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RESEARCH ARTICLE

Assessment of *Amaranthus hypochondriacus* L. efficacy on oxidative stress and reproductive parameters in fipronil intoxicated male albino rats

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Abstract

The therapeutic potential of *Amaranthus hypochondriacus* L. attributed to their diverse phytochemical makeup. Earlier studies have identified various bioactive phytoconstituents such as flavonoids, terpenoids, tannins, saponins, phytosterols and phenols in different parts of plant, including flower, seeds, leaves and stems. These phytochemicals have therapeutic importance, especially antioxidant, anti-inflammatory and cholesterol-lowering properties. The antioxidant activity of flavonoids and phenols could help the protection of oxidative damage. Although direct research on the therapeutic application of *A. hypochondriacus* treating reproductive disorders is limited, their active components suggest promising benefits. Further studies are required to determine their efficacy, appropriate dosage, safety parameters and underlying mechanisms of action. The aim of this study was to evaluate the *A. hypochondriacus* seed extract potential on oxidative and reproductive parameters in fipronil-intoxicated male Wistar rats. In the current investigation, group I given normal feed and water served as control, group II was administered with fipronil (at 24.25 mg/kg body weight), in hydroethanolic seed extract of *A. hypochondriacus* (HSEAH) (at 100 mg/kg body weight) in group III and combination of Fipronil (at 24.25 mg/kg body weight) and HSEAH (at 100 mg/kg body weight) was administered in group IV for 90 days in rats followed by estimating the parameters related to oxidative stress and reproductive toxicity parameters and histopathological examination. The results of study showed HSEAH treatment significantly (p<0.05) reduced the toxicosis caused by oxidative stress and elevated the levels of antioxidant enzymes such as reduced glutathione (GSH), lipid peroxidation (LPO) and catalase (CAT) in the various vital organ tissues. The present study offers an opportunity to explore the potential therapeutic properties of *A. hypochondriacus* in combating fipronil induced oxidative stress and reproductive toxicity.

Keywords: albino rats; *Amaranthus hypochondriacus*; fipronil toxicity; oxidative stress; reproductive toxicity

Introduction

Environmental pollution represents a pervasive and escalating threat to global health, with synthetic chemicals from agricultural and industrial activities constituting a major class of hazardous contaminants. Among these, pesticides are of particular concern due to their intentional release into the environment and their inherent biological activity. While crucial for crop protection and disease control, their persistence and non-target effects pose significant risks to ecosystems, wildlife and human health (1). Fipronil, a potent environmental pollutant, has garnered significant attention due to its widespread use in agriculture, veterinary medicine and pest control (2). This broad-spectrum phenylpyrazole pesticide is favoured for its effectiveness against a wide array of pests, ranging from insects to rodents. However, its pervasive use has raised concerns regarding its adverse impacts on ecosystems, non-target organisms and human health (3). Moreover, fipronil's toxicity extends beyond its acute effects on target pests, encompassing sub-lethal and chronic effects on non-target organisms. Studies have documented detrimental impacts on beneficial insects, aquatic organisms, birds and mammals, including humans (4). Fipronil exhibits potential toxicological effects on the nervous, reproductive and developmental stages, which may manifest even at low doses or chronic exposures (5). Among the many detrimental effects observed in man and animals, oxidative stress and reproductive toxicity are significant concerns. Fipronil have been documented to induce such oxidative stress, leading to reproductive dysfunction (6). The male reproductive system is a complex and finely regulated system, with spermatogenesis being a primary indicator of its functional state. This process, occurring within the seminiferous tubules of the testes, is highly susceptible to

oxidative stress and toxicant exposure (7). Sperm motility is a critical parameter of male fertility, as it is essential for successful ovum fertilisation. Impairments in motility are often a consequence of damage to the sperm plasma membrane and mitochondrial dysfunction, frequently induced by reactive oxygen species (ROS) (8). Reactive oxygen species production and the antioxidant defence system's capacity to neutralise them are out of balance, which leads to oxidative stress (9). This imbalance can lead to damage to lipids, proteins and deoxyribonucleic acid (DNA), ultimately affecting cellular function and viability (10). Reproductive toxicity, characterized by alterations in sperm quality, testicular histology and hormone levels, poses serious threats to male fertility and reproductive health (11). *Amaranthus hypochondriacus* L., commonly known as Prince's feather, is a grain crop belonging to the family Amaranthaceae. It has attracted researchers in recent years due to its important therapeutic benefits attributed to its rich nutritional composition and bioactive compounds, including amaranthin, polyphenols, tocopherols and flavonoids (12). Several investigations have reported the antioxidant properties of *A. hypochondriacus*, indicating its potential therapeutic effects against oxidative stress-induced pathogenesis (13). Amaranth seeds contain a complex of tocopherols, primarily α-tocopherol and γ-tocopherol. These are potent fat-soluble antioxidants that protect cellular membranes from lipid peroxidation, directly relevant to mitigating oxidative stress (14). Given the oxidative stress-inducing properties of fipronil and the antioxidant potential of *A. hypochondriacus*, there is a rationale to investigate the efficacy of *A. hypochondriacus* in mitigating oxidative stress and reproductive toxicity induced by fipronil exposure in male albino rats. Understanding the protective effects of *A. hypochondriacus* in this context could have significant implications for developing strategies to mitigate the adverse effects of pesticide exposure on mammalian health and fertility. This study aims to assess the efficacy of *A. hypochondriacus* on oxidative stress markers, sperm quality, testicular histology and hormonal levels in fipronil-intoxicated male albino rats.

