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Potential of *Macang* (*Mangifera foetida*) Bark Extract on Antioxidant Levels and Pro-Apoptotic Proteins in Rats (*Rattus norvegicus*) Exposed to Polystyrene Nanoplastics

(Potensi Ekstrak Kulit Macang (*Mangifera foetida*) pada Tahap Antioksidan dan Protein Pro-Apoptosis pada Tikus (*Rattus norvegicus*) Terdedah kepada Nanoplastik Polistirena)

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ABSTRACT

Nanoplastics in the environment primarily originate from contaminated aquatic animals, detergent waste, and cosmetic products, which can accumulate in the body through ingestion, inhalation, and skin exposure. This accumulation poses significant health risks, including reproductive health disruptions. This study aims to assess the potential of *Mangifera foetida* (*macang*) bark extract as an antioxidant to enhance immune and reproductive health in rats exposed to polystyrene nanoplastics (NP). Twenty-five male rats were divided into five groups: a control group, a negative control (10 µL/kg NP), and three treatment groups receiving 10 µL/kg NP with 50, 100, or 200 mg/kg *of M. foetida* extract for 35 days. The DPPH assay showed an IC_{50} value of 22.176 ppm, indicating strong antioxidant activity of the bark extract. Exposure to NP reduced the levels of endogenous antioxidant enzymes (SOD and CAT) and increased pro-apoptotic proteins (Bax and Caspase-3), affecting the histological structure and size of the testes. Treatment with *M. foetida* extract restored SOD and CAT levels while reducing Bax and Caspase-3 levels, improving histological structure by increasing the epithelium thickness and seminiferous tubule diameter. These findings suggest that *M. foetida* bark extract has potent antioxidant properties and can mitigate NP-induced toxicity, making it a promising protective agent.

Keywords: Antioxidant; apoptosis; health care; *Mangifera foetida*; nanoplastics

ABSTRAK

Nanoplastik dalam persekitaran biasanya berasal daripada kontaminasi haiwan akuatik, sisa detergen, dan produk kosmetik boleh terkumpul di dalam badan melalui pengambilan, penyedutan serta pendedahan kulit. Pengumpulan ini menimbulkan risiko kesihatan yang ketara, termasuk gangguan kesihatan reproduktif. Kajian ini bertujuan untuk menilai potensi ekstrak kulit kayu *Mangifera foetida* (macang) sebagai antioksidan dalam meningkatkan kesihatan imun dan reproduktif pada tikus yang terdedah kepada nanoplastik polistirena (NP). Dua puluh lima tikus jantan dibahagikan kepada lima kumpulan: kumpulan kawalan, kawalan negatif (10 µL/kg NP) dan tiga kumpulan rawatan yang menerima 10 µL/kg NP dengan 50, 100 atau 200 mg/kg ekstrak *M. foetida* selama 35 hari. Ujian DPPH mendedahkan nilai IC_{50} sebanyak 22.176 ppm, menunjukkan kekuatan aktiviti antioksidan ekstrak kulit kayu. Pendedahan kepada NP mengurangkan tahap enzim antioksidan endogenus (SOD dan CAT) dan meningkatkan protein pro-apoptosis (Bax dan Caspase-3) yang menjejaskan struktur histologi dan saiz testis. Rawatan dengan ekstrak *M. foetida* memulihkan tahap SOD dan CAT sambil mengurangkan tahap Bax dan Caspase-3, memperbaiki struktur histologi dengan meningkatkan ketebalan epitelium dan diameter tubul seminiferus. Penemuan ini menunjukkan bahawa ekstrak kulit *M. foetida* mempunyai sifat antioksidan yang kuat dan boleh mengurangkan ketoksikan yang disebabkan oleh NP, menjadikannya agen pelindung yang baik.

Kata kunci: Antioksida; apoptosis; *Mangifera foetida*; nanoplastik; penjagaan kesihatan

INTRODUCTION

Plastic waste is a major contributor to environmental pollution and poses a significant threat to global public health (Zeng et al. 2023). Currently, the use of plastic is becoming increasingly popular in industries, packaging, pharmaceuticals, and daily life due to its cheap cost, ease

of shaping, and versatility (Lebreton & Andrady 2019; Li et al. 2021). The accumulation of plastic waste continues to rise, yet the public's understanding of the impact of plastic materials is very limited (Chamas et al. 2020). In the environment, polystyrene plastic waste has the capacity to undergo a slow degradation process, transforming into microplastics (MP) (ranging from 1 µm to 5 mm) and nanoplastics (NP) (ranging from 1 nm to 1 µm) due to the prolonged impact of mechanical forces from water, exposure to ultraviolet radiation, and biological degradation (Xu et al. 2021).

Polystyrene nanoplastics (NPs) are deemed more perilous in comparison to microplastics due to their smaller size, capacity to penetrate biological membranes, and potential to induce severe health effects, including genotoxicity, infection, and carcinogenicity (Baş et al. 2023; Yee et al. 2021). NPs are primarily released into the environment through household disposal, particularly when using personal care products like facial scrubs and cosmetic formulations (Atugoda et al. 2023). When NPs enter the body, they can induce severe toxicity in cells and tissues, including oxidative stress, apoptosis, and inflammation (Gigault et al. 2018). Hence, there is a necessity for increased focus on NP in both environmental and organism studies.

