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Toxic and Antifertility Potential of Neem Seed and Andrographolide Based Bait against Male House Rat (*Rattus rattus* Linnaeus, 1758)

H. Kaur, M. Verma and B.K. Babbar*

Department of Zoology, Punjab Agricultural University, Ludhiana141 004, India
**E-mail: bhupinder@pau.edu*

***Abstract*:** Anti-reproductive and toxic properties of neem seed alcoholic hexane and andrographolide extract-based bait were investigated in house rat, *Rattus rattus.* Using these extracts three treated baits were prepared, the doses of both extracts were similar in these baits; difference was only in the method of their preparation. In treated bait 1, polymer was used to increase the palatability and bioavailability of secondary metabolites. In treated bait 2, both polymer and bio stabilizer (for thermo-, photo-stability, and bioavailability of secondary metabolites) were added. In treated bait 3, nanoemulsions of both extracts were used. Percentage acceptance of treated bait 2 was also maximum. However, percentage mortality was maximum with treated bait 3 due to Tween 20 added to it. The weight of reproductive organs, and sperm parameters were adversely affected in rats fed on treated bait 2. Histological studies revealed maximum interstitial space, percent seminiferous tubules with disorganized germinal epithelium, and minimum number of Leydig, germ, and Sertoli cells, in rats fed on treated bait 2. Both toxic and antifertility effect of treated bait 2 might be due to the increase in the palatability, bioavailability, and stability of secondary metabolites which can be used for long-term reduction of rodent population.

***Keywords*:** Andrographolide, Neem seed extract, Toxic agent, Antifertility agent, House rat

Rodents cause significant damage to a range of crops as well as under commensal situations (Brown et al 2013). They are extremely adaptable to any environment and have a very high reproductive potential, so they can keep their population at the maximum possible level (Pradhan and Talmale 2011). Rodenticide baits and trapping are commonly used management methods under field and commensal situations respectively. As chemical rodenticides are poisonous, they cause numerous ecological issues necessitating the use of plant-based products (anti-fertility agents and rodenticides) to manage the rodent population (Mandal and Dhaliwal 2007). Moreover, the reproductive potential of the residual population left after the application of these control methods increases so much that the population of rodents again reaches the same level in a short duration after the treatment. To check the sudden increase in the reproductive potential of rodents, antifertility agents based baits are required for the long-term management of rodents.

Plant-based anti-fertility agents and their products are used for ages to regulate the population by affecting spermatogenesis, oogenesis, and other reproductive processes. Flavonoids, Tannins, limonoids, and other compounds found in these plants are responsible for antifertility activities (Daniyal and Akram 2015, Samal 2016). These plant-based compounds are safe, eco-friendly, and have long-lasting effects (Shah et al 2016). Antifertility and toxic effects of *Azadirachta indica* and *Andrographis paniculata* are well known (Idu et al., 2023, Pandey and Rao 2018). Secondary metabolites in neem like azadirachtin, nimbin, nimbidin (Terpenoids), flavonoids, tannins, alkaloids, and phytosterols have toxic and antifertility effects, that could disrupt gonadotropin hormone and spermatogenesis (Kumar and Kumar 2014, Seriana et al., 2021). All the previous studies on antifertility effects were conducted using either pure compounds or by using extracts of polar or non-polar solvents as oral doses against rodents (Seriana et al., 2021). For the application of plant-based extracts as antifertility agents in fields against rodents, it was necessary to mix them in a bait.

Andrographolide and seeds of *A.indica* are well known for their bitter taste. Therefore, it was very difficult to make the rats feed on bait based on these extracts. Moreover, secondary metabolites in neem seed extract also degrade with time being photo-and thermo-labile (Madaki 2015). So, there was a need to formulate stable and palatable bait using these extracts against rodents to use both toxic and antifertility properties of andrographolide and NSAH (Neem seed alcoholic hexane) extracts under field conditions against rodents. Previous studies revealed that oral doses of NSAH extract and andrographolide have toxic and antifertility effects (Chawla 2018, Kaur 2019, Kavita 2021, Verma et al., 2023, Verma et al., 2024, 2025). However, when fed as baits to rats, their toxic and antifertility effects were not as pronounced as with oral doses, which might be due to less bioavailability of secondary metabolites in treated bait to rats because of the reduction in absorption of secondary

metabolites by dietary fats, proteins, and fibers (Bushra et al., 2011, Chawla 2018, Kaur 2019, Kavita 2021). Tran and Hinds (2013) reported that treatment with a higher concentration of promising plant extracts in conjugation with one another can lead to permanent sterility in rodents. As both plants have a different mode of action for their antifertility properties, both can be used in synergism to produce more antifertility effects (Al-Batran et al., 2013). Therefore, during the present investigation, neem seed extract and andrographolide were mixed in bait to synergize their toxic and antifertility effects against rodent pests.

