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Original Research Article

In vivo investigation of Roselle flower (*Hibiscus sabdariffa* L.) and Bee pollen (*Tetragonula laeviceps*) on carbon tetrachloride-induced spermatozoa damage

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Abstract

Purpose: *To determine the effect of roselle flower and bee pollen on carbon tetrachloride (CCl_4)-induced spermatozoa damage.*
Methods: *Roselle flowers (Hibiscus sabdariffa L.) and bee pollen (Tetragonula laeviceps) were obtained from Bali, Indonesia. A total of 24 BALB/c (Mus musculus) mice were used and randomly assigned into control, negative control (CCl_4-induced), positive control (CCl_4 + ascorbic acid) and treatment groups (25, 50, and 100 mg/20 g). Spermatozoa-induced damage was done using carbon tetrachloride (CCl_4). Sperm levels, movement, viability, and morphology of the spermatozoa were evaluated.*
Results: *Roselle and bee pollen granules at 50 mg/20 g (11.2 mg of roselle extract and 28 mg of pollen extract), demonstrated the most significant improvement in spermatozoa levels, movement, viability, and morphological characteristics compared to negative controls ($p < 0.05$).*
Conclusion: *Roselle and bee pollen granular combination offers a promising natural intervention for mitigating chemically induced reproductive toxicity. It suggests potential therapeutic applications in preserving male reproductive health and counteracting oxidative stress-related spermatogenic damage.*

Keywords: *Bee pollen, Granules, In vivo, Roselle, Spermatozoa*

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INTRODUCTION

Male infertility represents a critical global health challenge, affecting up to 40 % of couples struggling to conceive. Etiology of male reproductive dysfunction is multifactorial, encompassing a complex interplay of genetic, environmental, and physiological determinants [1]. Drugs such as analgesics (pregabalin, gabapentin), anti-inflammatories (sulfasalazine, methylprednisolone, colchicine), antineoplastics, and others also induce male infertility [2].

Oxidative stress emerges as a pivotal driver of male infertility, characterized by dysregulated equilibrium between reactive oxygen species (ROS) and antioxidant defense mechanisms. Excessive ROS generation induces comprehensive molecular damage to spermatozoa, compromising reproductive

potential. The intricate process of spermatogenesis (a sophisticated cellular differentiation occurring within seminiferous tubules) becomes particularly vulnerable to oxidative disruption [3]. Comprehensive sperm quality assessment relies on multifaceted parameters, including quantitative sperm levels, progressive motility, cellular viability, and morphological integrity. These metrics provide crucial insights into male reproductive functionality and potential fertility challenges [4].

Natural botanical interventions offer promising therapeutic strategies for mitigating oxidative stress-induced reproductive impairments. *Hibiscus sabdariffa* L. (Family: Malvaceae), renowned for its rich anthocyanin profile, demonstrates significant antioxidant capabilities. Experimental evidence demonstrates that the administration of roselle flower extract substantially enhances spermatozoa quantity and motility by attenuating lipid peroxidation in testicular tissues. Also, bee pollen from *Tetragonula laeviceps* (Family: Apidae) presents a sophisticated antioxidant matrix enriched with flavonoids, polyphenols, carotenoids, and vitamin C [6]. Its molecular mechanism involves neutralizing electrophilic species and intercepting free radical propagation, thereby providing robust cellular protection against mutagenic processes [7].

Preliminary studies indicate that bee pollen supplementation ameliorates sperm abnormalities, promotes accessory reproductive organ development, and potentially modulates testosterone hormone levels [8]. Granulation turns roselle flower and bee pollen extract into granules. Granules are more practical, stable, and moisture- and air-resistant than powders. Wet granulation improves flow and compatibility. Thus, this study investigates the effect of roselle and bee pollen extract granules on CCl_4-induced spermatozoa damage in male BALB/c mice (Mus musculus).