Nanoplastics induce oxidative stress as a toxic mechanism, resulting from an overabundance of reactive oxygen species (ROS), which disrupts the redox balance within cells. The accumulation of excessive ROS can cause oxidative damage and disrupt enzyme activities in organisms (Zhou et al. 2023). Studies conducted with polystyrene on male Sprague Dawley rats have shown that polystyrene reduces the activity of antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT) (Ijaz et al. 2021). Antioxidant enzymes function as the primary defence line, safeguarding biological molecules such as DNA, lipids, and proteins from damage by minimising the production of ROS (Ijaz et al. 2021).

The increased production of ROS induces mitochondrial dysfunction, triggering the release of pro-apoptotic factors and pro-inflammatory cytokines, and this cascade of events ultimately culminates in cell dysfunction or damage (Ferrante et al. 2022). Related research on polystyrene in male Sprague Dawley rats indicates that polystyrene enhances the expression of *Bax* and *Caspase-3* while reducing the expression of *Bcl-2* (Ijaz et al. 2021). Bcl-2 is recognised as an anti-apoptotic protein that inhibits apoptosis, whereas Bax is a pro-apoptotic protein with an antagonistic effect that promotes cell apoptosis. These proteins play a role in influencing the permeability of the mitochondrial membrane, leading to the release of cytochrome c into the cytoplasm and subsequent activation of Caspase-3 (Ijaz et al. 2021; Zhu et al. 2023). Examining the levels of apoptosis-related proteins in the myocardial tissues of goldfish also indicates that exposure to polystyrene nanoplastics significantly decreases Bcl2 levels and increases Caspase-3, Caspase-9, and Bax levels (Wu et al. 2022).

When ROS levels increase, the body responds by producing antioxidant enzymes such as CAT and SOD to neutralise ROS levels. However, if ROS production is very high, some ROS may remain (Widayati 2019). Antioxidants are compounds with the ability to reduce, suppress, and prevent the oxidation process by donating one or more electrons to free radicals, thereby quenching free radicals to address cellular health issues caused by NP as a source of oxidants; additional antioxidants are needed to neutralise the remaining ROS levels. NP exposure is often associated with oxidative stress, inflammation, genotoxicity, mutagenicity, decreased fertility of germ cells, and apoptosis in the reproductive system cells (Gao et al. 2023). However, the toxic effects induced by micro-nanoplastics can be inhibited with exogenous antioxidants through dietary supplement intake (Hayati et al. 2023, 2022).

One of the plants containing antioxidants is *Mangifera foetida* Lour (local name: *macang*). *Macang* fruit has a sour taste, coarse fiber, and a distinct turpentine aroma, indicating that *macang* is a source of antioxidants (Fitmawati et al. 2020). *M. foetida* possesses potential as a herbal medicine and is commonly found in forests. However, its existence is endangered by rapid and extensive deforestation. The interest in cultivation is diminishing due to its low economic value in the market and limited consumption by humans (Fitmawati et al. 2020). *M. foetida* contains mangiferin, which is 2.56% higher compared to other mango varieties (Sari, Purwaningsih & Krisnamurti 2020). Mangiferin is a group of polyphenolic compounds with strong antioxidant properties and various pharmacological activities. Quantitative analysis of antioxidants in wild mangoes from Sumatra, Indonesia, shows that *M. foetida*, along with its varieties such as limus, batu, and manis, contains gallic acid and quercetin, which have the potential as antioxidants (Fitmawati et al. 2020). However, the potential of *M. foetida* to neutralise the toxic effects of NPs has not been reported. Studies on alternative plant sources, such as red seaweed, have shown that they possess antioxidant properties capable of improving the immune system and reproductive health in rats exposed to nanoplastics (Triwahyudi et al. 2023). Hence, there is a need for research to assess the potential of *M. foetida* as an antioxidant source, aiming to neutralise the toxic effects of nanoplastics and enhance the health of rats exposed to NPs.

The objective of this study was to analyse the effectiveness of *M. foetida* extract on SOD, CAT, Bax, and Caspase-3 levels and the histological structure of rat testes exposed to NPs. Additionally, the study aims to support conservation efforts for *M. foetida* by preserving natural resources and enhancing its economic value. The exploration of *M. foetida* as an antioxidant source to neutralise the toxic effects of NPs and efforts to improve

the reproductive health of those exposed to NPs provide novel information that has not been researched before. The implications of the research results include utilising *M. foetida* as a herbal supplement.

MATERIALS AND METHODS

PREPARATION OF EXTRACT FROM *M. foetida* STEM BARK

The *M. foetida* plant was obtained from Setiajaya Village, Cibeureum District, Tasikmalaya City, West Java, and had passed plant identification testing (Biology Service Unit, Airlangga University). The stem bark was cleaned of impurities, washed thoroughly with water until clean, and then finely ground into a crude extract using a blender. Afterward, the crude extract was dried in an oven at a temperature of 40 °C. A 200 g portion of the crude extract was immersed in 2 L of 96% ethanol for five days with daily agitation. The macerate was then filtered using a filter paper and then concentrated using a rotary evaporator at a temperature of 50 °C until a dense extract was achieved, and further drying was carried out using dry ice.