MATERIAL AND METHODS

Experimental details: The study was conducted at Punjab Agricultural University (PAU), Ludhiana located at an intersection of 30˚55' N parallel of latitude and 75˚54' E line of longitude. Approval of Institutional Animal Ethics Committee, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana was obtained for the usage of animals during the 58th Meeting of IAEC held on 26.3.2021 vide Memo No. GADVASU/2021/IAEC/58/05. The male house rats, *R. rattus* were trapped with the help of single and multi-catch rat traps from the storehouses, grocery shops, fish markets, and poultry farms around PAU, Ludhiana. Adult rats (body weight 150-200g) with fully descended testes were selected out of the trapped rats. These rats were acclimatized in laboratory cages (each of size 36 x 23 x 23cm) at 25°C temperature, 30-50% humidity and 14 hour dark/10 hour light cycle for 15-20 days before the commencement of the experiment with food and water provided *ad libtium*. After acclimatization, healthy rats were again weighed and grouped for experimentation (Table 1). The treatment period was 25 days for group 2, 15 days for group 3 and 8 days for group 4. Treatment period varied because of the variation in the toxicity of different treated baits. At the start of experiment, treatment period was planned for 30 days. As rats started dying because of the toxic effect of synergistic bait, rats were necropsied earlier to study the antifertility effect of each treated bait. The toxic effect of NSA bait was not anticipated before starting the experiment. Plastic trays were kept under each laboratory cage to collect and dispose of animal faeces and urine.

Procurement of plant material and chemicals used: Mature dried seeds of *Azadirachta indica* were collected from Punjab Agricultural University, Ludhiana. Andrographolide was procured from Shubhasya Biotech, Bangalore, India. The bio-stabilizer (UV protector, antioxidant, and polymerization inhibitor) and polymer (Inert, non-poisonous, deep pored with stable matrix and occupying large surface area) required for the preparation of treated baits were provided by Orion Organics Private Limited, Ludhiana, India.

Preparation of the neem seed alcoholic hexane (NSAH) extracts and andrographolide powder alcoholic extract (APE)

Three types of NSAH extracts (1, 2 & 3) and 2 types of APE (1 and 2) were prepared.

NSAH extract 1: NSAH extract 1 was prepared as per the method of Subramanian et al (2019) (Fig. 1).

NSAH extract 2: To prevent the degradation of secondary metabolites in neem seeds, 5.3 ml of bio-stabilizer was added to 133g of neem seeds at the time of preparation of its powder to prevent the degradation of active ingredients in the neem seeds (Fig. 1).

NSAH extract 3: Nano emulsion of this neem seed extract was prepared by homogenizing concentrated stock of NSAH extract and 27ml of Tween 20 for 10 minutes (Fig. 1). This solution was then probe sonicated (amplification 25% and 20 KHz) for 30 minutes by keeping the NSAH extract in an ice bath to get the nano emulsion of NSAH extract (Ghotbi et al., 2014).

Andrographolide powder extract (APE 1): Andrographolide (2.67g) was dissolved in 27ml of warm ethanol to get the andrographolide powder ethanol extract (Chen et al 2010).

Nano emulsion of andrographolide powder extract (APE 2): Nano emulsion of andrographolide powder extract was prepared by dissolving 2.67g of AP in tocopherol and ethanol

Table 1. Layout of experiment

Groups	Feed	Duration of treatment (Days)	Number of rats used
Group 1 (Control)	Plain maize sugar (96:4) bait	-	5
Group 2 (Treated group 1)	Treated bait 1 (APE 1(2.67 g AP) and NSAH 1 (133 g NSP) adsorbed on polymer and mixed in maize and sugar (4 gm) to prepare 100g bait)	25	5
Group 3 (Treated group 2)	Treated bait 2 (APE 1 and NSAH 2 adsorbed in polymer and mixed in maize and sugar (4 gm) to prepare 100 g bait).	15	5
Group 4 (Treated group 3)	Treated bait 3 (APE 2 and NSAH 3 adsorbed in polymer and mixed in maize and sugar (4gm) to prepare 100g bait).	8	5

AP: Andrographolide powder; NSP: Neem Seed Powder; APE 1: Andrographolide Powder Alcoholic Extract; APE 2: Nano emulsion of APE 1; NSAH 1: Neem Seed alcoholic hexane (NSAH) extract 1(133g NSP); NSAH 2: NSAH 1 containing bio stabilizer; NSAH 3: Nano emulsion of NSAH 1

in a 1:1 ratio (4.67g of tocopherol and 4.67g of ethanol) and 4.67g of Tween 20 was then added to this solution. This solution was then homogenized for 10 minutes and then probe sonicated for 30 minutes to get the nano emulsion of andrographolide (Yen et al 2018).