EXPERIMENTAL

Materials

Roselle flowers (*Hibiscus sabdariffa* L.), and bee pollen (*Tetragonula laeviceps*) were obtained from Bali, Indonesia and identified in the National Research and Innovation Agency of Indonesia (BRIN), carbon tetrachloride (CCl_4) pro analytical grade (Merck, Germany), neutral buffer formalin (NBF; Sigma-Aldrich Chemical Company), ethanol 70 % pro analytical grade (Merck, Germany), aerosil, PVP K30, lactose, Manihot starch, ascorbic acid was pharmaceutical grade provided by the Laboratory of Pharmaceutical Technology, Udayana University, Bali, Indonesia.

Preparation of extracts

Roselle flower (1 kg) powder from Bali, Indonesia, was macerated in 10 L of 70 % ethanol. The powder underwent two consecutive extraction cycles. The solvent (10 L) was used for a 6 h active maceration with constant stirring, followed by an 18 h static extraction period. The maceration solution was filtered. Half the first extraction volume (5 L) was utilized to repeat the extraction. The extract was collected and evaporated at 50 °C with a rotary evaporator.

Thereafter, bee pollen (*Tetragonula laeviceps*; 2 kg) powder was macerated in 20 L of 70 % ethanol for 24 h with occasional stirring. This was repeated for 3 days. Extraction was repeated using the same solvent. The extract was carefully collected in a container and evaporated at 45 °C using a rotary evaporator and thereafter kept in air-tight containers for further use.

Preparation of granules

Granulation was executed using the wet granulation method with precise pharmaceutical engineering principles. The roselle flower extract and bee pollen extract were mixed with aerosol (drying agent) separately. After drying, both extracts were uniformly mixed in a mortar to create a mixture I. Another excipient was prepared with 6.98 g Manihot starch, 5.14 g lactose, and 1.4 g Polyvinylpyrrolidone K30 (PVP K30), which were mixed and processed as mixture II in a separate mortar. Mixture I was slowly added to Mixture II while grinding until homogeneous. Thereafter, 5 g purified water was gradually introduced to achieve a consistent and kneadable mass. The mixture was sieved through a No. 10 mesh, then oven-dried at 50 ± 2 °C) until moisture content was below 5 %. The formulation states that 50 g of the granules comprise 6 g roselle extract and 12 g bee pollen extract. A mouse dose was 50 mg/20 g granules which consists of 6 mg roselle extract and 12 mg bee pollen extract. This study also employed 25 mg/20 g granules (consisting of 3 mg hibiscus extract and 6 mg bee pollen extract), and 100 mg/20 g (12 and 24 mg, respectively). Each minimum and maximum effective of roselle extract and bee pollen extract were evaluated when setting dosages. The lowest effective dose for roselle extract for mice is 2.8 mg/20 g, while bee pollen is 5.6 mg/20 g. The highest effective dose of roselle extract is 14 mg/20 g, and bee pollen is 33.6 mg/20 g.

Animal treatment

A total of 24 male BALB/c mice (*Mus musculus*), aged 2 - 3 months and weighing 20 - 30 g, were acquired from the local animal research facility. The animals were randomly distributed into six groups (4 mice per group) and maintained under standard conditions (12 h light / dark cycle, room temperature and unrestricted access to food and water ad libitum) to acclimatize for 7 days. The animals were randomly assigned to 6 groups, namely untreated control group (KN), positive control group (K+), negative control group (K-), dose I group (P1), dose II group (P2), and dose III group (P3). The untreated control group (KN) served as the baseline reference, receiving no intervention throughout the 20-day experimental period. Positive control group (K+) underwent initial carbon tetrachloride-induced oxidative stress for 10 days, followed by treatment with ascorbic acid for 10 days (2.52 mg/20 g) to establish a comparative antioxidant intervention benchmark. Negative control group (K-) experienced carbon tetrachloride-induced oxidative stress for 10 days, followed by a 10-day non-interventional period to assess unmitigated toxicant effects. Dose group I (P1) received 25 mg/20 g of extract granules following 10 days of carbon tetrachloride exposure. The dose II group (P2) received 50 mg/20 g extract granules after 10 days of CCl_4 exposure. The dose III group (P3) received 100 mg/20 g extract granules after 10 days of CCl_4 exposure. All experimental interventions were administered orally, with a consistent daily dosing regimen over 20-day experimental timeline.