MEASUREMENT OF ANTIOXIDANT CONTENT IN THE EXTRACT

The DPPH assay method began with the preparation of a DPPH stock solution at a concentration of 1 mg/mL. This solution was then diluted with methanol to a concentration of 50 µg/mL. Next, a stock solution of *M. foetida* stem bark extract was prepared at a concentration of 1 mg/mL, which was then diluted using methanol to achieve concentrations of 200, 150, 125, 100, 75, 50, 35, 25, 15, 12.5, 10, and 6.25 µg/mL. A total of 200 µL of the diluted extract solution was added to a microplate, followed by the addition of 100 µL of the 50 µg/mL DPPH solution. The test solutions were incubated for 30 min at room temperature. The microplate was then placed in a microplate reader (Thermo Scientific Multiskan Go), and the absorbance was measured at a wavelength of 517 nm. The IC_{50} value for each extract was determined using the linear regression equation derived from the curve of sample concentration versus % inhibition. The antioxidant activity was calculated using the formula herewith and categorised according to Wardhana et al. (2023).

$$\text{Percentage inhibition} = ((A_{blank} - A_{sample})/ A_{blank} \times 100\%).$$

EXPERIMENTAL ANIMALS

Twenty-five adult male Wistar strain rats (*Rattus norvegicus*), aged 6-8 weeks and weighing 100-200 g, were obtained from the Faculty of Pharmacy, Universitas Airlangga, Indonesia. The rats were acclimatised for 14 days before treatment, residing in a controlled environment with a 12-h dark and 12-h light cycle, maintaining a room temperature of 25-27 ºC. The rats were housed in wire mesh-covered plastic cages, furnished with bedding, food pellets (Hi-Pro-Vite), and water bottles. Both food and water were provided *ad libitum.* Ethical approval for all animal care procedures was granted by the Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga, Indonesia (Certificate No. 1034/HRECC.FODM/VIII/2023).

TREATMENTS OF RATS WITH NANOPLASTIC AND PLANT EXTRACT

The study is categorised into five groups, including a control group (sterile distilled water), a negative control (10 µL/kg NP), and three treatment groups. The treatment groups receive a combination of polystyrene nanoplastics (NPs) (Sigma Aldrich, Germany) and *M. foetida* plant extracts (10 µL/kg NP with varying concentrations of plant extract: 50, 100, and 200 mg/kg). All groups were supplemented with 0.5% carboxymethyl cellulose (CMC). Treatments were administered orally once daily for 35 days.

BLOOD SERUM COLLECTION

Prior to blood serum collection, the rats were administered chloroform anaesthesia via inhalation. Blood was then collected intracardiacally using a 5 mL syringe, drawing approximately 3-4 mL of blood. The collected blood was transferred to a microtube and centrifuged at 3000 rpm for 10 min to separate the serum. The obtained serum was transferred to a new microtube and stored at 4 °C. The collected blood serum was used for enzymatic assays.

ENZYMATIC BIOCHEMICAL ASSAY

The levels of SOD, CAT, Bax, and Caspase-3 were measured using the ELISA kits for rats (Bioassay Technology Laboratory, China) following the manufacturer's protocol. Standard solutions were prepared by diluting the standard diluent and standard solutions to the following concentrations: SOD (80, 40, 20, 10, and 5 ng/mL), CAT (40, 20, 10, 5, and 2.5 ng/mL), Bax (4000, 2000, 1000, 500, and 250 ng/mL), and Caspase-3 (6.4, 3.2, 1.6, 0.8, and 0.4 ng/mL). A volume of 50 µL standard solution was added to the standard wells, while 40 µL of serum was added to the sample wells, followed by 10 µL of specific antibodies (anti-SOD, anti-CAT, anti-Bax, or anti-Caspase-3). Then, 50 µL of streptavidin-HRP was added to both the standard and sample wells. The plate was sealed and incubated for 60 min at 37 °C. After incubation, the plate was washed five times with wash buffer. For each of substrate solutions A and B, 50 µL were added to each well, followed by incubation in the dark for 10 min at 37 °C. Afterward, 50 µL of stop solution was added, and the optical density (OD) was measured at 450 nm using an ELISA reader within 10 min. The concentrations of SOD, CAT, Bax, and Caspase-3 in the samples were determined by comparing the OD values with the standard curve constructed from known standard concentrations.

TESTICULAR HISTOLOGICAL EXAMINATION

The testicular organ was placed in a 10% neutral buffered formalin (NBF) solution for 24 h. Subsequently, the testis was rinsed with distilled water and dehydrated using graded alcohol, starting with 70% alcohol, 90% alcohol, and finally absolute alcohol, each for 45 min. The testis then underwent the clearing stage using xylenes (xylene I and II) for 45 min each, followed by immersion in absolute xylene overnight. The testicular organ was embedded in a paraffin block before it was cross-sectioned to a thickness of about 4 µm using a microtome and stained with haematoxylin and eosin. Histological examination of the testis was conducted by observing the gonad structure, diameter, and thickness of the seminiferous tubule epithelium. The histological slides were observed using an Optilab Viewer 4 microscope camera (MTN016220424, Optilab Advance Plus, Indonesia), integrated with the Image Raster application on a laptop or computer.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 25.0 software. The one-sample Kolmogorov-Smirnov test was used to evaluate normality, and the Levene test was employed to verify the homogeneity of variances. Statistical significance was assessed through one-way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) for post hoc analysis. Results were considered significant if $p < 0.05$. The data related to the histological structure of rat testes were analysed descriptively.

