanol root extracts of V. amygdalina

Mineral analysis of the plant extracts revealed the presence of mineral elements namely, calcium (Ca), magnesium (Mg), lead (Pb), manganese (Mn), zinc (Zn), and potassium (K), sodium (Na). The quantity of each element in mg/kg in the extract was determined. The amount of each element present in every treatment dose was also calc Faulted and compared to the recommended daily requirement dose. The results indicated that the amount of potassium and lead (in all the three extract doses), as well as zinc and manganese (in the 87 and 150 mg/kg extract doses), exceeded the recommended daily allowance (Table 2).

Table 1: Retention time, name, class, molecular mass, formula, and percentage abundance phytochemicals identified in methanol extracts of Vernonia amygdalina

RT (min)	Chemical name	Chemical class	Molecular Formula	Mw (g/mol)	% Abundance
4.3264	2-Prpenoic acid, 2-methyl-	Fatty acid	$C_{12}H_{14}O_2$	190.24	1.06
5.1440	Dimethyl sulfone	Sulfones	$C_2H_6O_2S$	94.14	0.76
6.6892	Phenol	Phenols	C_6H_5OH	94.11	0.24
7.0457	Benzene, propyl-	Alkylbenzene	C_9H_{12}	120.19	0.31
7.0457	N-Benzyl-2-phenethylamine	Alkaloid	$C_{15}H_{17}N$	211.30	0.34
7.7939	Phenol, 2-methoxy	Polyphenol	$C_7H_8O_2$	124.1372	0.29
8.7390	Undecanol	Fatty alcohol	$CH_3(CH_2)_9CH_2OH$	172.31	2.87