Qualitative analysis of the NSAH extract: Qualitative analyses of the NSAH 1, 2, and 3 were carried out . Benedict's Reagent Test for glycosides confirms the presence of glycosides (Biu et al., 2009) while a ferric chloride test was conducted to check the presence of tannins (Biu et al., 2009). To test the presence of saponins, a foam test was performed (Kosma et al., 2011). Salkoniski reaction was performed for the presence of phytosterols (Sasidharan et al., 2011).The appearance of a brownish to the red colour indicated the presence of reducing sugars after adding and boiling an equal quantity of both benedict reagent and NSAH extract (Biu et al., 2009).To test for the presence of terpenoids and alkaloids, the salkoniski test and Mayer's reagent test were performed respectively (Khanal 2021).

Preparation of treated baits using andrographolide and neem seed alcoholic hexane extracts: For the present investigation, three treated baits 1, 2, and 3 were prepared using APE and NSAH extracts.

Treated bait 1: For the preparation of 100g of this bait, stock NSAH extract 1 & APE 1 were adsorbed in a polymer to mask the bitter taste of extracts. Polymer with NSAH 1 and APE 1 was coated with 1 ml sugar syrup and then mixed with maize flour and sugar (4g) to prepare 100g bait.

Treated bait 2: For the preparation of 100gm of this bait, stock NSAH extract 2 & APE 1 were adsorbed in a polymer. This polymer with NSAH 2 and APE 1 was coated with 1 ml sugar syrup and then mixed with maize flour and sugar to prepare 100g bait.

Treated bait 3: For the preparation of 100g of this bait, stock NSAH extract 3 & APE 2 were adsorbed in a polymer. This polymer with NSAH 3 and APE 2 was coated with 1 ml sugar syrup and then mixed with maize flour and sugar to prepare 100g bait.

Toxicity and consumption of treated baits : Adult male rats were divided into four groups with five rats in each group (Table 1). Group 1 was fed on plain maize sugar-based bait mixed in 96:4 ratio. Groups 2, 3 & 4 were fed on treated baits 1, 2 & 3 for 25, 15 and 8 days respectively under bi-choice conditions. Consumption of baits was recorded after every 24 hours. Daily bait consumption (g/100 g body weight), consumption of active ingredients in treated baits, and percent acceptance of treated baits were calculated (Dhar and Singla 2014).

$$\text{Daily bait consumption (g/l00g bw)} = \frac{\text{Daily bait consumption by rat}}{\text{Weight of rat}} \times 100$$

$$\text{Active ingredient consumed} = \frac{\text{Quantity of NSP or Ap powder taken}}{\text{Quantity of treated bait given}} \times \text{Consumption of treated bait (g/100g BW)}$$

$$\text{Percent acceptance} = \frac{\text{Consumption of treated bait}}{\text{Consumption of treated bait +untreated bait}} \times 100$$

Antifertility effect of treated baits: Immediately after the treatment periods (Table 1), male house rats of each group were necropsied to observe the effect of treated baits on the weight of their reproductive organ, sperm parameters, and histomorphology of testes and cauda epididymis.

Effect on sperm parameters: To examine the effect of treated baits on various sperm parameters, sperm motility, viability, abnormalities, and sperm concentration (million/ml) were recorded in the cauda epididymal fluid of untreated and treated rats. Cauda epididymis of three animals from each group was used for the study. Immediately after cutting, one drop of cauda epididymal fluid was kept on the microscopic slide and observed under a microscope to study sperm motility at 5 different sites of a microscopic field of each rat. For sperm concentration, the sperms were counted in twenty-five large squares of charged hemocytometer, and an average number of spermatozoa/square were calculated, which was then multiplied by 10^6 to obtain a million sperms per ml (MJF 2015). Hypo-Osmotic swelling test (HOS test) of caudal epididymal sperms was also performed to determine functionally viable sperms (Jeyendran et al., 1984). To study sperm abnormalities, one drop of cauda epididymal fluid was stained with trypan blue and, smear prepared. Hundred sperms/rat were recorded to calculate % sperm abnormalities, % viability and % sperms with coiled tail. Each parameter was studied at 400x magnification