RESULTS AND DISCUSSION

Nanoplastics (NPs) are polymer particles measuring less than 100 nm, known to have significant impacts on living organisms due to their small size, which enables them to cross various biological barriers (Ali et al. 2024). NP particles can enter body cells through exposure via the gastrointestinal tract, respiratory system, or skin, triggering oxidative stress (Basini et al. 2023; Lett et al. 2021). Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds the capacity of the body's antioxidant system to neutralise them (Pizzino et al. 2017). Exposure to NPs, particularly polystyrene, induces oxidative stress and directly increases ROS levels in the rat body. This was demonstrated by Babaei et al. (2022), which reported elevated serum ROS levels in male Wistar rats exposed to NP via oral administration at concentrations of 1, 3, 6, and 10 mg/kg over a 35-day period. Elevated ROS levels lead to cellular damage through lipid peroxidation, protein degradation, and DNA mutations, all of which can trigger apoptotic responses (Sharifi-Rad et al. 2020).

The antioxidant properties of *M. foetida* bark extract was determined using the DPPH method. The inhibition percentage values were calculated to obtain the IC_{50} value. The IC_{50} value of *M. foetida* bark extract is 22.176 ppm, which falls under the category of very strong antioxidants, confirming the extract's potential as an effective antioxidant agent (Wardhana et al. 2023) (Figure 1).

Macang is one of the mango species from the Anacardiaceae family found in the Indonesian region. The plant's kinship is likely to produce similar secondary metabolites and pharmacological effects. *Macang* content

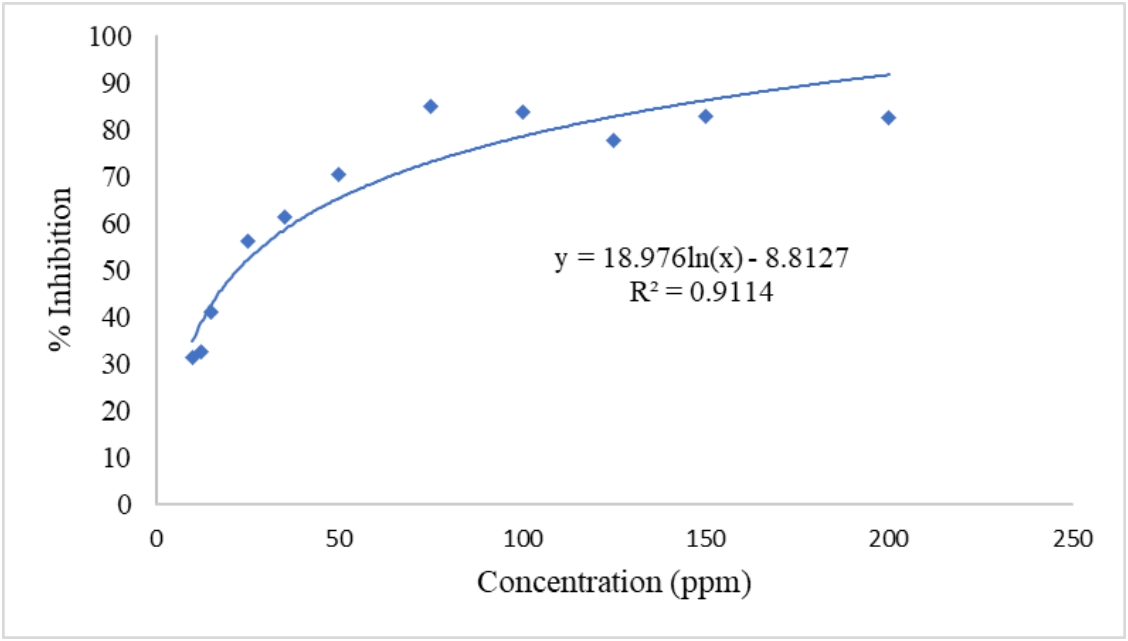


FIGURE 1. The antioxidant potential of *M. foetida* bark extract

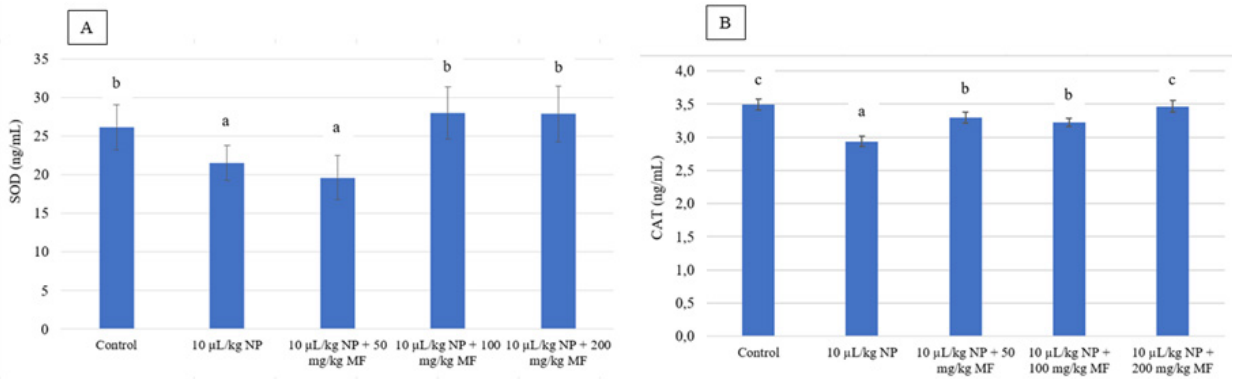
that acts as antioxidants includes phenolic compounds, flavonoids, carotenoids, and ascorbic acid (Nurviana, Karmindya & Suhendy 2021). *M. foetida* has the highest levels of mangiferin among mango types, which contains 667 secondary metabolite compounds, showing its potential use in phytopharmaceutical (Fitmawati et al. 2019). Mangiferin (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) is a polyphenolic compound with strong antioxidant properties and numerous pharmacological activities, and it has been shown to be non-toxic in acute and subchronic toxicity tests in rodents (Maulina 2021; Prado et al. 2015).