9.2603	Benzene, tert-butyl-	Aromatic hydrocarbon	$C_{10}H_{14}$	134.21	0.35
9.3038	Naphthalene	Aromatic hydrocarbon	$C_{10}H_8$	128.17	0.18
10.3245	1,2-Ethanediamine, N-N'-bis(phenylmethyl)-	Alkylamine	$C_{16}H_{20}N_2$	240.34	0.12
10.3245	Benzofuran, 2,3-dihydro-	Benzofurans	C_8H_8O	120.1485	0.10
10.6303	2-Undecanone	Ketone	$C_{11}H_{22}O$	170.29	3.04
10.9349	Naphthalene, 2-methyl-	Aromatic hydrocarbon	$C_{11}H_{10}$	142.2	0.21
11.0158	Decanoic acid, methyl ester	Fatty acid	$C_{11}H_{22}O_2$	186.2912	3.86
11.1121	Indole	Alkaloid	C_8H_7N	117.1479	0.45
11.1474	Thymol	Phenol: monoterpene	$C_{10}H_{14}O$	150.2176	7.37
11.1474	Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate	Phenols	$C_{12}H_{16}NO_2$	206.2609	2.35
11.2272	Phthalic anhydride	Phthalic Anhydrides	$C_8H_4O_3$ or $C_6H_4(CO)_2O$	148.11	0.31
11.3163	Phenol, 3,4-dimethoxy	Phenol	$C_8H_{10}O_3$	154.1632	0.47
11.5236	Hydroquinone	Phenol	$C_6H_6O_2$	110.1106	1.98
11.8282	Resorcinol	Phenol	$C_6H_6O_2$	110.1106	9.69
12.0345	Tetradecane	Acyclic alkanes	$C_{14}H_{30}$	198.3880	3.54
12.0542	Biphenyl	Biphenyls	$C_6H_5C_6H_5$	154.21	1.04
12.4324	Vanilin	Phenols	$C_8H_8O_3$	152.1473	6.32
12.6739	Benzaldehyde, 4-hydroxy	Phenols	$C_7H_6O_2$	122.1213	0.47
12.9806	Eugenol	Essential oil	$C_{10}H_{12}O_2$	164.2011	8.74
12.9806	Phenol, 2-methoxy-4-(1-propenyl)	Polyphenol	$C_{10}H_{12}O_2$	164.2011	0.59
13.3879	Benzene, 1,3-bis(1-methylethyl)	Benzenes	$C_{12}H_{18}$	162.2713	0.12
13.4408	Naphthalene, 2,3,6-trimethyl	Naphthalene	$C_{13}H_{14}$	170.25	0.20
13.4408	Naphthalene, 1,6,7-trimethyl	Naphthalene	$C_{13}H_{14}$	170.25	0.16
13.4408	Metharbital	Barbiturates	$C_9H_{14}N_2O_3$	198.2191	1.03
13.5413	Ethenone, 1-(2-hydroxyphenyl)-	Benzopyrazines	$C_8H_8O_2$	136.1479	0.45
13.7444	Phenol, 2,4-bis(1,1-dimethylelthy)	Polyphenol	$C_{14}H_{22}O$	206.329	2.84
14.0957	2-Propenoic acid, 3-phenyl-	Fatty acid	$C_9H_8O_2$	148.1586	2.48
14.0957	Di siloxane, hexamethyl	Organosilicons	$C_6H_{18}OSi_2$	162.3775	1.05
14.1301	Decani, 4-methyl-	Alkanes	$C_{11}H_{24}$	156.31	1.50
14.4823	1-Hexadecanol	Fatty alcohols	$C_{16}H_{34}O$	242.4406	2.12
14.4823	1-Hexadecene	Alkenes	$C_{16}H_{32}$	224.42	1.63
14.5589	Hexadecane	Acyclic alkanes	$C_{16}H_{34}$	226.4412	2.03
15.2056	Benzophenone	Aromatic ketone	$C_{13}H_{10}O$	182.2179	0.15
15.5714	9H-Pyridol[3,4-]indole,	Hydrogenated pyridines	$C_{12}H_{10}N_2$	182.2212	1.03
15.5714	2,4.6-Trimethoxyamphetamine	Phenethylamines (amphetamines)	$C_{12}H_{19}NO_3$	225.28	1.21
15.5714	Benzaldehyde, 4-hydroxy-3,5-dimethoxy	Phenols	$C_9H_{10}O_4$	182.1733	0.32
15.7248	Heptadecane	Alkanes	$C_{17}H_{36}$	240.5	1.49
15.7652	Ethenone, 1-(4-hydroxy-3,5-dimethoxypheny)-	Phenols/ Acetophenones	$C_{10}H_{12}O_4$	196.1999	0.38
16.0191	Methyl tetra decanoate	Fatty acid	$C_{15}H_{30}O_2$	242.3975	1.01
16.2347	Benzoic acid, 2,4,5-trimethoxy	Phenols	$C_{10}H_{12}O_2$	164.2011	0.23
16.7715	1-Octadecanol	Fatty alcohols	$C_{18}H_{38}O$	270.4937	1.38
16.8337	Octadecane	Fatty acyls	$C_{18}H_{38}$	254.4943	1.54
17.0925	1-Dodecanol	Fatty alcohol	$C_{12}H_{26}O$	186.33	1.49
17.231	Neoisomenthol	Monoterpenes	$C_{10}H_{20}O$	156.2652	1.03
18.5664	Di isobutyl phthalate	Phthalic acids	$C_{16}H_{22}O_4$	278.3435	0.37
18.5664	Dibutyl phthalate	Phthalic acids	$C_{16}H_{22}O_4$	278.3435	0.37

18.7767	n-Hexadecenoic acid	Fatty acid	$C_{16}H_{32}O_2$	256.4241	2.19
18.8089	Di isobutyl phthalate	Phthalic acids	$C_{16}H_{22}O_4$	278.3435	2.06
19.8338	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Fatty acid	$C_{19}H_{34}O_2$	294.4721	3.27
19.8929	9-Octadecanoic acid (Z)-, methyl ester	Fatty acid	$C_{19}H_{36}O_2$	296.4879	2.89
20.6566	Octadecanoic acid	Fatty acid	$CH_3(CH_2)_{16}COOH$	328.488	3.98
23.5096	Trimethylamine	Methylamines	C_3H_9N	59.1103	0.78
23.6930	Bis(2-ethylhexyl) phthalate	Phthalic acids	$C_{24}H_{38}O_4$	390.6	0.17

