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Therapeutic potentials of aqueous extract of *Piper nigrum* whole fruits against tramadol-induced oxidative stress and inflammation in rats

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ABSTRACT

Tramadol is a widely used opioid, high dose of which has been associated with oxidative stress and provocation of inflammation. This study aims to investigate the effects of aqueous extract of *Piper nigrum* (black pepper) whole fruits in ameliorating tramadol-induced oxidative stress and inflammation in male Wistar rats. The rats were induced using 60 mg/kg tramadol and then treated with different concentration of black pepper aqueous extract (PNAE); 250, 500, and 1000 mg/kg body weight as well as the reference medication Vitamin C (Vit.C), once daily for 14 days. The parameters related to inflammation and oxidative stress were examined. Secondary metabolite constituents found in the extract include tannins, phenols, flavonoids, saponins, alkaloids and glycosides. Administration of tramadol significantly (p<0.05) increased the level of inflammatory metabolites such as tumor necrosis factor-α and interleukin-1β, this was reversed insignificantly (p>0.05) by the Vit.C and BPAE at 250, 500, and 1000 mg/kg. Tramadol also significantly (p< 0.05) invokes oxidative stress by decreasing the level of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), while increasing the level of malondialdehyde (MDA), nitric oxide (NO) in liver and kidney tissues. Vit.C as well as at 250, 500, and 1000 mg/kg PNAE significantly (p< 0.05) reversed the level of MDA, GSH, SOD, CAT, thereby attenuating the effects of tramadol especially at the highest dose. These results revealed that the extract has therapeutic potential against oxidative stress and inflammation thereby attenuating and reversing the deleterious effects of tramadol in a dose dependent manner.

Keywords: Anti-inflammatory agents; Nitric oxide; Oxidative stress; *Piper nigrum;* Tramadol

1. Introduction

Oxidative stress is characterized by an imbalance between reactive oxygen species (ROS) production and the body's antioxidant defense system (Shankar and Mehendale, 2014). ROS are very reactive molecules secreted by living things as a result of environmental influences and regular cellular metabolism. Oxidative stress occurs when the ratio of oxidants to antioxidants is in favor of oxidants. This imbalance has the potential to destroy and change the activities of several cell structures, including proteins, lipids,

carbohydrates, and nucleic acids (Pizzino et al., 2017). Oxidative stress is implicated in the pathogenesis of various diseases, including drug-induced toxicity (Mohamed and Mahmoud, 2019). The immune system's reaction to an irritant, which might be bacteria, a foreign item, chemicals, radiation, or other impacts, is known as inflammation (Chen et al., 2020). It is typified by discomfort, heat, and swelling as symptoms. While chronic inflammation can linger for months or years, acute inflammation is transient and usually goes away in a matter of hours or days. Numerous illnesses, including cancer, heart disease, diabetes, and arthritis, are linked to chronic inflammation. Inflammation occurs when the body's white blood cells and the substances they produce protect the body from infection or heal an injury (Chen et al., 2020).

Tramadol is an opioid analgesic renowned for its dual mode of action, comprising μ-opioid receptor agonism and suppression of serotonin and norepinephrine reuptake. It is a widely used analgesic and has been reported to induce oxidative stress in animal models (Xia et al., 2020). Chronic tramadol exposure has been shown in previous studies to cause oxidative damage, inflammation, and apoptosis (Xia et al., 2020, Mousavi et al., 2021). For instance, research conducted on rats revealed that long-term tramadol use had neurotoxic effects that were mediated by inflammation, oxidative stress, and apoptosis (Mohamed and Mahmoud, 2019). Although oxidative stress and neurotoxicity have been linked to long-term tramadol usage, other research has shown that even brief administration of the drug can cause oxidative stress in a variety of organs (Vašková et al., 2016; Koohsari et al., 2020). Three days period is deemed appropriate for evaluating oxidative damage because acute tramadol administration can quickly change redox homeostasis, resulting in increased lipid peroxidation, decreased antioxidant enzyme activity, and an increase in oxidative stress markers. These changes are substantial within days of administration (Lagard et al., 2016; Mohamed and Mahmoud et al., 2019). This time period eliminates the possibility of long-term opioid dependency or tolerance interfering with the early metabolic changes associated with oxidative stress.

Furthermore, studies conducted on mice revealed that long-term tramadol treatment raised malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD) activity, which resulted in oxidative stress and cell damage (Mousavi et al., 2021). Despite its analgesic efficacy, tramadol can cause several adverse effects, therefore, investigating potential interventions to mitigate tramadol-induced oxidative stress is of great interest.