Specific quantitative testing for gallic acid and quercetin in wild mangoes from Sumatra using High-Performance Liquid Chromatography (HPLC) showed that *M. foetida* contains both bioactive compounds, which have antioxidant potential (Fitmawati et al. 2020). Antioxidants are substances capable of inhibiting and preventing the formation of free radicals or reactive oxygen species (ROS) during the lipid oxidation process (Ahmad, Elya & Mun'im 2017). Another study shows that the total phenolic and flavonoid contents of the bark were also higher compared to the leaves, indicating that the bark of *M. foetida* can be considered a potential source of antioxidants (Fitmawati et al. 2020). Flavonoids help to bind metal ions that can produce more harmful free radicals, thereby reducing ROS levels (Hritcu et al. 2017). The antioxidant compounds in *M. foetida* are useful for neutralizing reactive oxygen species (ROS) and inhibiting lipid oxidation processes caused by nanoplastic accumulation in the body.

The effect of *M. foetida* bark extract on SOD levels is presented in Figure 2(A). There was a significant difference (p = 0.001) in SOD levels after extract administration. The negative control group exhibited a 17.8% reduction in SOD levels (21.503 ± 2.269 ng/mL) compared to the positive control (26.154 ± 2.937 ng/mL), indicating that NP exposure (10 µL/kg) significantly decreased antioxidant capacity. Administration of *M. foetida* extract at 100 mg/kg and 200 mg/kg significantly increased SOD levels by 30.0% (27.972 ± 3.388 ng/mL) and 29.6% (27.863 ± 3.619 ng/mL) compared to the negative control. No significant difference was observed between these groups and the control, suggesting that *M. foetida* effectively restored SOD levels to normal.

The effect of *M. foetida* bark extract on CAT levels is presented in Figure 2(B). ANOVA showed a significant difference (P = 0.000) in CAT levels following extract administration. The negative control group exhibited a 16.1% reduction in CAT levels (2.938 ± 0.077 ng/mL) compared to the control (3.499 ± 0.083 ng/mL), indicating that NP exposure impaired antioxidant defenses. Administration of *M. foetida* extract at 50 mg/kg, 100 mg/kg, and 200 mg/kg significantly increased CAT levels by 10.7% (3.251 ± 0.052 ng/mL), 9.7% (3.223 ± 0.064 ng/mL), and 18.0% (3.467 ± 0.092 ng/mL) compared to the negative control. No significant difference was observed between the 200 mg/kg group and the control, indicating that this dose effectively restored CAT levels to normal.

This study showed that the introduction of 10 µL/kg NPs (size 100 nm) led to a reduction in the levels of endogenous antioxidant enzymes, specifically SOD and CAT. SOD is a crucial antioxidant enzyme that protects against oxidative stress by converting superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2), which is then neutralised by enzymes like catalase (CAT) (Zheng et al. 2023). NPs exposure increases ROS production, triggering SOD activity as a protective response. Fan et al. (2022) found that low concentrations of PS-NP (0.04-4 mg/L) in *Macrobrachium nipponense* for 21 days increased SOD, while higher concentrations (40 mg/L) decreased it. Similarly, in Wistar rats, NP exposure at 1 mg/kg for 5 weeks elevated both SOD and CAT activity (Babaei et al. 2022). These findings suggest



MF = *M. foetida* bark extract

FIGURE 2. The effect of *M. foetida* bark extract on (A) SOD and (B) CAT levels in rats exposed to NPs

that low NP concentrations activate antioxidant defences, while high concentrations impair them, leading to oxidative damage. In this study, SOD levels decreased after exposure to NPs at a concentration of 10 µL/kg, possibly because of the high ROS levels impairing its function. This led to a reduction in SOD activity, as seen in the negative control group. Similarly, studies on *Litopenaeus vannamei* and *Daphnia pulex* also reported decreased SOD contents after polystyrene NP exposure (Hsieh et al. 2023; Liu et al. 2020).

