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Testicular histomorphology, sperm and testicular morphometric evaluation of glyphosate exposed rabbit bucks treated with hydro-alcoholic leaf extract of *Newbouldia laevis*

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ABSTRACT. This study investigated the effect of glyphosate exposure on some male reproductive parameters and the potential protective effects of *Newbouldia laevis* leaf (NBL) extract. The 36 rabbits used for the study were randomized into four treatment groups (control, glyphosate-only, glyphosate + NBL 100 mg and glyphosate + NBL 200 mg) in a completely randomized design. From the results, glyphosate was found to significantly impair all measured semen parameters. Testicular morphometric analysis revealed a significant ($p < 0.05$) decrease in some testicular parameters due to glyphosate exposure. Histological examination of testes in the glyphosate-only group revealed severe disruptions in testicular architecture, likely contributing to the observed decrease in semen quality. Conversely, NBL treatment led to significant enhancement in spermatogenic activity, resulting in higher sperm count, motility, volume, libido, live sperm, and improved sperm morphology when compared to the herbicide-exposed group. NBL-treated groups exhibited significant ($p < 0.05$) increase in testicular circumference, width, and epididymal length. NBL extract also restored testicular architecture and ameliorated herbicide-induced histological changes. Therefore, the potential protective effects of *Newbouldia laevis* leaf extract on herbicide-induced reproductive dysfunction could pave the way for novel natural interventions to counteract the negative consequences of herbicide exposure.

Keywords: herbicide exposure; male reproductive parameters; phytochemicals; rabbits.

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Introduction

Glyphosate-based herbicides (GBHs) are widely used in agricultural production to control weeds. However, their widespread use has led to environmental contamination and the accidental exposure of non-target organisms, such as rabbits, to these chemicals. Glyphosate is mainly applied in fields and can persist several months in the soil. Since these plants contaminated with GBH are often consumed by animals or used in processing animal feed, concerns have been raised about its adverse effects on animal health. Soares, Silva, Duarte, Pena, and Pereira (2021) reported that residues of glyphosate have been detected in large number of samples, sometimes exceeding the legally permitted limits. Several studies have investigated glyphosate's potential adverse effects on a wide range of health issues including male reproductive function in different animal models (Dai, Hu, Tang, Li, & Li, 2016; Mutwedu et al., 2021; Okonko, Sam-Uket, & Ikpeme, 2021; Serra, Estienne, Vasseur, Froment, & Dupont, 2021) which are of particular interest in the present study, as they can negatively impact fertility and reproductive success. This exposure poses a significant threat to animals worldwide and conventional treatment approaches often have limitations, highlighting the urgent need for innovative and effective therapeutic options. Therefore, developing therapies of botanical origin for toxin exposure in animals holds tremendous potential for revolutionizing animal health. The rich repository of phytochemicals found in plants offers an array of therapeutic possibilities, which could complement or even replace conventional treatments.

Newbouldia laevis is a medicinal plant and has been studied for its potential protective effects against various toxicants, including heavy metals and environmental pollutants (Habu & Ibeh, 2015). The bioactive compounds present in *Newbouldia laevis*, such as flavonoids, alkaloids, and tannins (Obum-Nnadi, Okey-Ndeche, Nnagbo, & Ohabughiro, 2020), possess antioxidant and anti-inflammatory properties. These properties make *Newbouldia laevis* a promising plant for alleviating the testicular damage caused by herbicide intoxication.

The outcome of this study may have broader implications in the development of eco-friendly and sustainable approaches to safeguard reproductive health in wildlife, farm animals and humans.

Material and methods

Location of study: The research was conducted in the Department of Animal Science Teaching and Research Farm of the Nnamdi Azikiwe University, Anambra State, Nigeria.

Source of *Newbouldia laevis* leaves (NBL): The plant material for this study was sourced from Awka, in Anambra State, Nigeria and authenticated by a taxonomist in the Department of Botany, Nnamdi Azikiwe University, Awka. The leaves were air dried, finely ground using an electric blender and stored in an air-tight container.

Preparation of leaf extract: The ground leaf was weighed into a glass jar and extraction was carried out with aqueous-ethanolic solution in a ratio of 30:70% using maceration method. After 72 hours, a muslin cloth was used for filtration and the resulting hydro-alcoholic extract was finely filtered with Whatman filter paper. The obtained extract was concentrated using rotary vacuum evaporator and subsequently lyophilized.