Table 2: Effects of single therapeutic doses of *V. amygdalina* methanol extracts to female rats on the rate of conception, number of litters born, and the male: female litter count ratio

Dose in mg/kg body weight	Number of mated males/females	Number of females that delivered	Total Number of litters born	Average litter size per rat (Mean ± SD)	Number of male pups in the litter	Number of female pups in the litter	Ratio of males: females in litter
Control	2/10	10^{b}	104	10.4 ± 1.6^{b}	54	50	1.10 ± 0.12^{b}
Extracts doses (mg/kg body weight)							
50	2/10	8^{bc}	81	10.1 ± 2.4^{b}	58	23	2.52 ± 0.45^{a}
87	2/10	7^{bc}	83	11.9 ± 2.7^{ab}	56	27	2.07 ± 0.83^{a}
150	2/10	6^{c}	85	14.2 ± 3.9^{a}	54	31	1.74 ± 0.52^{a}

Results are expressed as the average of 10 rats in every group ± standard deviation (SD). Means with similar superscript letters within a column are statistically similar at a ρ-value of $\rho < .05$

Table 3: Mineral concentration and amount administered into each female rat at 50, 87, and 150 mg/kg body weight doses of methanol extracts of *V. amygdalina*

		mg/kg body weight			
Mineral	**Concentration (µg/g)**	**50**	**87**	**150**	**RDA (µg/day)**
Ca	2420.339	27.23	47.22	81.69	3214.3
Mg	25013.59	281.40	487.95	844.21	1350
Pb	3797.5	42.72	74.08	128.17	0.3214
Mn	432.5	4.87	8.44	14.6	7.392
Zn	1899	21.36	37.04	64.09	35.357
K	1463257.16	16461.64	28544.49	49384.93	11250
Na	20672.866	232.57	403.28	697.71	1607.1

Assume the average weight of the female rat is 225 g. Amount of mineral element administered in µg. The recommended daily allowance for: calcium is 1000 mg/day, magnesium is 420 mg/day for adult men and 320 mg/day for adult women, manganese is 2.3 mg/day for adult men and 1.8 mg/day for adult women (adequate intake), zinc is 11 mg/day for adult men and 8 mg/day for adult women, potassium is 3500 mg/day, sodium is 500 mg/day. Human daily intake for lead is 15-100 µg/day [46].