Materials and Methods

Chemicals and reagents

The chemical toxicant used in this study was fipronil (Jump®, 80 % w/w), which was procured from Bayer (India). All other chemicals and reagents used were of analytical grade. Diethyl ether was used as an anesthetic. For the biochemical assays, Ethylenediaminetetraacetic acid (EDTA), Phosphate Buffered Saline (PBS), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), hydrochloric acid (HCl), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), trichloroacetic acid (TCA), Tris(hydroxymethyl) aminomethane (Tris) buffer and hydrogen peroxide (H_2O_2) were employed. For reproductive and histopathological evaluations, Eosin Y, Nigrosin, Neutral Buffered Formalin (NBF), xylene and Hematoxylin and Eosin (H&E) stain were utilised. All these reagents were sourced from reputable suppliers, including Sigma-Aldrich (USA), Sisco Research Laboratories (SRL, India) and Himedia Laboratories (India).

Preparation of extract

Seeds of *A. hypochondriacus* were obtained from the department of Molecular Biology & Genetic Engineering, Govind Ballabh Pant University of Agriculture and Technology, Uttarakhand. The seeds were collected post-harvest in the month of September to ensure consistent phytochemical profile, as the time of collection is a recognised factor influencing plant constituents. Seeds were grown in polyhouse and plants were taxonomically authenticated by the National Botanical Research Institute (NBRI), Lucknow vide No. 109669 dated 29.09.2021. Plant specimen has been deposited in herbarium of NBRI, Lucknow. The hydroethanolic extracts were prepared by using of 500 g of seed powder, allowed to soak in 5000 mL hydroethanol solution (1:1) for 24 hr with frequent agitation at room temperature. The mixture was filtered by muslin cloth in Buchner funnel and then through Whatman filter paper no. 42. The residue was again soaked with fresh solvent for another 24 hr and this process was repeated two times. The extracts were obtained by drying the filtrate in fan incubator at a temperature of 40 °C. The final dried extracts were stored for later use at 4 °C in airtight glass containers. To ensure a consistent and fresh supply for the 90-day study, the extract was prepared in three separate batches at one-month intervals. Each batch was used for the subsequent month of dosing, thereby preserving its bioactivity throughout the entire experimental period.

Phytochemical analysis

A qualitative phytochemical analysis of the extract was performed using standard protocols (15) to identify the presence of major bioactive compound classes.

Test for alkaloids (Dragendorff's test)

Approximately 50 mg of hydroethanolic seed extract of *A. hypochondriacus* (HSEAH) was dissolved in 5 mL of 1 % aqueous hydrochloric acid. The solution was heated in a water bath for 5 min and filtered. To 2 mL of the filtrate, a few drops of Dragendorff's reagent were added. The formation of an orange or red precipitate was taken as evidence for the presence of alkaloids.

Test for flavonoids (Alkaline reagent test)

About 50 mg of the extract was dissolved in 5 mL of distilled water. To this solution, a few drops of dilute sodium hydroxide (NaOH) solution were added. The appearance of an intense yellow colour, which disappeared upon the addition of dilute hydrochloric acid (HCl), indicated the presence of flavonoids.

Test for tannins (Ferric chloride test)

A small quantity of HSEAH (50 mg) was dissolved in 5 mL of distilled water and filtered. To the filtrate, a few drops of 5 % ferric chloride ($FeCl_3$) solution were added. The formation of a bluish-black or greenish colour was considered a positive test for tannins.

Test for saponins (Froth test)

Roughly 100 mg of the extract was shaken vigorously with 5 mL of distilled water in a test tube. The formation of a stable, persistent froth (foam) of more than 1 cm in height that lasted for 10–15 min confirmed the presence of saponins.

Test for terpenoids (Salkowski test)

To 50 mg of the extract dissolved in 2 mL of chloroform, 2 mL of concentrated sulfuric acid (H_2SO_4) was carefully added along the side of the test tube to form a layer. The formation of a reddish-brown coloration at the interface was recorded as a positive result for terpenoids.

Test for phenols (Ferric chloride test for phenols)

To 2 mL of the extract solution (50 mg in 5 mL water), a few drops of neutral 5 % ferric chloride solution were added. The observation

of a dark green or blue colour was taken as a positive test for phenolic compounds.

Test for proteins (Biuret test)

To 2 mL of the extract solution, 1 mL of 10 % sodium hydroxide (NaOH) solution was added, followed by 1–2 drops of 1 % copper sulfate ($CuSO_4$) solution. The mixture was shaken gently. The appearance of a violet or purple colour indicated the presence of proteins.

Test for carbohydrates (Molisch's test)

To 2 mL of the extract solution, 2 drops of Molisch's reagent (an alcoholic solution of α-naphthol) were added. Then, 1–2 mL of concentrated sulfuric acid was carefully poured down the side of the test tube to form a layer. The formation of a red or purplish ring at the interface confirmed the presence of carbohydrates.

Experimental model

The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) vide approval number IAEC/CVASc./VPT/449 dated 12.12.2020. The therapeutic efficacy of *A. hypochondriacus* hydroethanolic seed extract was evaluated in 24 male Wistar rats of 6-week-old age, weighing between 90–100 g. The animals were housed under standard laboratory conditions (12 hr light and 12 hr dark cycle, 25 ± 2 °C, 50–60 % humidity). Throughout the experimental period, the rats were provided ad libitum access to water and a standard pellet diet (providing approximately 20–22 % protein, 4–5 % fat, 4–5 % fibre and essential vitamins and minerals) obtained from M/s. Godrej Agrovet Ltd., Maharashtra, India. Animals were divided into four groups of six rats each for conducting 90 days sub chronic study. Group I (Control Group) received a standard diet and ad libitum water. Group II (Fipronil-Intoxicated Group) orally administered fipronil at a dose of 24.25 mg/kg body weight per day. This group was used to establish the toxic effects of fipronil on oxidative stress and reproductive parameters. The dose of fipronil 24.25 mg/kg body weight was selected based on the sub-chronic toxicity study by earlier research, which established this dosage as effective in inducing significant oxidative stress and histopathological alterations in the liver and kidney of male albino rats over a 90-day period (16). This dose represents approximately $1/10^{th}$ of the reported oral LD50 for rats, a level commonly used in sub-chronic toxicological studies to ensure manifest toxicity without causing rapid mortality. Group III (HSEAH-Control Group) only administered the hydroethanolic seed extract of *A. hypochondriacus* (HSEAH) at a dose of 100 mg/kg body weight per day orally. This group served to evaluate the safety and any inherent effects of the extract itself. This dose was selected based on its established efficacy and safety profile in prior studies investigating the antioxidant and hepatoprotective properties of *A. hypochondriacus* extracts (17). Group IV (Fipronil + HSEAH Treatment) co-administered both fipronil (24.25 mg/kg body weight) and HSEAH (100 mg/kg body weight) per day orally. This group was essential for assessing the protective and ameliorative efficacy of the seed extract against fipronil-induced toxicity.