Experimental animals and management: A total of thirty-six (36) composite rabbit bucks, aged 26 to 30 weeks and weighing 1.56 - 1.70 kg were used for this study. The rabbits were housed individually in units in a hutch. Each hutch unit was provided with a feeder and drinker. The animals were quarantined for 2 weeks before the commencement of the research work. Throughout the research period, the rabbits were allowed *ad-libitum* access to water, compounded feed and forage.

Presentation of the glyphosate herbicide: In preparing the forage for the rabbits, 5 mL of Force Up® herbicide was thoroughly mixed in 0.5 L of water (manufacturer's recommendation for weed control). The mixture was applied to the forage by spraying and the contaminated forage was allowed to dry before being introduced to the rabbits.

Experimental Design: Thirty-six (36) rabbit bucks were randomly assigned to four (4) Treatment groups designated as: Control, Glyphosate, Glyphosate + 100 mg NBL and Glyphosate + 200 mg NBL. Each Treatment group consisted three (3) replication with three (3) rabbits per replicate in a completely randomized design. The administration of the extract preceded the introduction of the contaminated forage and the study continued for a duration of eighty-four (84) days

Data collection and evaluation

Semen collection**:** Two weeks before semen collection, a teaser rabbit doe was used to train the bucks to mount an artificial vagina (AV). On the 57th day following the administration of the test ingredients, the bucks were placed on a semen collection schedule of twice per week. One ejaculate was collected from each rabbit buck once between 08:00 to 13:00 hours (local time) on Mondays and Thursdays for 4 consecutive weeks.

Seminal evaluation: Ejaculate volume was determined by reading-off directly in millilitres from a calibrated glass collection tube attached to the AV. Sperm motility percentage score was subjectively assessed in a drop of fresh semen on a warm glass slide covered with a warm cover slip and examined using a microscope at ×40 magnification. Sperm cell concentration ($\times 10^6$ mm^{-3}) was determined using haemocytometer at a dilution of 1 in 100 in a 10% formaldehyde solution. Total sperm ($\times 10^6$ mm^{-3} per ejaculate) was determined by multiplying the semen ejaculate volume by the sperm cell concentration. Sperm morphological examination was done using eosin/nigrosin stain. Morphological abnormalities observed were; bent tail, shoe hock, coil tail, cytoplasmic droplet, headless and tailless spermatozoa. Live/dead sperm count was assessed in the same smears used for sperm morphological examination. Libido was estimated by observing the reaction time (s) which elapsed between the introduction of a doe to a buck and ejaculation.

Histomorphological studies: At the end of semen collection period, the experimental bucks were sacrificed using captive bolt followed by immediate exsanguinations. Thereafter, the testes were harvested, transversely cut and fixed in Bouin's fluid for 24 hours. The tissues were washed in ascending grades of ethanol (50, 75 and 100%) and cleared with xylene. They were embedded in paraffin wax with a Shandon Duplex automatic tissue processor and then sectioned using microtome at 4-5 µ thickness. Dewaxed sections were stained with Haemotoxylin and Eosin (H&E). The slides were covered with DPX (Distyrene, Plasticizer, and Xylene) mountant to increase refractive index of the stained preparation and then covered with slides to prevent scratches. All sections were examined under light microscope using ×10 and × 40 magnifications. Photomicrographs of the testicular tissues were taken with TSView7 photomicroscope for observation and documentation.

Statistical analysis: The data generated were subjected to statistical analysis of variance (ANOVA) at 5 % level of significance. Occurrence of significant means was separated using Duncan Multiple Range Test (DMRT).

Results and discussion

The findings from the analysis of libido and sperm characteristics in glyphosate exposed rabbit bucks treated with *Newbouldia laevis* extract as shown in Table 1 showed a significant ($p < 0.05$) decrease in libido, mean sperm motility, semen volume, total ejaculate, live/dead sperm ratio and sperm morphology in rabbits that were given glyphosate when compared to the control group. This decline in the libido and seminal profile of glyphosate-exposed rabbits may be attributed to decrease in blood testosterone, follicle-stimulating and luteinizing hormone levels (Owagboriaye et al., 2017), which are crucial for spermatogenesis. A study by Okonko et al. (2021) reported significant ($p < 0.05$) and dose dependent decrease in mean sperm motility, viability, sperm count and an increase in sperm head abnormality of rats exposed to glyphosate. Also, Nerozzi et al. (2020) reported that glyphosate is capable of significantly ($p < 0.05$) decreasing sperm motility, viability, mitochondrial activity and acrosome integrity.