Effect on histomorphology of reproductive organs: Testis and cauda epididymis were dissected out, free from fat, and fixed in neutral buffered formalin (NBF) for 72 hours followed by dehydration, clearing, and then embedding in paraffin wax (Humason 1979). Transverse sections of testis and cauda epididymis were cut at 5µm thickness and stained with Periodic acid Schiff (PAS) reagent and Haematoxylin respectively. Stained Slides were studied by using Magvision software. The diameter (µm) of seminiferous tubules, epididymis, and their lumen across the major and minor axis were recorded from three rats of each group. The seminiferous tubular diameter was taken for 3 tubules for each stage of seminiferous epithelial cycle (SEC). Several abnormalities of seminiferous tubules were identified and percent disorganized tubules were calculated. Different stages (13 stages) of the seminiferous epithelial cycle (SEC)

were identified (Lagarrigue et al., 2011). The number of spermatogonia, spermatocytes (leptotene, zygotene, pachytene, and diplotene), round spermatids, elongating and elongated spermatids, and Sertoli cells was assessed from seminiferous tubules of different stages of SEC (n=3 for each stage from each group of rats (n=3). Frequency of different stages of SEC in each group was also calculated. Hundred tubules for each group were assessed. Fetal and adult Leydig cells in interstitial tissue were identified and counted from each group of rats (n=3). The true count of almost round cells was calculated (Abercrombie 1946). Each type of cell counted from different stages of seminiferous epithelial cycle were then aggregated.

Where correction factor =

$$\frac{Section\ \ thickness}{Section\ \ thickness + \sqrt{\left(average\ \frac{diameter}{2}\right)2 - \left(average\ \frac{diameter}{4}\right)2}}$$

Statistical analysis: Significance of difference was determined at a 5% level of significance using SAS version 9.4.

RESULTS AND DISCUSSIONS:

Phytochemical studies of NSAH extracts: Qualitative analysis of all three extracts of NSAH (NSAH 1, 2, and 3) revealed the presence of glycosides, tannins, phytosterols, phenolic compounds, saponins, reducing sugar, terpenoids, and alkaloids. The presence of maximum secondary metabolites was in NSAH 2 followed by NSAH 1 and NSAH 3 (Table 2). NSAH extracts were prepared using ethanol and hexane to extract all the secondary metabolites (polar and non-polar like azadirachtin, nimbin, nimbidine, nimbidol, picrin, sialin etc.) with antifertility and toxic properties from the neem seeds (Kumar and Kumar 2014, Fokunang et al., 2019, Braga et al., 2021). The presence of more active components in NSAH 2 could be attributed to the addition of a biostabilizer to neem seeds, which prevented the degradation of secondary metabolites in seeds. The NSAH 3 had the highest rate of degradation of secondary metabolites. NSAH 3 is a nano emulsion of NSAH 1. Secondary metabolites in neem being thermolabile might degrade during the preparation of nano emulsion due to the generation of heat during sonication.

Consumption and toxic effect of treated baits: For the first five days of the treatment period, consumption of all three baits was significantly lower than that of plain bait, but thereafter, there was a non-significant difference between the consumption of plain and treated baits, indicating good palatability. The average percent acceptance and daily consumption of active ingredients (NSP & AP) was significantly more with treated baits 2 & 3 as compared to the treated bait 1 (Table 3, Fig. 2). Increased palatability observed was likely due to the bitter extracts' adsorption on deep-pored, non-reactive stable polymers, which masked the bitter taste and increased the baits' palatability. This polymer also has a large surface area. NSAH extract and andrographolide adsorbed on polymer spread on a large surface area, resulting in increased absorption and so bioavailability of secondary metabolites. Feeding of rats on treated baits 1, 2 & 3 resulted in 20% mortality in 17-25 days, 40% in 12-15 days, and 20-100 % in 2-8 days respectively indicating maximum toxic effect of treated bait 3 (Table 3). Earlier and more mortality with treated bait 2 as compared to bait 1 might be due to more stability and bioavailability of secondary metabolites with toxic effects, due to the addition of bio-stabilizer while preparing neem seed powder. This bio-stabilizer is a polymerization inhibitor and UV protector. The neem seed extract is resinous, it agglomerates rapidly resulting in the least absorption. The maximum toxic effect of treated bait 3 was due to the addition of Tween 20 in NSAH 3 and APE 2 (Eskandani et al., 2013). Previous studies revealed that direct oral administration of andrographolide powder (20, 40, 80 & 500 mg/kg bw) and NSAH extract (6.67g NSP) can cause 40-100% mortality in 24 hours (Kavita 2021, Kaur 2022). Delayed mortality in the present study with treated baits might be due to the still less bioavailability of active ingredients in treated bait to rats because of the reduction in absorption of active ingredients by dietary fats, proteins, and fibers (Bushra et al., 2011).