Ethical approval for this study was obtained from the Animal Ethics Committee of Udayana University (approval no. B/59/UN14.2.9/PT.01.04/2023) and complied with the International Guidelines for Animal Studies [9].

Spermatozoa suspension

Mice were terminated using chloroform and dissected to harvest the cauda epididymis. The cauda epididymis was cut into small pieces and placed in petri dishes containing 1 mL 0.9 % NaCl, and 2 mL 10 % neutral-buffered formalin (NBF) solution.

Spermatozoa counting

The sperm count was determined by adding 2 mL of 10 % NBF into the sperm suspension previously procured. One drop of spermatozoa suspension was carefully placed onto the Improved Neubauer Haemocytometer. The sample was examined under a microscope at x 100 and x 400 magnification. Calculation of sperm count was performed utilizing Eq 1 [10].

Total of Spermatozoa = $(N/4)10^6$ cauda epididymis …….. (1)

N: number of spermatozoa counted in 25 squares of the Neubauer hemocytometer.

Movement test

One drop of spermatozoa suspension was carefully placed into the Improved Neubauer counting chamber. The chamber was examined under a microscope (x 100 magnification). A total of 25 spermatozoa were counted, and the speed was determined using a stopwatch. The time taken for the counted spermatozoa to move forward to cover one micro hemocytometer was converted to micrometers per second and recorded [11].

Viability test

Viability of spermatozoa was assessed by examining the presence of live and dead spermatozoa under a microscope after the addition of eosin-nigrosine dye solution [12]. Spermatozoa suspension (5 µL) was placed on top of a microscopic slide and a few drops of eosin-nigrosine dye were added, creating a test sample. The sample was spread across the microscopic slide until a smear was formed. Test sample was left to dry for 15 min before a cover glass was placed over it. Viability observation was done under a microscope (x400). Live and dead cells were counted based on the color of the spermatozoa head until 100 spermatozoa cells in total had been counted. Pink-colored spermatozoa heads indicated dead cells, while white-colored spermatozoa heads indicated live cells.

Morphology studies

Spermatozoa morphology was analyzed by adding 5-10 µL of spermatozoa suspension on a microscope slide. Several drops of eosin-nigrosine dye were added creating a test sample. The sample was then spread across a microscope slide to create a smear and left to dry for 15 min. After drying, a cover glass was placed over the sample. Morphology was observed under a microscope (x 400). The observation focused on 100 spermatozoa cells, specifically looking for any abnormalities in the head, midpiece, and tail parts of the spermatozoa cells.

Data analysis

Data analysis was performed using the Statistical Packages for Social Sciences (SPSS, version 22.0, IBM, Armonk, NY, USA). Data analysis began with a normality test using Shapiro-Wilk test and a homogeneity test using the Levene method. Normally distributed data were compared using one-way analysis of variance (ANOVA) at a 95 % confidence level. Duncan's Multiple Range Test was used to test significance. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of *Roselle* flower and Bee pollen on spermatozoa

There was no significant difference in spermatozoa count at 25 mg/20 g, 50 mg/20 g, and 100 mg/20 g doses ($p > 0.05$; Table 1). Furthermore, negative control demonstrated significant decrease in spermatozoa levels, movement, viability, and morphology ($p < 0.05$). However, the 50 mg/20 g dose demonstrated a significant increase in spermatozoa levels, movement, viability, and morphology ($p < 0.05$), with values approaching positive control (Figure 1 and Figure 2).

DISCUSSION

The direct process of spermatogenesis determines the number and quality of spermatozoa. Disruptions during this process impair spermatogonium cell development and spermatozoa production. The increase seen following treatment with the granules is due to free radical-fighting antioxidants. The direct process of spermatogenesis determines the number of spermatozoa. Disruptions during this process impair spermatogonium cell development and spermatozoa production. Since testosterone is essential for normal spermatogenesis, it stops without testosterone, according to Johnson and Everitt [13]. Therefore, increasing testosterone hormone levels will boost spermatogenesis. The antioxidant activity of roselle and bee pollen primarily stems from ascorbic acid, β-carotene, and anthocyanins [14].