Collection of test samples

The sample was collected after 90^{th} day of experiment. On the last day of the experiment, animals were fasted overnight (for approximately 12 hr) but allowed free access to water prior to the induction of anesthesia and collection of blood samples. Rats were anaesthetised with diethyl ether and sacrificed humanely by cervical dislocation. After the course of treatment, the vital organs (viz. liver, kidney, brain, heart, spleen and testis) were surgically removed and blotted on tissue paper and processed. A 500 mg of tissue sample were weighed and added in 5 mL of ice-cold phosphate-buffered saline (PBS) (pH 7.4). The resulting homogenate was then stored at -80 °C until further analysis. For reduced glutathione estimation, an additional 200 mg of sample from each tissue was weighed individually and placed in 2 mL of 0.02 M EDTA solution. The 10 % homogenates were made under ice-cold conditions with the help of homogeniser and they were centrifuged for 10 min at 3000 rpm. The supernatant was used for the study. For reproductive health evaluation cauda epididymis were removed. Sperm were collected from cauda epididymis, by cutting into small pieces with scissors and flushing with PBS (pH-7.4) at 37 °C to take out the cauda epididymal fluid which is immediately used for sperm quality evaluation (18).

Determination of oxidative enzymatic biomarkers

Lipid peroxidation (LPO)

The sample was centrifuged for 10 min at 3000 rpm prior to use. The 25 µL of butylated hydroxytoluene (BHT), 250µL of thiobarbituric acid (TBA) and 250µL of HCl were combined with 250µL of supernatant. Before centrifuging, the mixture was kept to 95 °C for 10 min in a water bath. The supernatant was transferred into a quartz cuvette tube after centrifuging and the absorbance at 535 nm was measured.

$$\text{LPO (nmole.MDA/g)} = \frac{\text{OD of test}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample taken}} \times 10^9 \times \text{DF} \times \text{IT}$$

Here, EC stands for extinction coefficient, IT stands for incubation time (in hours), DF for dilution factor, MDA stand for malondialdehyde and OD for optical density.

Reduced glutathione

Reduced gluthathione was calculated by applying the 5-5' dithiobis 2-nitrobenzoic acid (DTNB) method to estimate free sulfhydryl groups. A 10 % homogenate was prepared using 0.02 M EDTA. After adding 1 mL of the homogenate supernatant, 0.8 mL of water and 0.2 mL of a 50 % trichloroacetic acid (TCA) solution, the mixture was incubated for 15 min at room temperature. After that, the mixture was centrifuged for 15 min at 3000 rpm and 0.4 mL of the supernatant was taken out. This was mixed with 0.2 mL of DTNB solution and 0.8 mL of tris buffer. Absorbance was measured at 412 nm within the 5 min against blank.

$$\text{GSH (mM/g)} = \frac{\text{OD of test}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample taken}} \times \text{DF} \times 1000$$

Here, EC stands for extinction coefficient, DF for dilution factor and OD for optical density.

Catalase (CAT)

Five microliters of tissue homogenates were added to 1 mL of phosphate buffer in an eppendorf tube and the contents were then transferred straight into the cuvette tube. Then, 1 mL of

hydrogen peroxide was added. The reaction was initiated and the OD was measured at 240 nm against blank every 30 sec for 3 min.

$$\text{CAT (U/mL)} = \frac{\triangle OD / time}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{amount of sample taken}} \times \frac{1}{\text{Amount of tissue homogenate}}$$

Reproductive health assessment

Sperm motility (%)

A drop of cauda epididymal fluid was placed on a sterile glass slide and examined under a microscope to determine the motile sperm. The percentage of progressively motile sperms among total sperms in one visible microscopic field at 100X power was used to score sperm motility.

Sperm viability (%)

One drop of cauda epididymal fluid was taken on a glass slide and added one drop of 0.1 % eosin solution in 0.9 % saline kept at 37 °C to determine the percentage of viable sperm count. It was covered with a coverslip after 2 min. At 400X magnification power, the number of live, transparent-headed spermatozoa and dead, pink-headed spermatozoa per 100 sperms of an animal was recorded.

Sperm density

The Neubauer hemocytometer, which is frequently used to count red blood cells was used to measure the density of spermatozoa. The cauda epididymal fluid was diluted in a pipette containing 0.02 % eosin in 0.09 % saline solution at a ratio of 1:200. After charging the haemocytometer chamber with diluted cauda epididymal fluid, it was examined under a 40X optical microscope. The spermatozoa that were inside the square and those that touched two of its sides in five secondary squares, 4fourat each corner and one in the center, each with 16 tertiary squares were counted. To calculate the number of spermatozoa in millions/mL, the average of the total number of sperm in five secondary squares was multiplied by 10^7.

Sperm morphology

Eosin/nigrosin stain was used for the evaluation of the morphology of the spermatozoa. The 40 µL of sperm suspension and 10 µL of 1 % eosin Y and nigrosin taken in a test tube and mixed properly. For staining, the sperm suspension was incubated for 45–60 min at room temperature. The percentage of abnormal sperm morphology was calculated by counting the number of normal and abnormal sperm per 100 sperm per slide of each animal at 400X magnification power. Spermatozoa on each slide were analyzed under a light microscope.