Table 1. Libido and semen indices of glyphosate intoxicated rabbit bucks treated with *Newbouldia laevis* extract.

Parameters	Control	Glyphosate	Glyphosate + (NBL 100 mg)	Glyphosate + NBL (200 mg)	SEM
Libido (sec)	15.91^{b}	24.47^{a}	$9.64b^{c}$	8.51^{c}	2.13
Motility (%)	76.70^{a}	31.55^{b}	73.70^{a}	82.41^{a}	8.25
Concentration (10^6 mm^{-3})	42.22^{ab}	16.89^{b}	57.45^{a}	52.78^{a}	8.49
Volume (mL)	0.60^{a}	0.41^{b}	0.52^{ab}	0.64^{a}	0.06
Total ejaculate (10^9 mm^{-3})	25.39^{a}	5.69^{b}	29.57^{a}	33.66^{a}	4.30
Dead sperm (%)	27.92^{b}	57.60^{a}	20.03^{b}	24.39^{b}	4.49
Live sperm (%)	72.08^{a}	42.40^{b}	79.97^{a}	75.61^{a}	4.49
Normal morphology (%)	79.71^{b}	56.91^{c}	79.31^{b}	88.57^{a}	1.68
Abnormal morphology (%)	20.29^{b}	43.09^{a}	20.69^{b}	11.43^{c}	1.68
Cytoplasmic droplet	3.61^{ab}	6.75^{a}	3.11^{b}	0.36^{b}	1.00
Shoe hock	$8.17b^{c}$	16.35^{a}	9.55^{b}	6.47^{c}	0.56
Bent tail	6.96^{b}	15.59^{a}	6.88^{b}	4.59^{b}	0.80
Coil tail	1.56	4.01	0.67	0.00	1.35
Headless	0.00	0.39	0.00	0.00	0.19
Tailless	0.00	0.00	0.48	0.00	0.24

[a,b]Means bearing different letters of superscript within the same row differ significantly ($p < 0.05$).

Conversely, the mean values of semen parameters in NBL treated groups revealed significant ($p < 0.05$) improvements which resulted in increased spermatogenic activity, with higher sperm count, motility, volume, libido, live sperm and improved sperm morphology compared to the herbicide-intoxicated group. In another study on the effects of ethanolic leaf extract of *Newbouldia laevis* on cisplatin-induced gonadotoxicity in Wistar Rats by Ataman and Osinubi (2014), significant ($p < 0.05$) improvements were observed in sperm count, sperm motility and percentage live/dead sperm of Wistar rats that was treated with l00 mg kg^{-1} of the extract.

The improved reproductive processes obtained in this study could be connected to the findings of Simeon, Sunday, and Anaduaka (2014). They found that the administration of *Newbouldia laevis* leaf extract could potentially help restore hormonal balance, including levels of testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are crucial for maintaining healthy reproductive function.

Table 2 presents the result of average measurements derived from paired testicular morphometric analysis, which typically involved the assessment of various parameters related to testicular size, weight, volume, width, circumference and length. Testis circumference, width and epididymis length of rabbits treated with glyphosate +NBL (200 mg) were significantly ($p < 0.05$) higher than those in the glyphosate-only group but similar to the control and glyphosate +NBL (100 mg) groups. Also, the testis density of rabbits in glyphosate-only group showed higher significant ($p < 0.05$) difference with rabbits in the glyphosate + NBL (100 mg) group but significantly similar with rabbits in the control and glyphosate + NBL (200 mg) groups.