Table 1: Mean level, movement, viability, and morphology of spermatozoa in treatment groups (mean ± SD)

Treatment	Level of Spermatozoa (million/cauda epididymis)	Movement of Spermatozoa (µm/s)	Viability of Spermatozoa (%)	Morphology of Spermatozoa (%)
(KN)	29.31±0.36^{b}	31.39±2.39^{b}	61.25±6.19^{a}	16.50±1.47^{a}
(K+)	48.19±1.28^{e}	43.98±1.16^{d}	79.62±3.32^{c}	29.12±0.75^{b}
(K-)	16.22±1.53^{a}	17.41±1.39^{a}	48.75±1.32^{b}	9.12±1.25^{c}
(P1)	35.44±1.95^{c}	31.89±2.49^{b}	63.37±4.02^{a}	19.00±1.47^{d}
(P2)	41.81±1.62^{d}	37.06±2.18^{c}	71.37±1.70^{d}	25.75±1.32^{e}
(P3)	37.22±1.28^{c}	32.31±4.05^{b}	65.12±2.09^{a}	20.75±2.02^{d}

$^{abcde}P < 0.05$) within each column based on post hoc Duncan's test. KN: mice were only given feed and water *ad libitum* K-: mice were only induced with CCl_4 0.04 ml/20 g. K+: mice were induced with CCl_4 0.04 mL/20 g and ascorbic acid 2.52 mg/20 g. P1: mice were induced with CCl_4 0.04 mL/20 g and given a combination granular dose of 25 mg/20 g. P2: mice were induced with CCl_4 0.04 mL/20 g and given a combination granular dose of 50 mg/20 g. P3: mice were induced with CCl_4 0.04 mL/20 g and given a combination granular dose of 100 mg/20 g

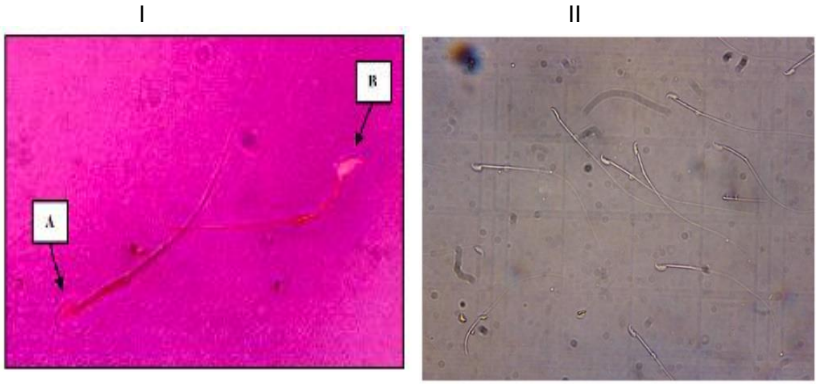

Figure 1: Viability of spermatozoa, (I) Number of spermatozoa (II). Level of spermatozoa. ***Key:*** living spermatozoa (A) dead spermatozoa (B), under a microscope (mag 400x)