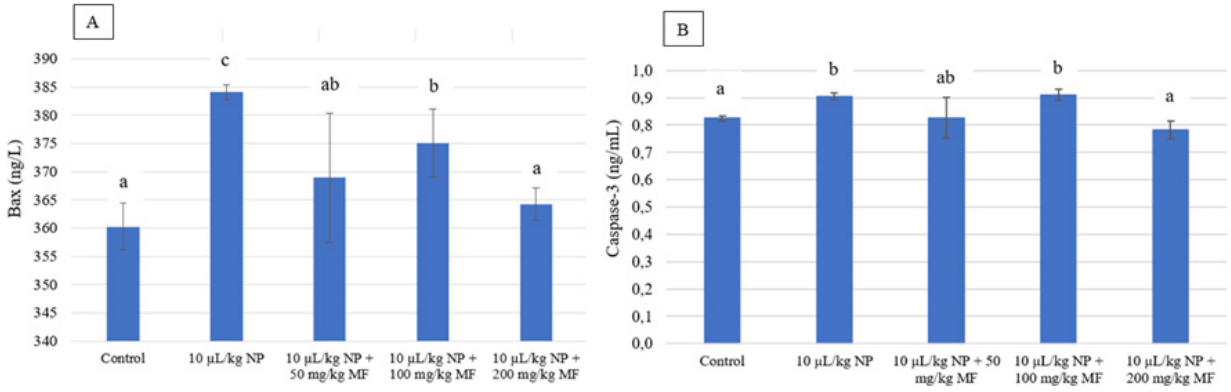
Catalase (CAT) is another antioxidant enzyme that helps protect cells from oxidative stress by decomposing hydrogen peroxide (H_2O_2) into water and oxygen, thus preventing the accumulation of toxic H_2O_2 in cells (Yuzugullu Karakus 2020). If H_2O_2 produced from SOD activity is not quickly removed, it can transform into highly toxic hydroxyl radicals through the Fenton reaction (Sies 2020). Based on the results, NPs exposure reduced CAT levels in the negative control group. This suggests that NP exposure causes dysfunction in the antioxidant system, leading to a decreased capacity for H_2O_2 detoxification. The reduced CAT levels may be caused by excessive H_2O_2, which leads to more severe oxidative damage in cells. A study by Ijaz et al. (2024) also supports this finding, showing a significant reduction in CAT levels in rats exposed to 50 µg/kg polystyrene NPs.

The increase in SOD and CAT levels after the addition of *M. foetida* extract suggests that the toxicity of NPs can be neutralised by the addition of exogenous antioxidants. Antioxidants in *M. foetida*, such as mangiferin, act as electron donors capable of neutralising superoxide radicals (O_2^-), thereby reducing the workload of SOD enzymes in the body (Estuningtyas et al. 2019). As a result, SOD enzymes can function normally again, preventing further oxidative damage. Additionally, these compounds work synergistically with the CAT enzyme to detoxify H_2O_2 by converting it into water and oxygen, maintaining free radical balance in the body (Minniti et al. 2023). Moreover, the bioactive compound mangiferin found in *Mangifera* species has the ability to activate the Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2) pathway, which increases the expression of antioxidant genes such as *SOD* and *CAT* (Khalil et al. 2024).

The effect of *M. foetida* bark extract on Bax and Caspase-3 levels is shown in Figure 3(A) and 3(B), respectively. A significant difference ($p = 0.000$) was observed following the extract administration. Bax levels in the negative control group (384.138 ± 1.329 ng/L) were 6.62% higher than in the control group (360.292 ± 4.163 ng/L), indicating increased pro-apoptotic activity due to NPs exposure (10 µL/kg). Extract administration at 50 and 200 mg/kg significantly reduced Bax levels, with the 200 mg/kg dose (364.203 ± 2.914 ng/L) showing the greatest reduction (5.19%) and restoring levels closest to normal. These results suggest that *M. foetida* bark extract mitigates NP-induced apoptosis, with higher doses provide greater protection.

Meanwhile, NPs exposure (10 µL/kg) increased Caspase-3 levels by 9.8% (0.906 ± 0.012 ng/mL) compared to the control (0.825 ± 0.009 ng/mL), signifying enhanced pro-apoptotic activity. Administration of *M. foetida* extract significantly reduced Caspase-3 levels. The 50 mg/kg dose (0.827 ± 0.074 ng/mL) lowered Caspase-3 by 8.7%, while the 200 mg/kg dose (0.783 ± 0.032 ng/mL) resulted in a 13.6% reduction compared to the negative control. Notably, Caspase-3 levels in the 200 mg/kg group were not significantly different from the control, indicating that this dose effectively counteracted NP-induced apoptosis.

Nanoplastics can trigger oxidative stress, leading to mitochondrial damage and activation of the intrinsic apoptotic pathway. This pathway involves pro-apoptotic proteins such as Bax and Caspase-3 (Xiong et al. 2024). The study found a significant increase in Bax levels in the negative control group exposed to NPs, indicating



NP=Nanoplastic, MF = *M. foetida* bark extract

FIGURE 3. The effect of *M. foetida* bark extract on (A) Bax and (B) Caspase-3 in rat exposed to nanoplastics

the activation of the pro-apoptotic pathway in response to damage caused by elevated ROS production. Uncontrolled ROS accumulation can alter mitochondrial membrane permeability, leading to the release of pro-apoptotic factors such as Bax. Under severe oxidative stress, like NPs exposure, Bax is activated and translocates to the outer mitochondrial membrane. Bax interacts with the membrane, causing cytochrome c release into the cytosol and triggering the activation of Caspase-9 and Caspase-3, which are crucial in apoptosis execution (Korotkov 2023).