Semen analysis is widely recognized as a crucial aspect of assessing male fertility. Interestingly, although testicular size is a readily measurable parameter, it has been somewhat overlooked in predicting the same fertility characteristics. The seminiferous tubules, comprising over 80% of testicular size, play a pivotal role in sperm production, and smaller testicles have been associated with reduced sperm production within these tubules (Tijani, Oyende, Awosanya, Ojewola, & Yusuf, 2014). Consequently, testicular size can be linked to both the quantity and quality of semen, which ultimately determine an animal's fertility. Intriguingly, a notable association was observed in the results of semen evaluation indices presented in Table 1 and testicular measurements in Table 2, thus, suggesting that testicular size may be utilized as an indicator for predicting male fertility as reported by Bellurkar et al. (2020). Tijani et al. (2014) documented that male animals with smaller testicular volumes tended to have reduced sperm production. Similarly, Manuel, Ugboma, and Nwankwo (2017), Abu, Kolade-Yunusa, Atim, Obakeye, and Dakum (2021) and Apriansyah (2022) found a significant direct relationship between testicular volume, sperm morphology, motility, concentration and total testosterone serum concentration. Also, Ansa and Imasuen (2015) found strong positive associations between the weight of testes and both gonadal sperm reserves and ejaculate concentration. They also observed a similar correlation between testis circumference and ejaculate concentration, as well as between epididymal weight and extragonadal sperm reserves.

Table 2. Mean testicular morphometry of glyphosate exposed rabbit bucks administered hydro-alcoholic leaf extract of *Newbouldia laevis*.

Parameters	Control	Glyphosate	Glyphosate + (NBL 100 mg)	Glyphosate + NBL (200 mg)	SEM
Testes Length (cm)	2.80	2.63	3.07	2.93	0.19
Testes Weight (g)	1.82	1.42	1.70	1.77	0.17
Testes Circumference (cm)	3.25^{a}	2.73^{b}	3.00^{ab}	3.47^{a}	0.15
Testes Width (cm)	1.27^{ab}	1.03^{c}	1.15^{bc}	1.35^{a}	0.06
Testes Volume (cm^3)	1.70	1.07	1.80	1.75	0.21
Testes Density ($g\ cm^{-3}$)	1.07^{ab}	1.53^{a}	0.95^{b}	1.03^{ab}	0.16
Relative testes weight (%)	0.10	0.08	0.10	0.10	0.01
Epididymis Length (cm)	16.40^{ab}	13.80^{b}	23.90^{ab}	30.40^{a}	4.41
Epididymis Weight (g)	1.08	0.85	1.07	1.35	0.16
Vas deferens Length (cm)	8.29	7.43	7.27	8.35	1.04
Vas deferens Weight (g)	0.27	0.10	0.23	0.25	0.06

^{a,b}Means bearing different letters of superscript within the same row differ significantly ($p < 0.05$).

In Table 2, with the exception of vas deferens, the measured testicular morphometric indices indicate a decrease in the group exposed solely to glyphosate herbicide. This outcome aligns with the findings of Owagboriaye et al. (2017), who reported testicular degeneration in male albino rats when orally exposed to glyphosate. Furthermore, the observed higher significant ($p < 0.05$) difference in testis density ($1.53\ g\ cm^{-3}$) in the glyphosate-only group, compared to the result of other morphometric parameters, clearly supports the finding by Abdullahi, Musa, and Jibril (2012), who found no significant correlation between testis density and nearly all studied morphometric characteristics. Additionally, Abdullahi et al. (2012) reported a negative correlation between testes density and some testicular characteristics such as testes volume and scrotal circumference. This led to the conclusion that testicular characteristics cannot be predicted from testis density.

Positively, the groups receiving *Newbouldia laevis* leaf extract demonstrated higher testicular circumferences, width and epididymal length and this effect became more pronounced with an increase dose of the extract. While no statistically significant ($p < 0.05$) difference was observed in the testis weight and volume results, the higher numerical increase in the groups treated with glyphosate + NBL (100 mg) and glyphosate + NBL (200 mg) suggests a potential beneficial effect of *Newbouldia laevis* leaf extract in mitigating glyphosate toxicity. Consequently, this finding largely implies that *Newbouldia laevis* extract could have a beneficial effect on testicular morphometry, which serve as crucial indicator of testicular health and function. These effects are likely attributable to the presence of phytochemicals in the plant, which may possess antioxidant properties, thus protecting testicular tissue from oxidative damage caused by glyphosate herbicide and ultimately supporting testicular health. This observation substantiates the assertion by Enye et al. (2015) that *Newbouldia laevis* has the potential to enhance testicular size by mitigating the dysfunction caused by glyphosate. Also, in a study conducted by Ataman and Osinubi (2014), it was found that administering 100 mg kg^{-1} of ethanolic leaf extract of *Newbouldia laevis* resulted in significant improvements in the testicular and epididymal weight, testicular volume, structural integrity of the testis and epididymal tissues in Wistar rats subjected to cisplatin-induced gonadotoxicity.