Effect of treated baits on body weight and reproductive organs: There was a significant reduction in the final body weight of the treated groups as compared to the untreated group (Fig. 3). Weight of the male reproductive organs (testis) also reduced significantly in the treated rats as compared to the untreated rats but a non-significant

Table 2. Qualitative analysis of neem seed alcoholic hexane extracts (NSAH)

Secondary metabolites	NSAH 1	NSAH 2	NASH 3
Glycosides	++	++++	+++
Tannins	+	+++	-
Phytosterols	+++	++++	++
Phenolic compounds	+++	++++	+
Saponins	++++	++++	+++
Reducing sugar	+++	+++	++
Terpenoids	++	+++	+
Alkaloids	++++	++++	+++

NSAH 1: Neem Seed alcoholic hexane (NSAH) extract 1(133g NSP), NSAH 2: NSAH 1 containing bio stabilizer , NSAH 3: Nano emulsion of NSAH1 , ++++: Very high;+++: High; ++: Low; +: Very low

difference in weight was among treated groups, although the reduction in the weight of reproductive organs was more in rats fed on treated bait 2 as compared to the rats fed on other treated baits (Table 3). This indicated the maximum antifertility effect of treated bait 2, which might be due to the stabilization of secondary metabolites having both toxic and anti-fertility properties. Changes in the color (blue-black) of reproductive organs and other organs of the body in group 4 rats indicated the maximum toxic effect of treated bait 3 due to the addition of Tween 20 in this bait.

Effect on sperm parameters: Drastic and significant reduction in percent sperm motility, viability, HOS +ve sperms , and sperm concentration (millions/ml) was recorded in group 3 rats (3.00, 14.00, 22.0, 0.12 respectively) followed by group 2 rats fed on treated baits 2 and 1 respectively as compared to the untreated group (89.4, 86.80, 78.33, 2.69 respectively). Sperm abnormalities like head-tail separation, coiled tail, abnormal head, irregular shape, bent mid-piece, and double tail were also recorded. These sperm abnormalities (%) increased significantly in group 3 rats followed by group 2 rats (Table 4). The treated bait 2 was fed to rats for a shorter duration (15 days only) as compared to treated bait 1 (25 days), however, effects on sperm parameters were almost similar. Therefore, results for sperm parameters indicated the more antifertility effect of treated bait 2 in shorter duration as compared to treated bait 1. The effect on sperm parameters was more drastic in rats fed on treated bait 2 than that reported earlier with individual extracts (Kaur 2019). Most sperms were immotile and dead, with abnormalities reaching 83.33% after only 15 days, likely due to the polymer and bio stabilizer enhancing palatability, stability, and bioavailability, as well as the synergistic action of the compounds. In contrast, treated bait 3 showed limited impact, possibly due to thermo-degradation of antifertility metabolites during nano emulsion preparation. Previous studies also reported adverse effects: neem leaf meal (NLM) in rabbits improved sperm traits only at low inclusion (≤0.85%) but reduced quality at higher levels (Ogbuewu et al., 2022) and aqueous wood-ash extract of *A. indica* decreased sperm motility, viability, and concentration in mice (Auta and Hassan 2016).Neem leaf extract also reduced motility and increased abnormalities in rats (Mishra and Singh 2005). These parameters are clearly indicating the effect on sperm parameters (immotile, dead and abnormalities reaching up to 80%) was severe with treated bait 2 as compared to treated bait 1 and 3.