Histopathological examination

Small portions of testis taken from animals of each group were fixed overnight in ten percent neutral buffered formalin (NBF) to preserved tissue from putrefactive and autolytic conversion and to maintain the cellular components. Then tissue was washed in running tap water for 24 hr, dehydrated in series of different concentration of ethyl alcohol, cleared in xylene and embedded in paraffin wax. Six-micron thick sections were prepared by microtome. Hematoxylin and eosin were used to stain these sections and were then examined using a light microscope.

Statistical analysis

The sample size of n = 6 per group was determined based on established standards for sub-chronic *in vivo* toxicity studies. For the analysis of variance (ANOVA) that was used, this sample size (total N = 24) provided sufficient degrees of freedom to detect statistically significant differences ($p<0.05$) with good power for large effect sizes, which were typically expected in this toxicological challenge model. Results of the study were analysed by IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA) by applying t-test, one way ANOVA and two-way ANOVA for reproductive parameter and oxidative stress parameters respectively and expressed as Mean ± SE. The differences were considered statistically significant at $p<0.05$ or lower as determined by Tukey's and Newman-Keuls multiple comparison test. Graphs of the study were developed by using the Graph Pad Prism 9 software program (San Diego, CA, USA).

Results and Discussion

Extract analysis

The percent yield of the *A. hypochondriacus* seed extract was 4.08 % w/w dry matter and brownish yellow in colour. The qualitative phytochemical studies of HSEAH revealed the presence of several major classes of bioactive compounds including alkaloids, flavonoids, phenols, terpenoids, proteins, carbohydrates and fats or fixed oils. These results confirm that HSEAH is a complex mixture rich in phytoconstituents widely recognized for their antioxidant and therapeutic properties, which align with the observed biological activities in this study.

Clinical observation

The present study evaluated *A. hypochondriacus* seed extract potential on oxidative and reproductive parameters in fipronil intoxicated male albino rats in which no mortality could be observed in any of the treatment group. However, feed intake was reduced and hyperactive behavior showed in fipronil treated group. Hyper active behaviour in fipronil treated rats maybe due to fipronil and its metabolite fipronil sulfone binding to γ-Aminobutyric acid type A ($GABA_A$) receptors which diminishes the hyperpolarising chloride current they cause hyperexcitability of the neuron resulting seizures and convulsion in animals (19).

Oxidative stress biomarkers

Oxidative stress biomarkers (Fig. 1–3) LPO, GSH and CAT were determined in testis, liver, kidney spleen, brain and heart tissues of rats exposed to 24.25 mg/kg body weight of fipronil for 90 days. *In vivo* oxidative stress related parameters, various tissues of fipronil intoxicated groups reflected the changes due to extreme oxidative damage. But these changes were found to be improved in the HSEAH treated rats. The significantly ($p<0.05$) decreased LPO levels in the organs of HSEAH treated groups may be due to reduction in the oxidant mediated stress in these tissues. The reduced levels of GSH in various organs of fipronil treated groups may be considered as oxidative damage due to fipronil intoxication. The catalase activity in tissues also followed the same trend as GSH in fipronil treated animals, probably as a response to fipronil intoxication stress. The elevated levels of these enzymes in HSEAH treated groups can be considered as an indication of the antioxidative property, which can in turn lead to inhibition in the progression of toxicity. Fipronil and its

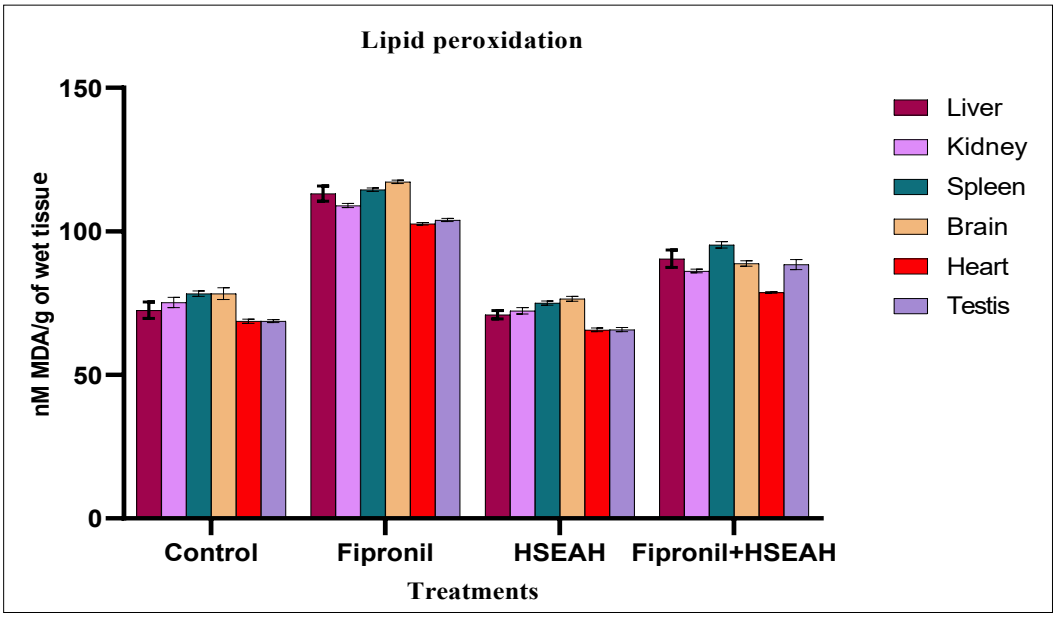

Fig. 1. Effect of hydroethanolic seed extract of *Amaranthus hypochondriacus* on lipid peroxidation (nM MDA/g of wet tissue) following oral administration fipronil for 90 days in rats. Data are presented as mean ± SE (n = 6; p<0.05).