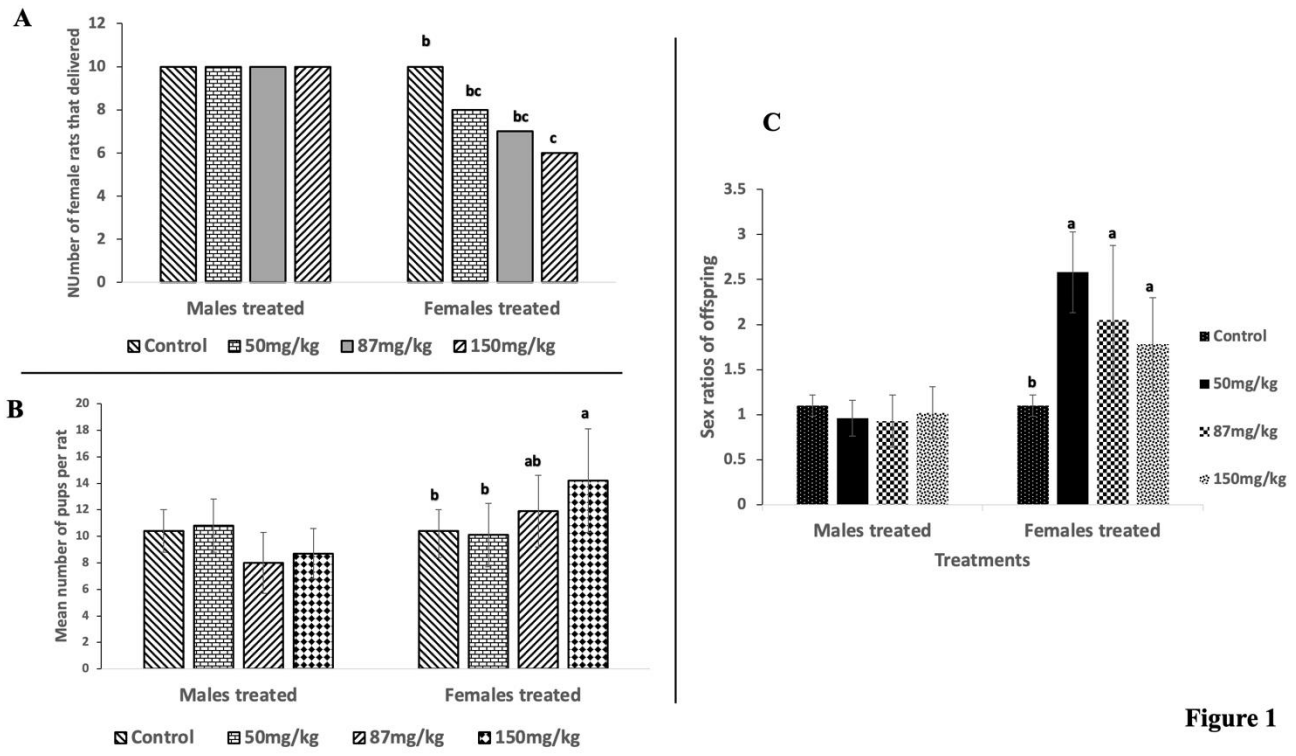

Figure 1: Effects of methanol extracts of *V. amygdalina* to male and female rats on the number of female rats that delivered (A), the average number of pups born by each rat (B), and the male: female litter count ratio. Results are expressed as Mean ± standard deviation (SD) for ten animals for each group. Means with similar superscript letters within a column are statistically similar at a *P*-value of p≤0.05