Effect on testicular histomorphology: To determine the effect of treated baits on testicular histomorphology, the total number of different germ and Sertoli cells in different stages (1-13) of the seminiferous epithelial cycle was determined (Table 5). Compared to the treated groups, the number of different germ and Sertoli cells in different stages of SEC was

Table 3. Comparison of consumption of plain and treated baits, weight of reproductive organs and toxicity among groups

Groups N=5 (Treatments)	Consumption (g/100gm BW)					Weight (g/100g bw)			Percent mortality (Time to death)
	Pre-treatment period (Plain bait)	Treatment period		AIC (AP)	AIC (NSP)	Testes	Corpus and caput epididymis	Cauda epididymis	
		Plain bait	Treated bait						
Control	4.99	4.9^{a1}	4.29^{a1}	-	-	0.77^{a}	0.16^{a}	0.26^{a}	-
Treated bait 1	4.53	4.31^{a1}	2.35^{b2}	0.063^{a}	3.13^{a}	0.64^{ab}	0.18^{a}	0.14^{a}	20 (17 to 25 days)
Treated bait 2	4.98	3.86^{a1}	2.95^{b2}	0.07^{b}	3.93^{b}	0.40^{b}	0.08^{a}	0.10^{a}	40 (12 to 15 days)
Treated bait 3	4.22	3.76^{a1}	2.87^{b2}	0.077^{ab}	3.84^{ab}	0.57^{ab}	0.28^{a}	0.18^{a}	100 (2-8 days)

All values are Mean±SE;; a, b indicate significance difference (p≤0.05) along the columns; 1,2 indicate significant difference (p≤0.05) between the consumption of plain and treated baits, NSAH 1: Neem Seed alcoholic hexane (NSAH) extract 1(133g NSP); NSAH 2: NSAH 1 containing bio stabilizer; NSAH 3: Nano emulsion of NSAH 1 Treated bait 1: APE 1 and NSAH 1 adsorbed in polymer and mixed in maize and sugar (4gm) to prepare 100g bait; Treated bait 2: APE 1 and NSAH 2 adsorbed in polymer and mixed in maize and sugar (4gm) to prepare 100g bait; Treated bait 3: APE 2 and NSAH 3 adsorbed in polymer and mixed in maize and sugar (4gm) to prepare 100g bait Plain bait: Maize: Sugar-96:4; AP: Andrographolide powder; NSP: Neem Seed Powder; APE 1: Andrographolide Powder Alcoholic Extract; APE 2: Nano emulsion of APE 1;

Table 4. Effect of baits on sperm parameters

Groups (Treatments)	Treatment period (days)	Motility (%)	Concentration (million/ml)	Viability (%)	HOS+VE (%)	Sperm abnormalities (%)
Control	-	89.4^{y}	2.69^{y}	86.80^{y}	78.33^{y}	12.66^{y}
Treated bait 1	25	4.25^{x}	0.35^{x}	19.75^{x}	21.66^{x}	76.00^{x}
Treated bait 2	15	3.00^{x}	0.12^{x}	14.00^{x}	22.0^{x}	83.33^{x}
Treated bait 3	8	88.5^{y}	1.10^{y}	82.50^{y}	75.0^{y}	23.00^{y}

See footnote of Table 3 for treatment details

significantly higher in the control group. Overall, treated bait 2 has a substantial effect on the number of different types of spermatogonia (stem cells for spermatogenesis) and Sertoli cells (somatic cells and play a very important role in spermiogenesis). These findings indicated that bio stabilizer added in treated bait 2 increased the stability and thus Bio efficacy of extracts and can cause permanent sterility in rats if further stabilized since it had the maximum effect on germ and somatic stem cells. The total number of seminiferous tubular cells reduced in the treated groups as compared to the control group. Reduction in the total number of cells and spermatids was more in treated group 3 in later stages of SEC. Histomorphological studies also revealed that 16.64% of seminiferous tubules of untreated group 1 were disorganized (Fig. 4). However, the percentage of seminiferous tubules with disorganized epithelium increased in treated groups and was maximum in group 3 rats indicating that treated bait 2 has a maximum effect on the organization of germinal epithelium . The percent area of interstitial space was minimum in the control group as compared to the treated groups (Fig. 5, 7), being maximum in rats of Group 3 due to the maximum shrinkage of seminiferous tubules. The number of adult Leydig cells and fetal Leydig cells was significantly more in group 1 as compared to the treated groups. Among treated groups, the number of adult Leydig cells and fetal Leydig cells was also significantly less in group 3 followed by groups 2 and 4 (Fig. 6), indicating the maximum antiandrogenic effect of treated bait 2. Thasmi et al (2021) reported that when rats were treated with neem leaf extract @ 200mg/kg bw, the number of Leydig cells decreased resulting in a low level of testosterone in male rats. When neem leaf meal was given to rats (15%), the number of Leydig cells decreased. An increase in interstitial space and reduction in LH and FSH levels was also reported when rats were treated with neem oil @1.2 ml/animal (Shaikh et al., 2017).