As presented in Figure 1, light microscopic examination of testes sections at lower (×10) and higher (×40) magnifications of the Control and NBL extract treated groups showed generalized normal architecture with spermatids filled seminiferous tubules (ST). At higher magnification, the seminiferous tubules of the NBL extract groups are capped by tufts of late spermatids attached by their heads in the lumen and the spermatogonia resting on the basement membrane. The glyphosate-only treated group revealed disorganized epithelium and abnormal spermatogenic columns with almost unrecognizable sections of shrunken tubules, separated from each other with widened interstitial space and had different shapes with irregular outlines. At higher magnification, the disruption of the seminiferous tubules in the glyphosate-only group shows lumen filled with degenerated/sloughed spermatogenic cells and aggregates of necrotic spermatids, degeneration of sertolic and germ (spermatogenic cells) with pyknotic multinuclei.

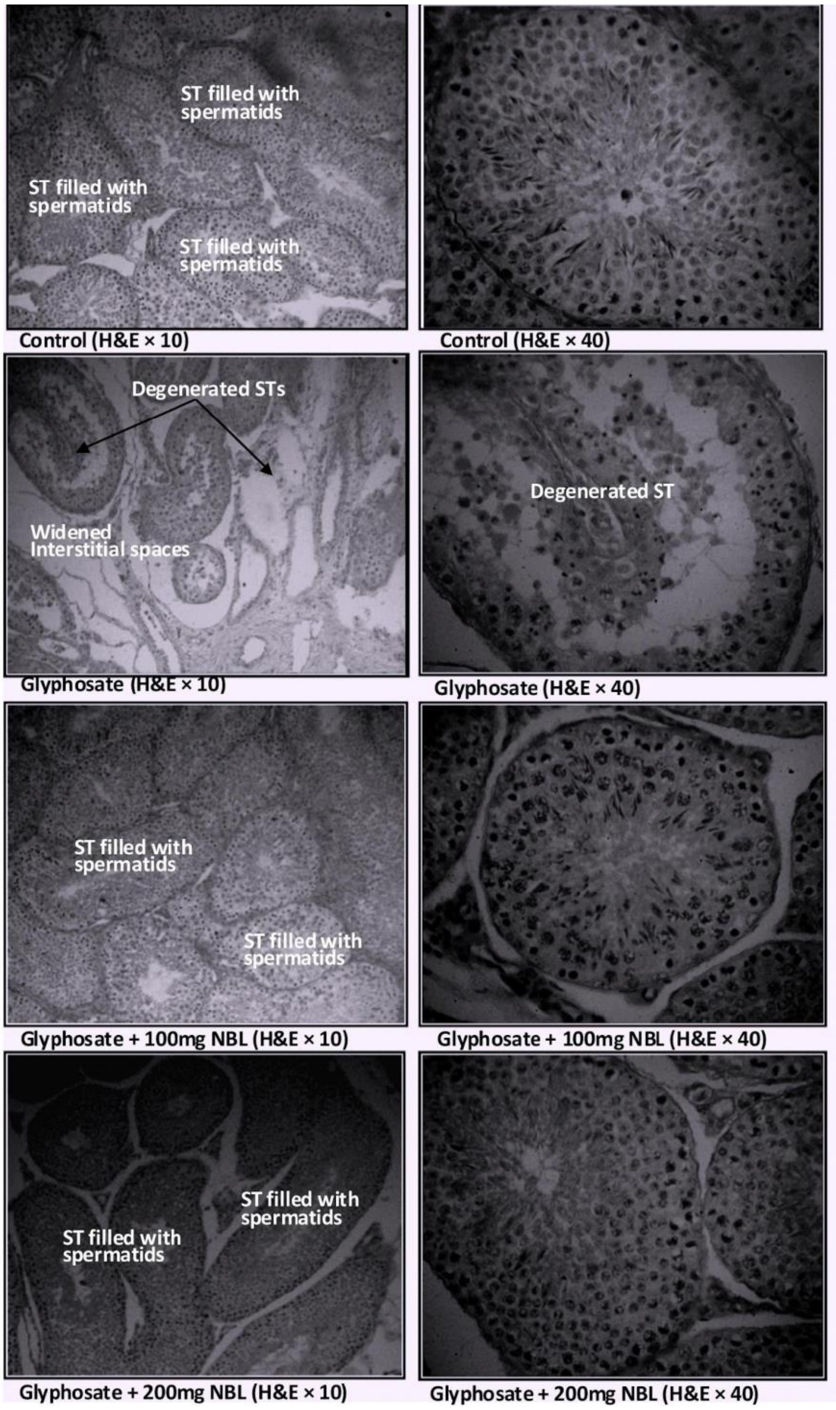

Figure 1. Histomorphological photomicrographs of glyphosate intoxicated testis treated with *Newbouldia laevis* extract.

These findings conforms with earlier observations of Owagboriaye et al. (2017), Avdatek, Birdane, Türkmen, and Demirel (2018) and Hashim, Bashir, Yasin, Rashad, and El-Gharbawy (2022) who independently reported several severe histomorphological changes in the testes of glyphosate treated animals.

Apparently, these degenerative changes in the seminiferous tubules may have disrupted spermatogenesis in this study leading to the observed decreased motility, libido, volume, viability, concentration of sperms and also the production of abnormal sperms which is capable of affecting male fertility.

On the other hand, the histological abnormalities viewed in the testes of herbicide intoxicated rabbit bucks treated with *Newbouldia laevis* leaf extract showed significant restoration of testicular architecture. The extract was observed to ameliorate the herbicide-induced histological changes in the testes, including reduced interstitial space, seminiferous tubule damage, and disruption of germ cell layers.