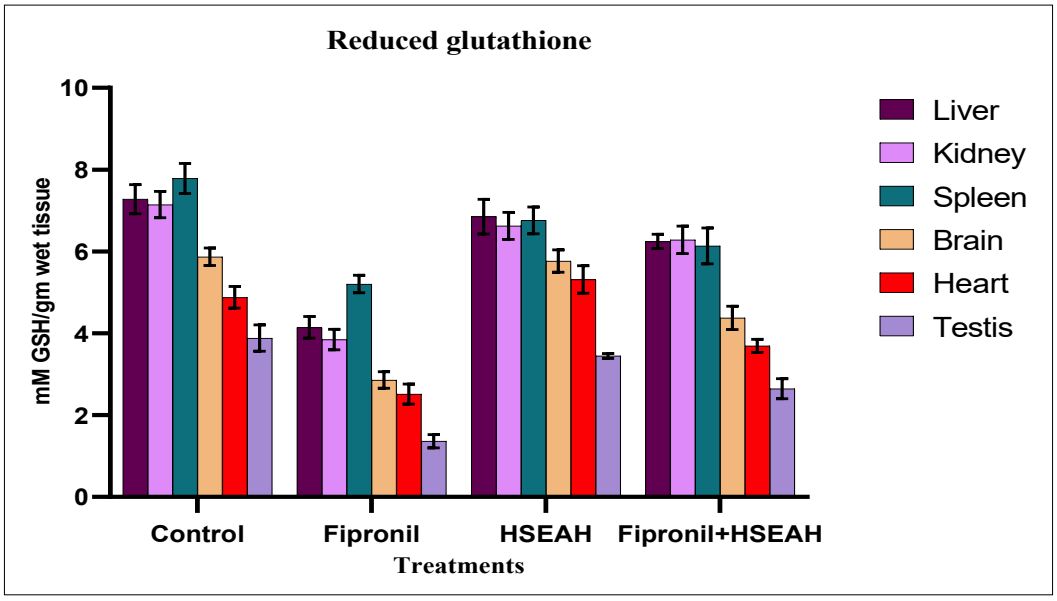

Fig. 2. Effect of hydroethanolic seed extract of *Amaranthus hypochondriacus* on GSH (mM GSH/gm wet tissue) following oral administration fipronil for 90 days in rats. Data are presented as mean ± SE (n = 6; p<0.05).

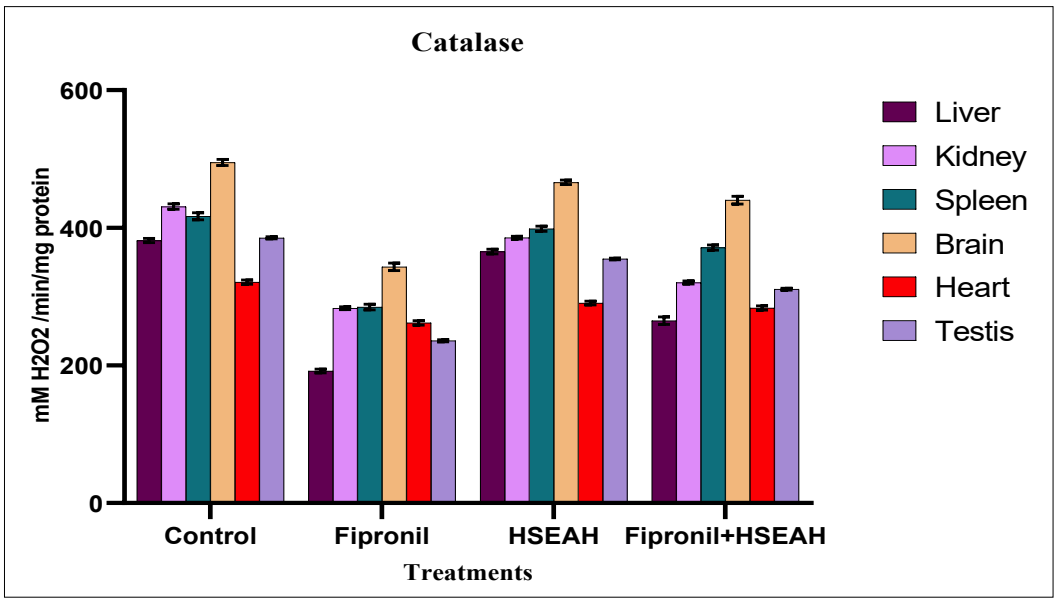

Fig. 3. Effect of hydroethanolic seed extract of *Amaranthus hypochondriacus* on catalase (mM H2O2 utilised/min/mg protein) following oral administration fipronil for 90 days in rats. Data are presented as mean ± SE (n = 6; p<0.05).

sulfone metabolite are known to induce oxidative stress not only through GABA-A receptor disruption but also by directly impairing mitochondrial function, leading to excessive superoxide generation (20). This ROS burst depletes primary antioxidants like GSH and can directly inhibit enzymatic activity, including that of catalase (21). The significant restoration of these parameters in the HSEAH co-treatment group (IV) can be mechanistically linked to the direct free-radical scavenging capacity of its constituent flavonoids (e.g., rutin, quercetin) and tocopherols, which intercept ROS before they can damage cellular components. Furthermore, compounds like squalene may upregulate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (22), a key transcriptional regulator of antioxidant response elements (ARE), thereby promoting the *de novo* synthesis of GSH and antioxidant enzymes like catalase, thus restoring the cellular redox balance. Findings on sub chronic toxicity in earlier studies also revealed that fipronil treatment caused oxidative stress in the vital organs of male rats, which is evident from the generation of LPO and a reduction in GSH (16). However, the degree of LPO increase in our study was more pronounced, potentially due to the longer exposure period. Lipid peroxidation is known to disturb the membranes cellular integrity and induces toxicosis in various vital organs of animals (23). Therefore, it has used as biochemical markers of fipronil induced oxidative stresses and suggested as one of the molecular mechanisms involved in pesticides-induced toxicity (24). Both enzymatic and non-enzymatic, antioxidants collaborate to mitigate the effects of ROS in tissues and actively guard against oxidative cell damage and work as a free radical scavenger. Superoxide dismutases (SOD) expedites the dismutation of superoxide anion into a less reactive molecule (H_2O_2), which is then quickly transformed into oxygen and water by CAT (25). Our result was in accordance with previous researchers who reported a catalase level GSH and LPO altered by sodium arsenite in male Wistar rats. The subsequent reversal of these biomarkers' values by HSEAH to near-normal levels demonstrates a protective efficacy (17).