In the treated groups, seminiferous tubular diameter and luminal diameter decreased markedly in all the stages of SEC, indicating shrinkage of seminiferous tubules. However, due to the disruption of the germinal epithelium, its thickness increased in treated groups 2, 3, and 4 (Fig. 7). Earlier studies also reported a reduction in the diameter of seminiferous tubules in male rats when fed orally on neem seed extract @159 mg/kg bw (Daniyal and Akram 2015). When male quails were fed on neem seed (40%) based bait, the diameter of seminiferous tubules of treated males decreased significantly (Gois et al 2019). In groups 2 & 4 rats, vacuolization of germinal epithelium was prominent. Pathological changes observed in group 3 rats include tubular atrophy, exfoliation and depletion of germ cells, shrinkage of seminiferous tubules, the occurrence of pyknotic cells, and vacuolization in seminiferous tubules indicating impaired spermatogenesis (Fig. 7). Previous studies supported our finding as an aqueous extract of neem leaf (200mg/kg bw/day for 30 days) caused vacuolization in seminiferous tubules (Santra and Manna 2009). In some of the seminiferous tubules, cellular associations were also disturbed due to the drastic reduction in the number of various germ cells and exfoliation of germinal epithelium. The treated bait 2 affected spermatogenesis and spermiogenesis, by targeting stem germ cells (spermatogonia and Sertoli cells), and Leydig cells due to synergistic effect of stabilized secondary metabolites of NSAH extract and andrographolide in treated bait 2 leading to permanent sterility in male house rats. Spermatogenesis occurs under the influence of FSH and Leydig cells produce testosterone under the influence of LH. Reduction in the number of different types of testicular cells during the present study clearly indicated hormonal disturbances in treated rats leading to severe effect on both spermatogenesis and spermiogenesis (Santi et al., 2020). Spermatogonia in the testis are the most primitive spermatogonial stem cells (SSCs), which play an important role in maintaining highly productive spermatogenesis by self-renewing and continuously generating daughter spermatogonia that differentiate into spermatozoa (Kubota and Brinster 2018). In the absence of Sertoli cells, haploid round spermatids are unable to form their tails and remain immotile, preventing them from going through the spermiogenesis process. Sertoli cells give metabolic assistance and sustenance to growing sperms. Because spermatogonia are precursors of spermatogenesis and

Table 5. Effect of baits on true count of cells of seminiferous tubules

Groups	Number of cells per seminiferous tubule									Total number of cells
	SG	PL	L	P	Z	D	RS	EL	SC	
Control	22.13^{a}	20.93^{a}	36.96^{a}	40.87^{a}	28.72^{a}	30.28^{a}	91.32^{a}	62.27^{a}	4.17^{a}	338
Treated bait 1	15.47^{ab}	14.30^{b}	28.59^{ab}	27.68^{a}	21.19^{a}	21.64^{b}	62.97^{b}	45.16^{b}	3.44^{b}	240
Treated bait 2	5.23^{b}	3.20^{c}	23.67^{b}	31.00^{a}	21.66^{a}	21.74^{b}	64.15^{b}	44.49^{b}	1.63^{c}	217
Treated bait 3	12.67^{ab}	11.70^{b}	29.75^{ab}	30.04^{a}	26.59^{a}	28.49^{ab}	70.53^{ab}	53.50^{ab}	3.51^{b}	267

SG: Spermatogonia, PL: Preleptotene, L: Leptotene, P: Pachytene, Z: Zygotene, D:Diplotene, RS: Round cells, EL: Elongating cells, SC: Sertoli cells

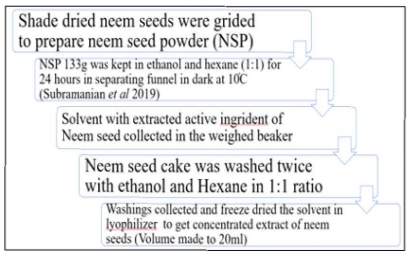

Fig. 1 Method of preparation of neem seed alcoholic hexane (NSAH) extract.