This study found a significant rise in Caspase-3 levels in the negative control group after nanoplastic exposure, along with the elevated Bax levels. An increase in Caspase-3 levels indicates the activation of the intrinsic apoptotic pathway. The release of cytochrome c from the mitochondria initiates the formation of the apoptosome, which activates Caspase-9. This leads to the activation of Caspase-3, a key protease responsible for apoptosis execution (Nwaechefu et al. 2024). Caspase-3 breaks down target proteins, causing the degradation of structural and enzymatic proteins, ultimately resulting in programmed cell death (Sahoo et al. 2023).

Polystyrene NPs with a size of 100 nm have the capability to penetrate cells, induce oxidative stress and inflammation, disrupt normal cellular function, and trigger apoptosis (Hu & Palić 2020; Jung et al. 2020). Exposure to polystyrene NP in the myocardial tissue of goldfish showed a significant decrease in Bcl2 levels and a significant increase in Caspase-3, Caspase-9, and Bax levels (Wu et al. 2022). Exposure to two distinct sizes of NP (25 nm and 70 nm) induces a notable upregulation in the expression of pro-apoptotic proteins, including DR5, Caspase-3, Caspase-8, Caspase-9, and cytochrome C, in A549 human alveolar epithelial cells (Xu et al. 2019).

In the treatment groups receiving the *M. foetida* bark extract, the reduction in Bax levels indicates that the extract prevents the activation of the mitochondrial pathway that triggers cytochrome c release, thereby reducing apoptosis. By inhibiting Bax activation and cytochrome c release, *M. foetida* extract also blocks the activation of Caspase-9 and Caspase-3. The antioxidant compounds in the extract may reduce DNA and mitochondrial damages, thus preventing apoptosis-induced cell death (Fairley et al. 2023). In the group treated with the highest concentration (200 mg/kg), Caspase-3 levels significantly decreased by 13.6% (0.783 ± 0.032 ng/mL) compared to the negative control (0.906 ± 0.012 ng/mL), indicating that the extract effectively protects cells from NP-induced apoptosis.

Histological observations of rat testes following exposure to NPs show alterations in testicular structure (Figure 4). Haematoxylin and eosin (H&E) staining illustrates that spermatogenic cells at all developmental stages in the control group exhibit a well-organised and dense arrangement (Figure 4(A)). Conversely, in the negative control group, spermatogenic cells within the seminiferous tubules appear disorganised and less densely packed (Figure 4(B)). Administration of *M. foetida* extract at concentrations of 50 mg/kg (Figure 4(C)), 100 mg/kg (Figure 4(D)), and 200 mg/kg (Figure 4(E)) demonstrates an improvement in testicular structure, restoring it to a normal appearance.

Elevated ROS levels resulting from NP exposure can accumulate and trigger lipid peroxidation, leading to cell membrane damage (Li et al. 2020). This process generates malondialdehyde (MDA), a toxic byproduct that can induce DNA mutations and interact with proteins, thus altering their structure (Ayala, Muñoz & Argüelles 2014). Lipid peroxidation not only damages cellular components but also impacts the reproductive system, causing reductions in tubular diameter and epithelial thickness. These changes are linked to apoptosis, initiated by a decline in mitochondrial membrane permeability as a consequence of lipid peroxidation.

The effect of *M. foetida* bark extract on epithelial thickness and seminiferous tubule diameter is presented in Figure 5. Based on ANOVA analysis, there was a significant effect of *M. foetida* extract on seminiferous tubule epithelial thickness (p=0.000) (Figure 5(A)). NPs exposure in the negative control group reduced epithelial thickness by 57.5% (48 ± 3 µm) compared to the control (113 ± 5 µm), indicating substantial epithelial damages. The 50 mg/kg dose increased thickness by 27.1% (61 ± 2 µm), the 100 mg/kg dose by 52.1% (73 ± 4 µm), and the 200 mg/kg dose by 106.2% (99 ± 6 µm). The 200 mg/kg group exhibited the highest recovery, approaching normal epithelial thickness, suggesting the extract's potential protective effect against NP-induced damage.

Similarly, the *M. foetida* bark extract had a significant effect (p=0.000) on seminiferous tubule diameter (Figure 5(B)). NP exposure in the negative control group reduced tubule diameter by 14.0% (240 ± 3 µm) compared to the control (279 ± 3 µm), indicating structural damages. However, administration of *M. foetida* extract at 100 mg/kg (272 ± 4 µm) and 200 mg/kg (268 ± 1 µm) significantly restored tubule diameters. These findings suggest that *M. foetida* bark extract, particularly at higher concentrations, mitigates NP-induced structural damage in seminiferous tubules.

The reduction in epithelial thickness in the seminiferous tubules is caused by a decrease in spermatogenic cell count due to nanoplastic exposure. Ijaz et al. (2023) reported a decrease in spermatogenic cells in groups exposed to 100 nm NPs compared to the control group. Amereh et al. (2020) reported significant differences in tubular diameter and epithelial thickness between the control and nanoplastic-exposed groups (1, 3, 6, and 10 mg/kg), with the exposed groups showing reduced tubular size and epithelial thickness. Similarly, Triwahyudi et al. (2023) found a decrease in tubular diameter and epithelial thickness in rats exposed to nanoplastics at a concentration of 2 µL/kg body weight for 35 days compared to the control group.