Reproductive health assessment

The effects of fipronil on reproductive health assessment after 90th day in rats are presented in Table 1 and Fig. 4 and 5. Reproductive toxicity study parameters, sperm motility, sperm viability and sperm density decreased and sperm head tail separation and other sperm abnormalities were increased significantly (p<0.05) in fipronil treated group in comparison of control group animals. However, rats treated with HSEAH animals showing improvement of these reproductive parameters in comparison of control group. The sperm morphology, fipronil treated group II rat sperm shows higher number of defected and dead sperm. Group III rat treated with HSEAH showed normal sperm morphology and vitality. Whereas fipronil combination with

Table 1. Effect of hydroethanolic seed extract of *Amaranthus hypochondriacus* on reproductive health parameters

		Reproductive health assessment				
Groups	**Treatments**	**Sperm motility (%)**	**Sperm viability (%)**	**Sperm density (million/mL)**	**Sperm head tail separation (%)**	**Other sperm abnormalities (%)**
I.	Control	70.5 ± 1.45^a	66.5 ± 3.06^a	301 ± 6.6^b	15.3 ± 1.26^b	15.8 ± 1.3^b
II.	Fipronil	41.5 ± 3.7^b	32.3 ± 2.33^c	189 ± 8.51^c	31.3 ± 1.93^a	35.2 ± 2.32^a
III.	HSEAH	74.7 ± 2.4^a	68.2 ± 2.39^a	338 ± 8.03^a	13.3 ± 1.26^b	14.7 ± 1.8^b
IV.	Fipronil+ HSEAH	48.2 ± 2.12^b	42.3 ± 2.14^b	279 ± 6.76^b	28.3 ± 1.5^a	28.2 ± 2.43^a

Values in the table are mean ± S.E. (n = 6); Values having different superscripts (a, b, c) differ significantly (p<0.05) when compared within a column.

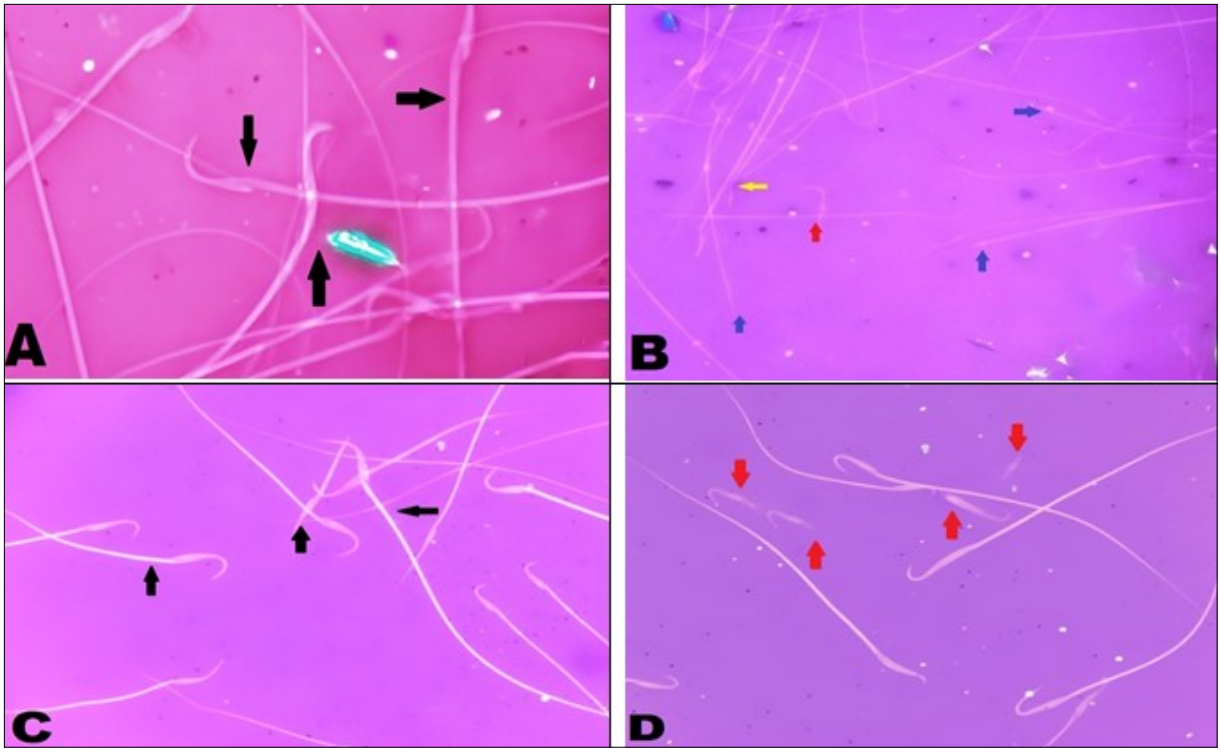

Fig. 4. Light microscopic images of sperm morphology in different treatment groups: A - Light microscope photograph of control group I rat showing normal appearance of sperm (black arrow); B - Group II rat received fipronil (at 24.25 mg/kg body weight alone) showing severe sperm abnormalities separation of head (red arrow) and tail (blue arrow), bending of neck (yellow arrow); C - Group III rat received HSEAH (at 100 mg/kg body weight alone) showing normal appearance of sperm (black arrow); D - Group IV rat received fipronil (at 24.25 mg/kg body weight) plus HSEAH (at 100 mg/kg body weight) showing mild separation of head (red arrow).