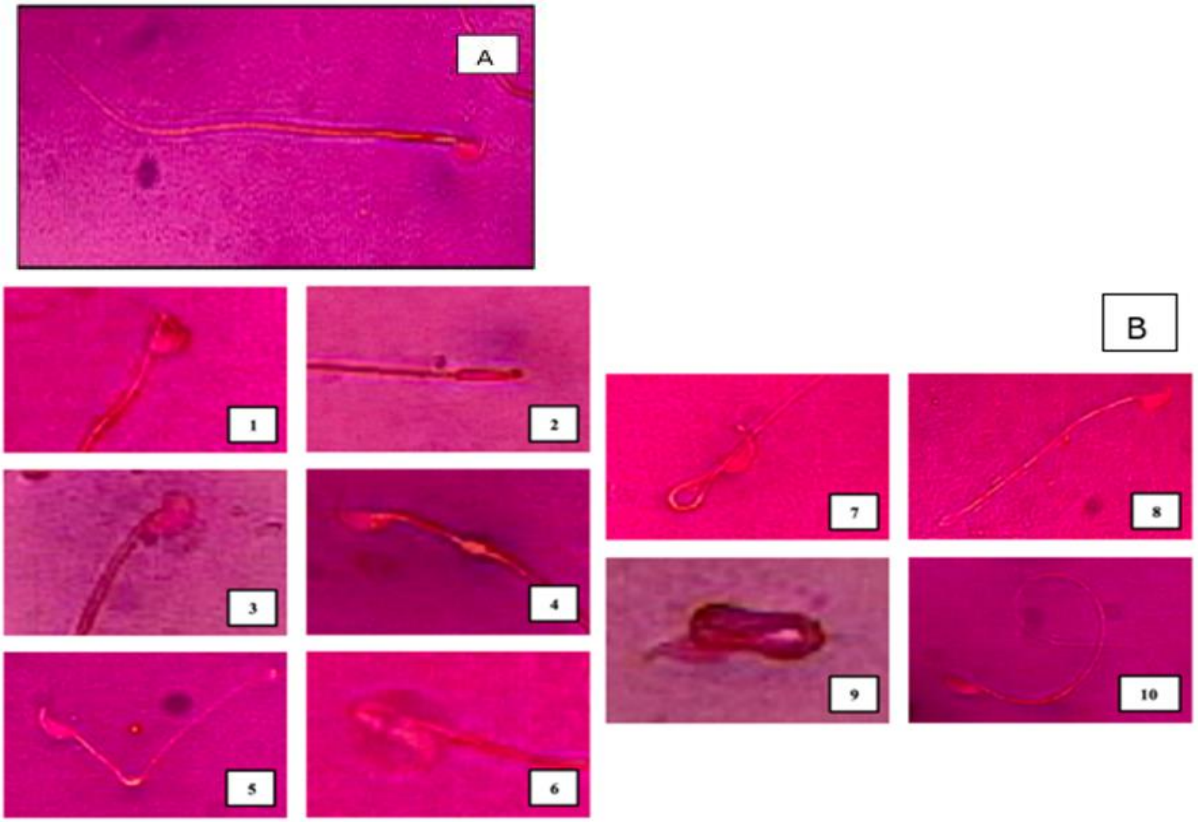

Figure 2: Features of spermatozoa. Normal morphology of spermatozoa (A). Head region abnormality (B1-B3), cytoplasm residue abnormality (B4), midpiece abnormality (B5-B7), tail abnormality (B8-B10)

Antioxidant compounds increase testosterone levels. However, if the levels are too high, this may lead to excessive testosterone secretion, which directly affects the hypothalamus by reducing the secretion of Gonadotropin Releasing Hormone (GnRH). This condition subsequently leads to a decrease in LH and FSH secretion by the anterior pituitary. Less LH prevents Leydig cells from making testosterone, while less FSH affects the production of androgen-binding protein (ABP) by Sertoli cells. Low testosterone and FSH levels affect spermatogenesis and reduce spermatozoa [15].

This finding is consistent with previous reports which revealed that ascorbic acid in doses exceeding 2000 mg may transform into pro-oxidants and reduce the number of Leydig and Sertoli cells. Furthermore, high levels of carotenoid compounds may also have a pro-oxidant effect, possibly due to autoxidation [16].