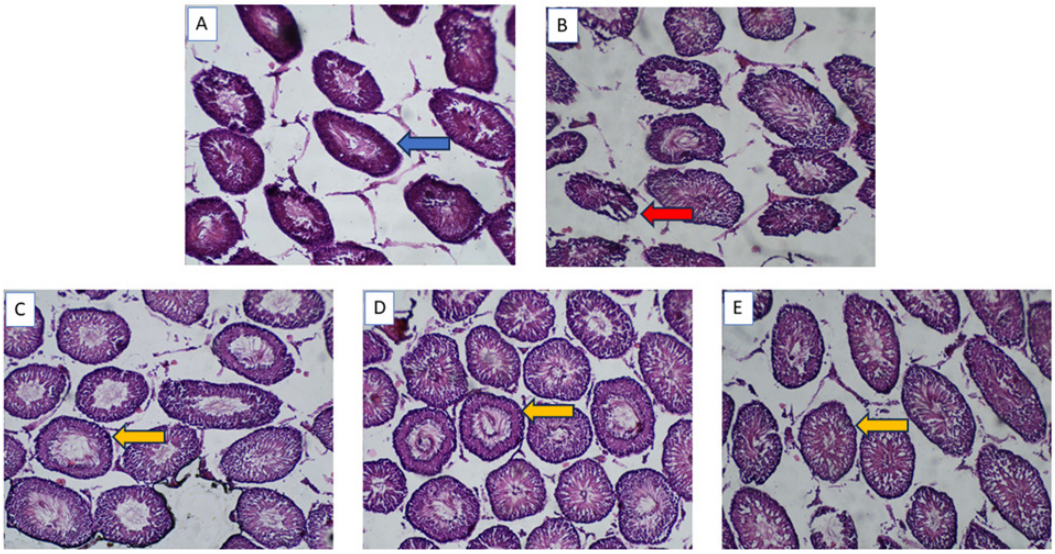
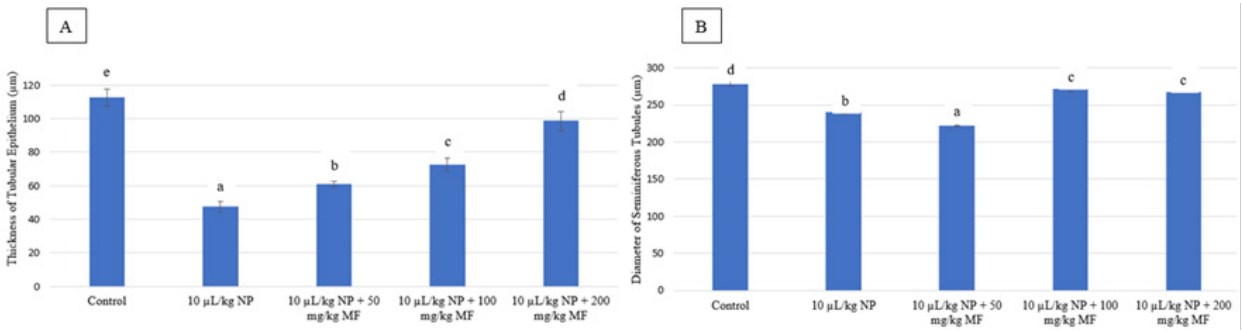


FIGURE 4. Histological structure of rat testis. (A) Control, (B) Negative control (NP 10 µL/kg), and treatment groups combining NP 10 µL/kg and varying concentrations of *M. foetida* bark extract (C) 50 mg/kg, (D) 100 mg/kg, and (E) 200 mg/kg. Magnification 100x. Spermatogenic cells are well-organised and dense (blue arrow), disorganised and less dense (red arrow), *M. foetida* bark extract restores the normal testicular structure (yellow arrow)



NP=Nanoplastic, MF = *M. foetida* bark extract

FIGURE 5. (A) Epithelial thickness and (B) diameter of seminiferous tubules in the testis after exposure to NP and various concentrations of *M. foetida* bark extract

The enhancement in epithelial thickness and seminiferous tubular diameter due to *M. foetida* extract may caused by the antioxidant compounds, such as mangiferin. Mangiferin can reduce oxidative stress by inhibiting ROS, which typically trigger the pro-apoptotic pathway (Rahmani et al. 2023). Mangiferin has been shown to block cytochrome c release from mitochondria, a crucial step in mitochondrial apoptosis. Additionally, mangiferin can decrease Bax expression by modulating signaling pathways such as AKT and NF-kB, which play important roles in regulating apoptosis (Jangra et al. 2021; Pal, Sinha & Sil 2013).

CONCLUSIONS

This study shows that the administration of *M. foetida* bark extract to rats exposed to NPs can enhance the activity of antioxidant enzymes, namely SOD and CAT. The increased activity of these enzymes indicates that *M. foetida* bark extract is capable of reducing oxidative stress induced by NP exposure, which is associated with increased production of reactive oxygen species (ROS). Additionally, the administration of *M. foetida* also plays a role in lowering the levels of pro-apoptotic proteins such as Bax and Caspase-3, indicating that the extract can protect cells from apoptosis triggered by nanoplastics. Histological

results also show structural improvement in tissues from rats exposed to nanoplastics, further supporting the evidence that *M. foetida* bark extract has potential as a protective agent against damage caused by nanoplastics. Further research can explore the molecular mechanisms related to the bioactive compounds in *M. foetida* and their effects on various environmental stressors. Additionally, further clinical trials are needed to assess the effectiveness of this extract as a cellular protective agent against damage caused by microplastic and nanoplastic pollutions.

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