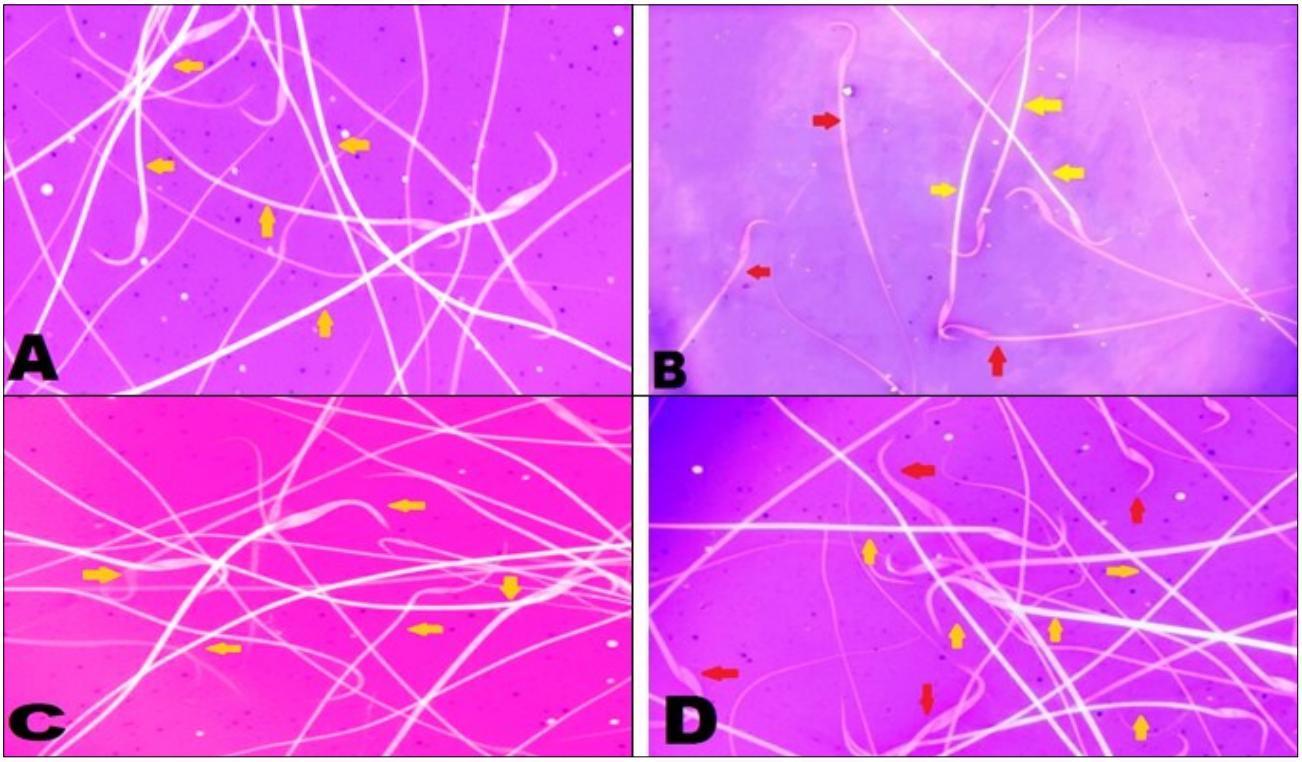

Fig. 5. Light microscopic images showing sperm viability in different treatment groups: A - Control group I rat showing normal live sperm (yellow arrow); B - Group II rat received fipronil (at 24.25 mg/kg body weight alone) showing severe dead (red arrow) and live sperm (yellow arrow); C - Group III rat received HSEAH (at 100 mg/kg body weight alone) showing normal live sperm (yellow arrow); D - Group IV rat received fipronil (at 24.25 mg/kg body weight) plus HSEAH (at 100 mg/kg body weight) showing less dead (red arrow) and live sperm (yellow arrow).

seed extract treated rat group sperm shows less defects and dead sperm as compared to control group. Normal structural morphology is important for the integrity of the long tail, sperm capacitation and generation of energy during sperm passage to fertilise the oocyte. Alteration in sperm morphology may be mainly due to the direct effects of the increase in nitric oxide (NO) levels in veins. Controlled NO concentrations is essential for normal physiological functions of sperm (26). Fipronil reduced epididymal sperm count in rat due to activity of the glutathione peroxidase enzyme increased and that of catalase was reduced in the testis. Also, a reduction in GSH and an increase in the concentration of MDA were observed in the animals treated with fipronil (27). Hydroethanolic seed extract of *A. hypochondriacus* treatment showed significantly increased sperm density in fipronil intoxicated rats; it indicates the protective effect of the extract in reversing the fipronil and its metabolites induced reproductive parameter. This effect may be due the presence of rutin, quercetin, tocopherols and squalene in the extract, which are collectively known for their potent antioxidant activities (28). Previous researchers investigated squalene effect on reproductive performance of boars. Feeding of squalene significantly increases the reproductive performance and serum testosterone levels in boars. Higher doses of squalene also increased semens' volume and motility and increased the size of litter as compared with controls (29).