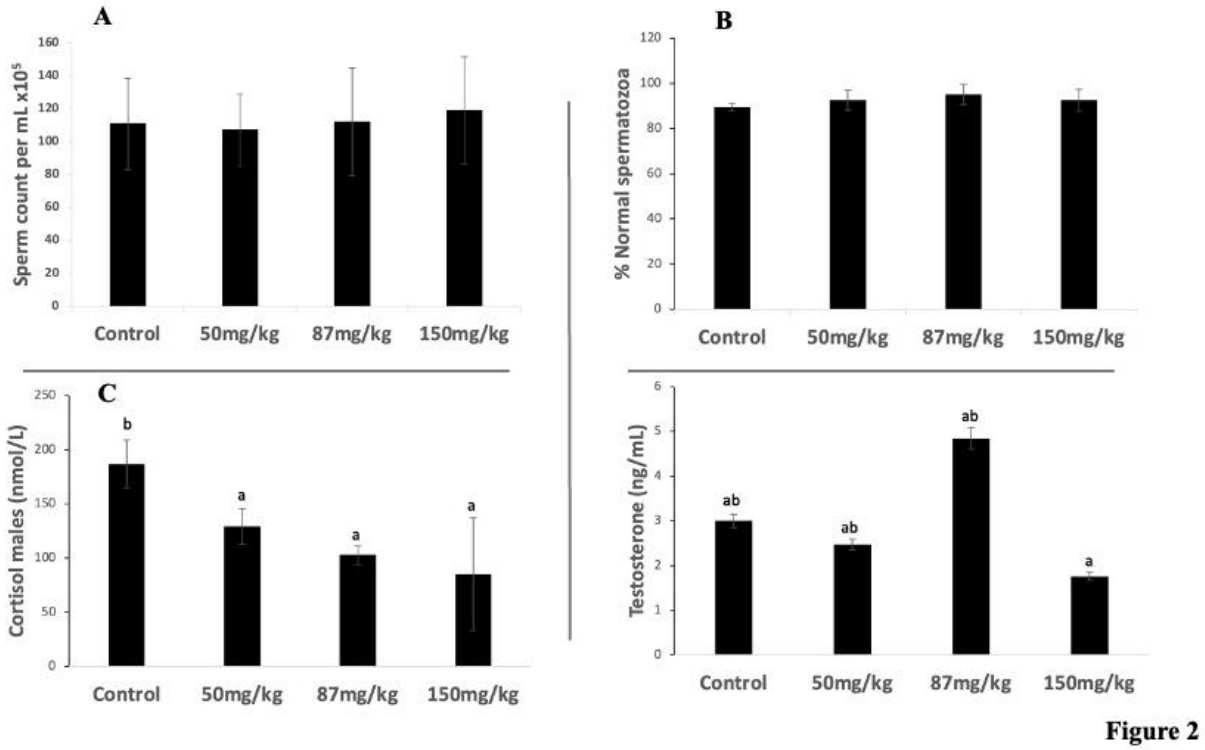

Figure 2: Effects of methanol extracts of *Vernonia amygdalina* to male rats on factors that may contribute to the sex selection. (A) Sperm count (B) Percentage of normal spermatozoa (C) Serum testosterone levels (D) Serum cortisol levels. Results are expressed as Mean ± standard deviation (SD) for five animals for each group. Means with similar superscript letters within a column are statistically similar at a *P*-value of p≤0.05