Piper nigrum, commonly known as black pepper in English, *Iyere* in Yoruba, *Uziza* in Igbo and *Barkonobaki* in Hausa languages of Nigeria, is a popular spice worldwide (Turrini *et al*., 2020). This flowering vine belongs to the Piperaceae family and is grown for its fruit, which is often dried and used as a flavoring or spice. It is one of the most often used spices in cuisines all over the world and is used extensively as a spice (Turrini et al., 2020). The chemical ingredient, piperine, which is distinct from the capsaicin found in chilli peppers, is what gives it its spicy flavour. According to recent research, the pericarp of black pepper includes minerals, antioxidants, and flavor and bioactive components that may have positive effects on health (Lee et al., 2020). It contains various bioactive compounds, including piperine and piperamides, which have demonstrated several pharmacological properties such as antioxidant and anticancer effects (de Souza et al., 2016). Research has demonstrated that in oxidative stress experiments, piperine guards against oxidative damage by suppressing free radicals and reactive oxygen species, reducing lipid peroxidation, and influencing cellular thiol status and other antioxidant enzymes (Haq et al., 2021). Furthermore, piperine has pleiotropic qualities that include anti-inflammatory, anti-cancer, antioxidant, and antihypertensive actions (Haq et al., 2021). Hence, this study aimed to investigate the protective effects of *P. nigrum* whole fruits on tramadol-induced oxidative stress and inflammation in male Wistar rats.

2. Materials and methods

Plant material and authentication

Fresh *P. nigrum* whole fruits were bought from an herb seller at a market (*Oja Ipata*) in Ilorin West local government, Kwara State, Nigeria.

The leaves were identified and authenticated by a botanist at the University of Ilorin Herbarium, Ilorin, Nigeria. A voucher sample was deposited under "UILH/001/1575/2023".

Experimental animals

Forty-eight healthy male Wistar rats (*Rattus norvegicus*) weighing 139.20 ± 3.25 g were supplied by Markeen Global Ventures at Ilorin, Nigeria. The animals were fed rat pellet and tap water, cared for and housed in well-ventilated hygienic aluminium cages, temperature (25℃ – 27℃), photoperiod (12 hours light and dark cycle), and relative humidity (45% – 50%). The study was conducted in line with the guidelines of National Institute of Health on the handling and use of laboratory animals (NIH Publication No. 80-23) as approved by Al-Hikmah University Ethical Review Committee (HUI/UERC/2023/015). The institution's policy on animal use and care was closely adhered to in order to provide for the animals' care and handling.

Drugs and chemicals

Tramadol was a product of Peace Standard Pharmaceutical Ind. Ltd, Ilorin, Kwara State, Nigeria, while a Vitamin C was obtained from Unicure Pharmaceutical Ltd, Ijebu-ode, Ogun State, Nigeria. ELISA kits for both tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL 1β) were products of Elabscience Cambridge, MA, USA. The supplementary reagents used during this experiment were the highest quality and made in accordance with specifications using distilled water and calibrated measuring flasks.

Preparation of extract

To prepare *P.nigrum* aqueous extract (PNAE) The whole pepper fruits were rinsed under running tap water and air-dried at room temperature for 96 hours. Prior to extraction, the dried fruits were pulverised using an electric blender (Master Chef Blender, MC-BL 1980, Guangdong, China), and kept in an airtight container. 100 g of the powdered sample was extracted in 1000 ml of distilled water (1/10 w/v) at 25 ºC with persistent shaking for 48 h, after which was filtered using a Whatman No. 1 filter paper. The resulting filtrate was concentrated in a rotary evaporator (Model RE 52A Zhengzhou, Henan, China) to give a yield of 16 g. The yield obtained was reconstituted in distilled water to give the required doses of 250, 500 and 1000 mg/kg body weight (estimated based on data from the ethnobotanical survey). The percentage (%) yield was calculated mathematically as;

% Yield =(Weight of the crude extract (g)/Weight of dried powdered sample (g)) ×100

Screening of secondary metabolites

Five grams (5g) of whole *P. nigrum* fruit were dissolved in 40 milliliters of distilled water and then subjected to phytochemical screening to check for the presence of tannins, phenols, flavonoids, saponins, alkaloids, glycosides, terpenoids, and anthraquinones. The flavonoid test (Edeoga et al., 2005) involved adding magnesium ribbon and concentrated hydrochloric acid to the extract, resulting in a pink-red coloration if positive; the phenol test (Harborne, 1973) used ferric chloride solution, which produced a greenish-blue, violet, or blue-black color; tannins were detected using ferric chloride (Harborne, 1973; Trease and Evans, 1989), which produced a brownish-green or blue-black reaction; and the saponin test (Odebiyi and Sofowora, 1978) involved heating powdered extract in distilled water, followed by filtration and additional analysis. Terpenoids were identified by mixing the extract with sulfuric acid and chloroform, which produced a reddish-brown interphase; the anthraquinone test (Edeoga et al., 2005) involved heating the extract with ferric chloride and hydrochloric acid, followed by ether extraction and ammonia treatment, where a pink or deep red coloration confirmed its presence; and alkaloids were tested (Harborne, 1973) by adding hydrochloric acid, followed by Wagner's and Mayer's reagents, which produced an orange precipitate.