Histopathological examination

Histopathological examination of testis revealed group II rats showed severe congestion and degenerative changes in spermatids, detachment of germinal layer from basement membrane, massive edema in between the seminiferous tubules, necrosis in spermatids and sloughing in some germinal layers (Fig. 6). No alterations were found in rats of groups I and III. Similar changes with varying intensity found in groups IV rats. Severe lesions in group II followed by groups IV rats indicating the ameliorative effect of HSEAH. Pesticide toxicity has been shown to impair redox equilibrium as well as the development of oxidative damage. Several investigations have demonstrated that increased ROS generation during fipronil toxicity causes redox homeostasis disruption (30). The testicular pathogenesis caused by fipronil in this investigation could be due to rise in levels of MDA and NO due to the high production of reactive oxygen metabolites, particularly hydroxyl radicals. Lipid peroxidation plays a role in the disruption of cellular membrane integrity and has been linked to hepatic injuries (31). Reactive oxygen species may damage cell membranes and other biological components, causing protein oxidation, caspase-3 activation, lipid peroxidation and DNA damage, all of which can result in cell failure (32). Treatment with HSEAH restored the level of altered oxidative and reproductive parameters towards normally showing its ameliorative efficacy. It may be due to presence of tocopherols, terpenoids and higher concentration flavonoid such as kaempferol, diglycoside, rutin, quercetin and squalene in the extract (33).

Conclusion

This study presents the first evidence for the efficacy of *Amaranthus hypochondriacus* seed extract in mitigating fipronil-induced oxidative stress and reproductive toxicity in a rat model. Our findings demonstrate that HSEAH co-administration significantly ameliorated fipronil's detrimental effects, as evidenced by the restoration of key antioxidant enzymes (GSH, CAT), reduction in lipid peroxidation (LPO) and marked improvement in sperm quality and testicular histology. These beneficial effects can be attributed to the potent antioxidant constituents such as flavonoids, squalene and tocopherols present in the extract. Our results have significant implications, suggesting that plant-based bioactive compounds can serve as a sustainable and effective strategy to counteract occupational and environmental chemical exposures.

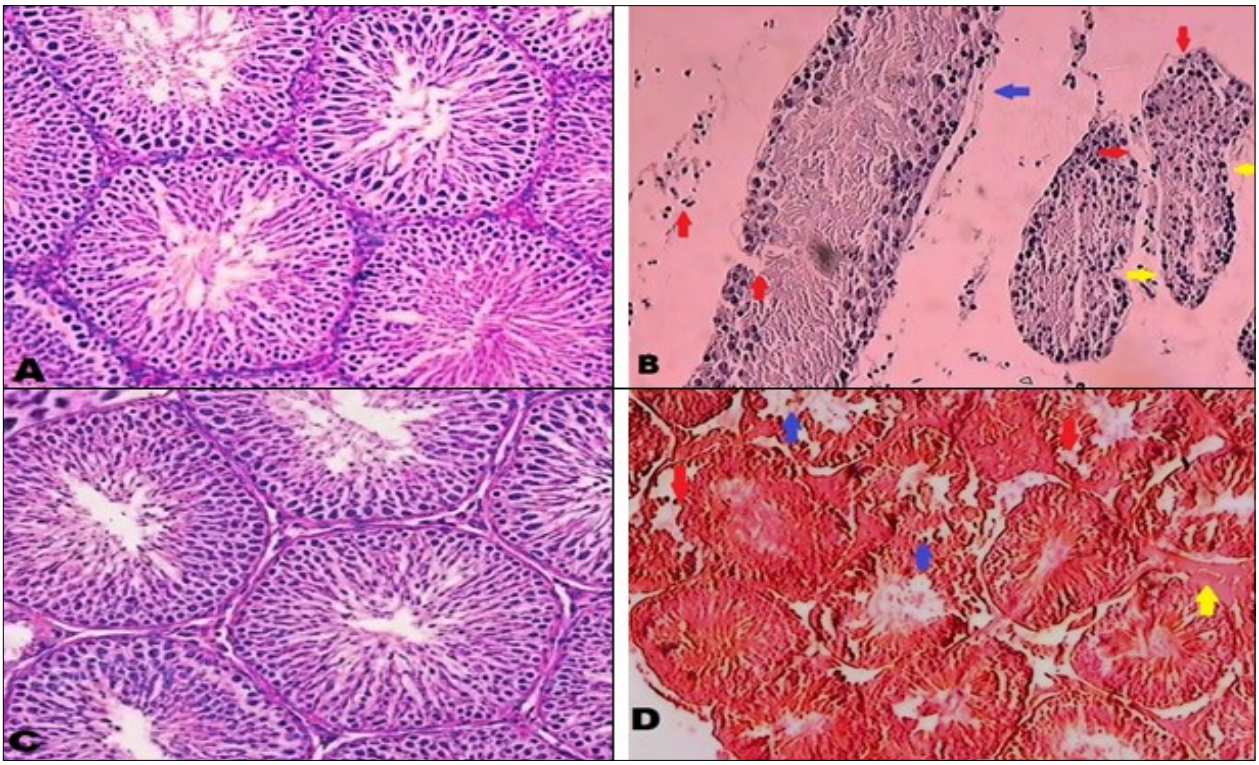

Fig. 6. Light microscopic images of testicular histology in different treatment groups: A - Control group I rat testis showing normal histological appearance of seminiferous tubule and germinal layer; B - Group II rat received fipronil (at 24.25 mg/kg body weight alone) showing severe degenerative changes in the spermatids (red arrow), edema in between seminiferous tubule (yellow arrow) and sloughing of the germinal layer (blue arrow); C - Group III rat received HSEAH (at 100 mg/kg body weight alone) showing normal histological appearance of seminiferous tubule and germinal layer; D - Group IV rat received fipronil (at 24.25 mg/kg body weight) plus HSEAH (at 100 mg/kg body weight) showing mild degenerative changes in the spermatids (red arrow) and edema in between seminiferous tubule (yellow arrow).

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Authors' contributions

MKV conceptualised the study, designed the methodology, conducted the experimental work and prepared the original draft of the manuscript. SPS provided overall supervision and contributed essential resources. AHA was responsible for data curation and graphical representation of the results. NA contributed to software handling and data validation. MB participated in the histopathological investigation. DP assisted in refining the methodology and validating the findings. NP contributed to the statistical analysis and edited the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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