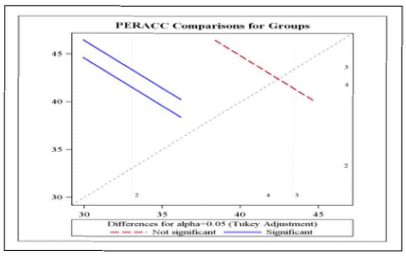

Fig. 2: Comparison of percent acceptance (PERACC) of treated baits among different groups of rats

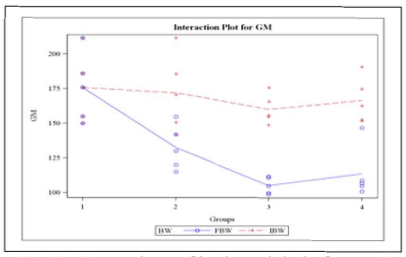

Fig. 3: Comparison of body weight before and after the treatment period among groups. IBW & FBM: Initial & Final body weight

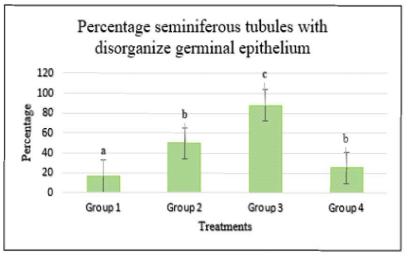

Fig. 4 Percent seminiferous tubules with disorganized germinal epithelium

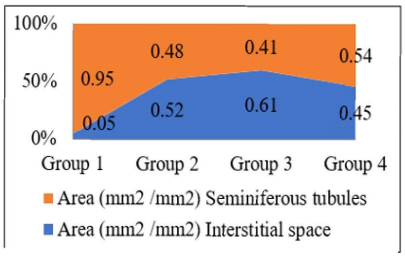

Fig. 5 Percentage area occupied by interstitial tissue and seminiferous tubules in different groups

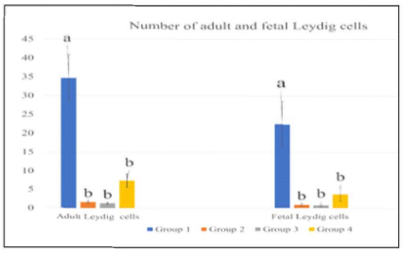

Fig. 6: Effect of treated baits on the number of adult and fetal Leydig cells

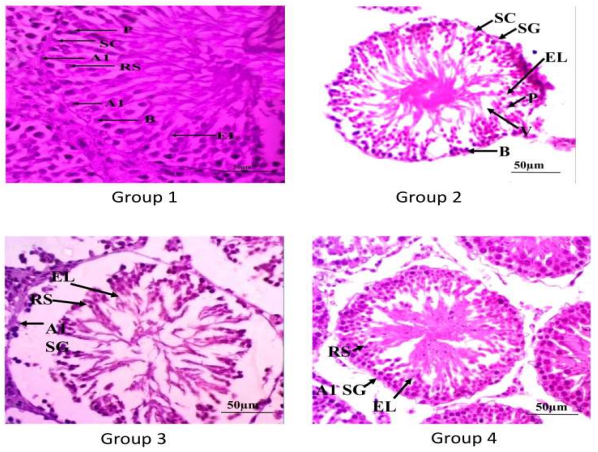

Fig. 7. Seminiferous tubules in different groups (PAS stained slides) showing vacuolization in groups 2 and 4 and exfoliation of germinal epithelium, pyknosis and reduction in the number of round spermatids in group 3, Magnification-400X

Sertoli cells supply nutrients and a favorable environment for growing spermatozoa, their absence or reduction in quantity in rats might result in lifelong infertility (Griswold 2018). Andrographolide affected spermatogenesis in male rats by preventing cytokinesis of the dividing spermatogenic cell lines. Sertoli cells were completely damaged and the spermatotoxic effect was also observed (Akbarsha and Murugaian 2000). Another study reported reduction in sperm density and the number of primary and secondary spermatocytes in rats treated with neem oil (Purohit et al., 2008). Ethanolic extract of all parts of *A. paniculata* given orally to adult male DDI mice @ 45mg/30 g bw impaired spermatogenesis and damaged Sertoli cells (Halim et al., 2005).

CONCLUSIONS

There was minimum antifertility effect of treated bait 3, which might be due to the thermo-degradation of secondary metabolites in the extracts at the time of preparation of their nano emulsion due to production of heat during probe sonication. Both the antifertility and toxic effect of treated bait 2, might be due to the synergistic effect of neem seed extract and andrographolide, addition of bio-stabilizer in this bait, which increased the thermo- and photostability and bioavailability of secondary metabolites in the bait. Adsorption of extracts in polymer masks the taste and also increased the bioavailability of secondary metabolites increasing its efficacy. As treated bait 2 is palatable and has both toxic and antifertility effects, can be successfully applied under field conditions for the long-term management of rodent pests. However, further studies are required to determine, the efficacy, the shelf life and stability of treated bait 2 under real field conditions.

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