Using established methods, the secondary metabolites that were detected were quantitatively analyzed: A powdered sample was extracted with acetic acid in ethanol, then filtered, concentrated, and precipitated using ammonium hydroxide to determine the alkaloid content (Adeniyi et al., 2009). After drying and weighing the precipitate, the recovery % was determined. In order to quantify flavonoids, 80% aqueous methanol was used for several extractions, followed by filtering, evaporation, and weighing of the dried sample (Boham and

Kocipai-Abyazan, 1974). The process of tannin estimation (Makkar et al., 1993) involved boiling the sample in distilled water, filtering it, reacting it with Folin-Dennis reagent and Na2CO3, and then measuring the tannic acid content using spectrophotometry at 760 nm. In order to analyze saponins, aqueous methanol extraction, filtering, charcoal treatment, acetone precipitation, and weighing of the dried residue were all required (Obadoni and Ochuko, 2001). Glycosides were measured (El-Olemy et al., 1994) by first extracting the sample with alcohol, then using lead acetate and disodium hydrogen phosphate to precipitate undesirable chemicals one after the other. To determine the glycoside content as digitoxins, the filtered filtrate was reacted with Baljet's reagent, and absorbance was measured at 495 nm. For additional research, these quantitative assays estimated the number of bioactive components in the plant extract.

Induction of oxidative stress

Male Wistar rats that had been acclimatized for two weeks were induced into oxidative stress by the oral administration of 60 mg/kg body weight (b.wt)of tramadol (prepared daily in distilled water (DW)) using a plastic cannula for 3 days (Lagard et al., 2016; Mohamed and Mahmoud et al., 2019). Following the induction, blood samples were collected from rats in both the control and the tramadol-treated group and biochemical analysis such as levels of malondialdehyde (MDA) (Tekin and Seven et al., 2002) and nitric oxide (NO) (Dutta et al., 2022) were determined to confirm the induction of oxidative stress before initiating treatment with PNAE

Animal grouping and administration of extract and reference Drug

In a fully randomized design, a total of 48 male rats were divided into six groups (A to F), with 8 animals per group as follows: Group 1 (Control) (DW): Rats received 0.5ml of distilled water, group 2 (tramadol): Rats received 60 mg/kg tramadol to induce oxidative stress and inflammation and administered 0.5 mL of distilled water, group 3 (tramadol + Vitamin C): Rats induced into oxidative stress and inflammation and administered 0.5 mL of 250 mg/kg b.wt Vit. C, groups 4 (tramadol + PNAE (250 mg/kg): Rats induced into oxidative stress and inflammation and administered 0.5 mL of 250 mg/kg b.wt PNAE, groups 5 (tramadol + PNAE (500 mg/kg): Rats induced into oxidative stress and inflammation and administered 0.5 mL of 500 mg/kg b.wt PNAE, groups 6 (tramadol + PNAE (1000 mg/kg)): Rats induced into oxidative stress and inflammation and administered 0.5 mL of 1000 mg/kg b.wt PNAE

Using a plastic oropharyngeal cannula, the different animal groups were treated as described above once daily (08:00–08:45 h) for 14 days. DW.

Preparation of serum and tissue supernatants

The serum and tissue (supernatant) were prepared as described by Nurudeen et al. (2023). On the 15th day, rats were briefly anaesthetized, and when they become unconscious, the jugular veins were cut, and 5 mL of the blood was collected into sterile, dry centrifuge tubes. The samples were kept at room temperature for 15 minutes to allow the blood to coagulate. After centrifuging at 503 × *g* for 10 min using the Uniscope Laboratory Centrifuge (Model SM800B, Surgifriend Medicals, Essex, UK), clear serum was then extracted using a Pasteur pipette and frozen at -20°C. Furthermore, the tissue supernatants were also prepared. Briefly, the liver and kidney were blotted with a laboratory absorbent paper used to remove excess moisture, cut very thinly and homogenised in an ice-cold 0.25 M sucrose solution (1:5 w/v). The homogenates were centrifuged at 894 × *g* for 15 min and the supernatant was frozen at -20°C before biochemical assays were carried out within 24 h of preparation.