The possible mechanisms by which glyphosate could have exerted these adverse effects are by blood testis barrier (BTB) integrity disruption and testicular oxidative stress (Cavalli et al., 2013; Liu, Li, Lu, Wong, & Wang, 2022). Interestingly, a recent study by Liu et al. (2022) revealed a marked increase in the expression of genes linked to oxidative stress within the testes after exposure to glyphosate. This heightened the generation of reactive oxygen species (ROS) and led to a reduction in the levels of proteins associated with the blood-testis barrier (BTB) in primary Sertoli cells. Consequently, effectively managing the functionality of the testes in cases of glyphosate-induced testicular dysfunction would necessitate the presence of a substance possessing antioxidant capabilities.

Ataman, Osinubi, and Baxter-Grillo (2015) and Mocchegiani and Malavolta (2019) have documented that ascorbic acid, selenium, potassium and zinc are significant constituents of *Newbouldia laevis*. These constituents have been recognized for their potential to enhance sperm parameters, guard against DNA damage, and consequently foster fertility (Abad et al., 2013; Hajjar, Soleymani, and Vatanchian, 2020). Also, *Newbouldia laevis* have been reported to contain saponin, tannin, glycosides, alkaloid, flavonoid, and phenol (Ushie et al., 2021; Ujah, Ugochukwu, & Alozieuwa, 2022). These phytochemicals found in *Newbouldia laevis* are documented to possess antioxidant potentials (Banjarnahor & Artanti, 2015; Nafiu & Ashafa, 2017; Rani, Arora, Kaur, & Manhas, 2018) that could have probably protected the testis from glyphosate deleterious effects. It is therefore plausible to propose that these constituents within *Newbouldia laevis* might have exhibited protective effects.

Conclusion

The data obtained in this study clearly indicates that the exposure of male rabbits to glyphosate herbicide adversely affected sperm quality and testicular histology. However, promising evidence suggests that *Newbouldia laevis* leaf extract may offer a potential solution to mitigate these toxic effects. By exerting antioxidant properties, the extract appears to protect the male reproductive system, maintaining healthy testicular environment and enhancing sperm quality. Further research, including dose-response studies and long-term effects, is necessary to fully understand the potential of *Newbouldia laevis* leaf extract as a protective agent against herbicide-induced reproductive toxicity in male rabbits. This could lead to the development of natural and eco-friendly alternatives for mitigating herbicide-induced reproductive impairments in non-target organisms.

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