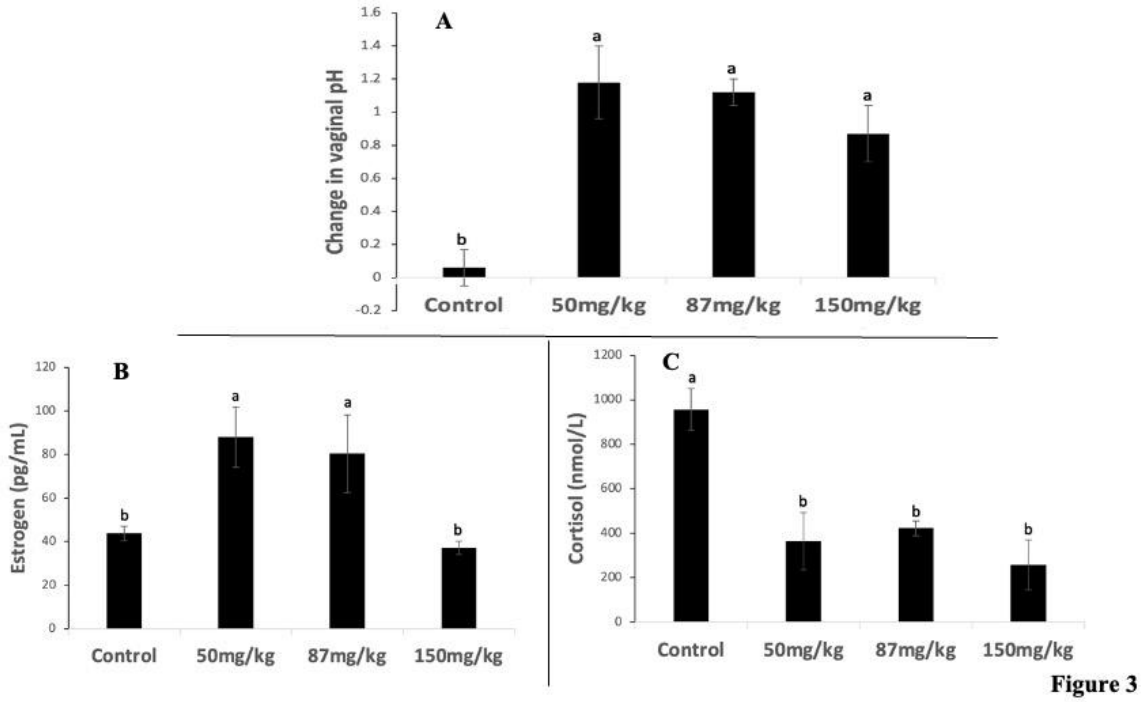

Figure 3: Effects of methanol extracts of *Vernonia amygdalina* to female rats on factors that may contribute to the sex selection. (A) Change in vaginal pH (B) Serum estrogen levels (pg./mL) (C) Serum cortisol levels (nmol/L). Results are expressed as Mean ± standard deviation (SD) for five animals for each group. Means with similar superscript letters within a column are statistically similar at a *P*-value of p≤0.05

DISCUSSION

Factors influencing the gender of an offspring can be divided into paternal/male-driven and maternal-driven factors. Known male-driven factors of sex selection include specific hormone levels, changes in the ratio of X: Y-chromosome bearing sperm, and morphological and/or physiological differences between the X- and Y-chromosome bearing spermatozoa as well as epigenetic influences. In this study, treatment of male rates with *V. amygdalina* extracts did not cause any significant changes in serum testosterone levels, sperm count, or morphology, and also the sex ratio of offspring sired by the treated male rats was not significantly affected. However, various studies have shown that sperm quality is highly dependent on testosterone levels and both have been associated with an increased ratio of male: female offspring. For example, a study conducted by Romandie *et al.*, [23] in *Cervus elaphus* (red deer) indicated that semen obtained from more fertile males with a higher percentage of normal spermatozoa sired more sons than daughters while less fertile males sired fewer sons and more daughters. Bulls with high testosterone levels had more Y-chromosome-bearing spermatozoa than X-chromosome-spermatozoa which were attributed to the increased number of male offspring [24]. Other studies by Kim *et al* [25] showed that suppression of testosterone levels caused azoospermia in eight weeks in animal models where apoptosis of testicular tissue and germ cells is induced via the caspase 3 pathway. Since the extracts did not cause any of these parameters in male rats this could be a probable reason why there was no change in the male to female ratio of the offspring.

Treatment of female rats with various doses of *V. amygdalina* showed that the number of female rats that delivered was significantly reduced in the group that received the 150 mg/kg dose but the average number of pups born was significantly increased. The decreased number of animals that delivered observed in the 150 mg/kg group unlike in the groups that received lower doses of the extract could be associated with the reproductive toxicity of the high doses of *V. amygdalina* extracts as reported by Ayelet and colleagues, 2006 [26]. In their study, it was showed that high doses of *V. amygdalina* extracts caused reduced implantation as well as increased uterotonic, a mechanism that led to abortion. Since more than half of the animals in the group conceived and delivered, it is therefore probable that this reduction in the number of females that delivered could be due to abortifacient properties of the plant. On the other hand, the increased number of pups per rat could be due to stimulation of superovulation in the female rats. Studies have shown that diet supplementation with *V. amygdalina* resulted into increased fecundity, fertilization, hatching rate as well as survival of larvae in *C. Gabrielinos* [27]. This could be attributed to the strong antioxidant properties of the plant that have been earlier reported to cause increased reproductivity in rats [28].