Sperm movement is determined by observing the speed of sperm as they traverse a single microhemocytometer box. The assessment of sperm movement focuses on sperm that exhibit rapid movement, as this movement is directly linked to the time it takes for the sperm to reach and fertilize the ovum during the reproduction process. The decrease in sperm movement is mediated by carbon tetrachloride (CCl_4), creating carbon trichloride (CCl_3) and carbon trichloride oxide (CCl_3O_2), which are free radical sources. Oxidative stress is caused by reactive free radicals containing unpaired electrons that react swiftly with DNA, protein, and lipids. The plasma membrane of mitochondria that creates Adenosine triphosphate (ATP) is damaged by lipid peroxidation from oxidative stress. Fibril contractions of sperm tail which move spermatozoa decrease as ATP availability decreases [5]. This tail movement propels spermatozoa forward. Roselle flower and bee pollen boost mitochondrial energy output. Antioxidant effect of Roselle results from ascorbic acid, β-carotene, and anthocyanins [14]. However, bee pollen contains flavonoids, carotenoids, and ascorbic acid. These compounds prevent CCl_4-induced ROS oxidation in the spermatozoa membrane and mitochondrial DNA, thus promoting ATP generation. They also lower testicular malondialdehyde (MDA) levels which is a result of lipid peroxidation that indirectly induces cell damage. Lower levels of MDA improve spermatozoa motility and reduce abnormalities [5,17].

Assessment of spermatozoa viability includes quantifying the number of cells exhibiting intact and impaired membranes, which is achieved with

the use of eosin-nigrosin staining. Normal spermatozoa cells are white or pink, indicating intact membranes. Crimson or dark crimson spermatozoa cells indicate broken membranes and are abnormal. Viability measures spermatozoa plasma membrane integrity. A high amount of ROS in the body might break the unconjugated double bond between PUFA methylene groups. This disruption may affect plasma membrane integrity. Findings from this study revealed that the combined granules restored spermatozoa from carbon tetrachloride injury independent of dosage. Only the granules at 50 mg/20 g restored spermatozoa from tetrachloride-induced damage and had a significant effect that exceeded control group. With increase in granular dose from 50 mg/20 g to 100 mg/20 g, normal spermatozoa vitality decreased significantly due to excessive antioxidant activity in the body, arising from the antioxidant chemicals in the granules becoming prooxidants that cause oxidative stress, thereby decreasing viability.

Sperm morphology involves counting and examining normal and aberrant spermatozoa Observations were recorded and represented as a percentage. Each spermatozoa sample was observed twice. Duncan's test was used to examine the data, which met the percentage difference requirements. The investigation showed that granules at various doses protect spermatozoa from oxidative damage. Significant differences were found between the control group and those treated with different granular doses (P1, P2, and P3) which implies that granules at all doses improve spermatozoa. It also shows that the granule increases sperm morphology quality, with the highest results observed at 50 mg/20 g.

Oxidative stress damages the plasma membrane and mitochondria, causing sperm morphological defects. Oxidative damage from excessive ROS generation damages cell membranes and impairs sperm maturation in the seminiferous tubules before storage in the epididymis tail. Normalcy of the head, midpiece, and tail is essential for examining sperm morphology. A single sperm cell injury suggests a morphological abnormality. However, the study found that the granules significantly reduced normal sperm morphology at 100 mg/20 g compared to 50 mg/20 g. However, higher doses of roselle flower and bee pollen granules may be harmful. This dosage contains the highest quantity of each ingredient that should improve spermatozoa quality (14 mg/20 g mice for roselle flower extract and 33.6 mg/20 g mice for bee pollen extract) [18].

CONCLUSION

Roselle flower and bee pollen granules demonstrate significant protective effects on spermatozoa with improved sperm morphology quality, count, movement and viability. The study reveals the therapeutic potential of combining roselle and bee pollen extracts in preserving male reproductive health, suggesting promising avenues for future studies in reproductive medicine and oxidative stress management.

DECLARATIONS

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Ethical approval

The experimental protocol was approved by the Udayana University Animal Ethics Committee (approval no. B/59/UN14.2.9/PT.01.04/2023) and conducted in strict accordance with international guidelines for the care and use of laboratory animals.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All the authors made substantial contributions to the conception and design of the study, data acquisition, analysis, and interpretation. All authors critically reviewed and approved the final draft of the manuscript for publication.

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