Several known maternal-driven factors influence the gender of an offspring. These include maternal diet [26], vaginal pH [30], maternal hormone levels [31], health, and body size [32] among others. In this study, the focus was directed to how these factors affect the process of fertilization by differential influence on the X- and Y-chromosome-bearing sperm. The results of the study showed that there was a significant elevation of vaginal pH in all the groups treated with *V. amygdalina* methanol extracts coupled with an increase in the male to female ratio of the offspring sired by the treated animals. It is well known that the X- and Y-chromosome-bearing spermatozoa are favored differently by different pHs and, therefore, there is a strong correlation between the vaginal pH at the time of mating and the sex ratio of the offspring which selectively affects the motility, capacitation, and survival of the X- and Y-spermatozoa [33]. For example, Shuttles [30] suggested that vaginal douching to lower or raise the pH before coitus would favor a female or male offspring, respectively. Additionally, a study conducted by Whyte *et al* [34] indicated that vaginal pH, steroidal hormone levels, and consequently offspring ratio are highly dependent on the amount of fat fed to mice before conception (diestrus phase) and after conception (metestrus phase). This shows that the pH of the vagina during conception affects the offspring's gender in mammals. We therefore suggested that the elevation of vaginal pH in females treated with methanol extracts of *V. amygdalina* could be attributed to the observed increase in the sex ratio of rat offspring but it is not clear the specific phytochemicals or minerals present in the extracts that would be responsible for this observation and their mechanisms of action.

It was also observed that the estrogen levels in female rats were significantly elevated upon administration of the 50 mg/kg and 87mg/kg doses of *V. amygdalina* compared to the untreated group

which can be attributed to the elevation of sex ratio in the offspring sired by the treated rats. These results are consistent with findings by Zhang [35] who reported that when mouse embryos were incubated with high levels of 17-β estradiol the number of males was significantly increased. High levels of estrogen and testosterone are known to increase the sex ratio of offspring while high levels of gonadotropins and progesterone lower the sex ratio [36]. Other findings by Fukuda *et al* [37] showed that the levels of estrogen and testosterone levels are higher when ovulation occurs in the right ovary which would support a previous suggestion by Schooner [38] that right-sided ovulations lead to the birth of more sons than daughters. Krakow [39] summarized possible mechanisms through which humoral factors (progesterone and steroid hormone) adjust the offspring sex ratio in mammals as follows: vaginal pH, mucus viscosity, semen composition selectively affects sperm motility while cumulus oophores micro-milieu, regulation of resorption by progesterone, and induction to 2nd meiotic division affect acrosome reaction threshold, resorption threshold, and sperm monsoonal content, respectively. It is therefore evident that maternal estrogen level is a key factor in the determination of the offspring's sex ratio, and based on this knowledge we suggested that high levels of estrogen increase the sex ratio of offspring and this is one of the mechanisms by which *V. amygdalina* causes an increase in sex ratio in rats.

We evaluated the effect of *V. amygdalina* extracts on stress hormones levels (cortisol) in female rats. The results showed a significant decrease in serum cortisol levels in all the female rats treated with methanol extracts at 50, 87, and 150 mg/kg body weight of *V. amygdalina* compared to that of the untreated group. Glucocorticoid hormones play a major role in energy reserve allocation which determines the levels of glucose and fatty acids in circulation. The plant's extract could have directly influenced the activity of reproductive tissues in the female rats thereby regulating the secretion and activity of steroid hormones as well as gonadotrophins. This is because glucocorticoid hormones receptors and glucocorticoid degrading enzymes are present in reproductive tissues and, most importantly, including sperm cells, suggesting that glucocorticoid hormones can directly affect the offspring sex ratio [40]. Fetal cortisol level is one of the mechanisms that determine and maintains *in-utero* sex ratios [41]. In the study, it was noted that female rats that sired offspring's of even sex ratios had the highest cortisol levels. Maternal cortisol levels are indicators of maternal stress levels. Evidence linking offspring sex ratio, maternal stress, and consequently energetic condition has been established in various studies but the physiological mechanisms involved have not been elucidated yet. Additionally, the elevation of glucocorticoid hormones leads to a decrease in testosterone levels and consequently lower sex ratios of offspring [40]. The plant's extract could have also reduced selective death of fetuses is a bet-hedging strategy in which females adjusted offspring sex ratio in stochastic environments via various hormonal pathways. This is consistent with findings by Love *et al* [42] who reported that elevation of cortisol hormone in European starlings (*Sturnus vulgaris*) resulted in increased selective death of male embryos while the hatched males were lighter in weight, had slower postnatal growth rates, and decreased immunity compared with offspring's born to the control groups. With this understanding, it is possible that *V. amygdalina* methanol extract influenced the sex ratio in rats by altering the maternal cortisol and estrogen hormones level.

A Gas Chromatography coupled with a Mass Spectrometry assay of *V. amygdalina* methanol root extract revealed the massive abundance of various antioxidants such as phenols and polyphenols, alkaloids, fatty acids, among others. These phytochemicals have been shown to possess strong antioxidant activities may be attributed to the sex selection activity and especially in favor of the Y-chromosome-bearing sperm which has been observed to be more prone to various forms of stress such as heat stress [43] and thus, the role of cellular stress in sex ratio alterations. *In-vitro* studies by Zhu *et al* [44] showed that the addition of antioxidants such as pyrroloquinoline quinone and coenzyme Q 10 significantly reduced oxidative stress in sperm cells resulting from oxidative phosphorylation during the high-speed linear sperm motility. Other recent studies by Vasconcelos and colleagues [45] have shown the role of antioxidants such as eugenol in the promotion of the synthesis of endogenous antioxidants such as superoxide dismutase (SOD), catalase, glutathione, and peroxiredoxin. It is therefore probable that the antioxidant phytochemicals present in the plant extracts played a significant role in the elevation of sex ratio of rat offspring in the treated groups.

Besides that, dietary minerals have also been shown to alter the sex ratios of offspring. Therefore, mineral analysis of the plants' methanol extracts was evaluated and the results are tabulated in Table 3. The levels of potassium and calcium were relatively high considering their low daily dietary requirements of 11250 µg/day and 1607.1 µg/day respectively [46]. When a diet rich in potassium and sodium was fed to female rats, the number of male and female pups recorded at birth was 91 and 63, respectively, (sex ratio = 1.44) compared to 66 males and 97 females (sex ratio = 0.68) when the diet was rich in calcium and magnesium minerals [47]. This further supports our hypothesis that the mineral composition of *V. amygdalina* has the potential to influence the sex ratio of rat offspring.

Additionally, a study conducted by Uehara and others [48] showed that Toll-like receptors 7 & 8 are only expressed in the X-chromosome-bearing sperm, and their activation causes reduction of motility in the X-chromosome only thus conferring functional differences between the X- and the Y-spermatozoa. Activation of these receptors causes inhibition of the enzyme hexokinase activity and therefore selectively lowers the motility of X-sperm cells by interfering with ATP production in these cells [48]. Since sperm motility is activated after ejaculation, it is therefore probable that the activity of the *V. amygdalina* methanol extract was via the activation of the Toll-like receptors; therefore, computationally simulated molecular docking studies of the phytochemicals in the plant and the Toll-like receptors 7 & 8 are recommended.

CONCLUSION

Although the plants did not show significant male-driven sex selection activity, when the study was repeated with the administration of similar doses to females, the ratio of males to females in the litter count was significantly increased in all the groups treated with the extracts. This indicates that the plant has the potential to influence the gender of offspring before or during conception in female rats. In conclusion, the findings of our study taken together indicate that high levels of vaginal pH, maternal serum estrogen and cortisol hormone are some of the mechanisms by which *V. amygdalina* causes an increase in sex ratio of offspring in rats. Further studies are recommended to elucidate the mechanisms by which the extracts cause changes in vaginal pH, hormone levels, phytochemicals, and mineral components selectively affect the X- and Y-spermatozoa in aspects of mitochondrial respiration, acrosome integrity, and internal pH leading to increased sex ratios in female rats.

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Author contribution

Njagi Eliud: conceived, designed, and supervised all aspects of the study, Mwonjoria John: contributed to the study design, and supervision of data collection, and Wambugu Enoc: contributed to data collection, analysis, and manuscript preparation.

Data availability

Any data supporting the results of this study may be obtained from the corresponding author upon request.

Conflict of Interest

None declared.

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