Evaluation of inflammatory activity and redox profile

The activity levels of inflammatory cytokines such as TNF-α and IL-1β in the serum were determined using ELISA kits, in accordance with the manufacturer's instructions. Oxidative stress-associated factors such as MDA (Nelson, 2004), reduced glutathione (GSH) (Ellman, 1959), superoxide dismutase (SOD) (Misra and Fridovich, 1972), catalase (CAT) (Beers and Sizer, 1952), nitric oxide (NO) (Miranda et al., 2001) and total protein (Gornall et al., 1949) concentrations in the tissues were determined.

Data analysis

The data generated from the study were presented as the mean ± standard error (SE) of the mean of eight replicates and su received 60 mg/kg bjected to a one-way analysis of variance (ANOVA). The data were considered statistically different at (p< 0.05) using GraphPad Prism version 6.01 (GraphPad Software, Inc., San Diego, California, United States).

Phytochemical analysis

The phytochemical screening of PNAE revealed the presence of tannins, phenols, saponins, flavonoids, glycosides and alkaloids. Tannins were found to be the most prevalent secondary metabolite, while flavonoids were found to be the least abundant (Table 1).

Oxidative stress induction

Following the administration of 60 mg/kg body weight of tramadol, after 3 days, the activity levels of MDA and NO significantly (p< 0.05) increased in the liver of tramadol-induced rats when compared to the normal control group (Table 2). The activity of TNF-α showed significant (p< 0.05) increased while IL-1β showed significant (p< 0.05) decreased when administered tramadol.

Modulatory effects of PNAE on inflammatory cytokines

When administered Vit.C, as well as PNAE at 250, 500, and 1000 mg/kg, the activity of TNF-α and IL-1β showed significant reversal (p < 0.05). The dosage at 1000 mg/kg was comparable to both reference drug and the control group (Table 3).

Modulatory effects of PNAE on oxidative stress markers

The levels of MDA, NO as well as the liver and kidney protein increased (p < 0.05) significantly while the level of GSH, SOD and CAT decreased (p< 0.05) significantly when administered tramadol. Administration of the PNAE at 250, 500 and 1000 mg/kg bwt significantly (p< 0.05) decreased the level of MDA, NO as well as the liver and kidney protein while GSH, SOD and CAT increased (p < 0.05) significantly in a dose dependent manner. The decrease in the levels of MDA, NO, liver and kidney protein as well as the increase in SOD, CAT and GSH were comparable to the control group (Table 4).

Modulatory effects of PNAE on protein levels

The level of liver and kidney protein increased (p < 0.05) significantly while serum protein shows no significant difference (p> 0.05) when administered tramadol. Administration of the PNAE at 250, 500 and 1000 mg/kg body weight significantly (p< 0.05) reversed the level of liver and kidney protein which is comparable to the control group (Table 5).

Table 1. Secondary metabolites of PNAE

Secondary metabolites	Concentration (mg/g)
Tannins	129.031±0.031
Phenols	36.147±0.002
Flavonoids	4.467 ±0.001
Saponins	30.083 ±0.111
Alkaloids	10.058±0.024
Glycosides	15.494±0.131
Terpenoid	ND.
Anthraquinones	ND.

Values are the mean of three replicates ± S.E, ND.: Not Detected

Table 2. Assessment of oxidative stress markers in Tramadol-induced oxidative stress in male Wistar rats

Parameters	Control group	Tramadol induced group
MDA (μmol/ml)	1.512±0.114[a]	3.237±0.093[b]
NO (mg/dl)	7.97±0.686[a]	14.775±0.527[b]

Values are means of three replicates ± SEM; Values with different superscripts across the row are significantly (p<0.05) different from others

Table 3. Effects of PNAE on inflammatory cytokines markers in male rats

Parameters	Group A	Group B	Group C	Group D	Group E	Group F
(TNF-α)	137.89±11.73[a]	164.21±5.75[b]	135.89±12.62[a]	143.66±0.93[a]	140.85.04±2.9[a]	137.01±4.85[a]
(IL-1β)	46.45±3.69[a]	30.40 ±6.27[b]	47.46±3.24[a]	35.46±4.61[c]	40.96 ±3.14[a]	45.02±5.26[a]

Values are means of eight replicates ± SEM; Values with different superscripts across the row are significantly (p< 0.05) different from others. Group A: control, group B: administered 60 mg/kg Tramadol , group C: treated with 250 mg/kg Vit.C, group D: treated with 250 mg/kg b.wt PNAE, group E: treated with 500 mg/kg b.wt PNAE, group F: treated with 1000 mg/kg b.wt PNAE***;*** TNF-α: Tumor Necrosis Factor-alpha; IL-1β: Interleukin-1 beta

Table 4: Redox profile male rats induced oxidative stress by tramadol following administration PNAE

Parameters (µmol/mg protein)	Group A	Group B	Group C	Group D	Group E	Group F
MDA liver	1.36±0.08[a]	2.31±0.07[b]	1.30±0.28[a]	1.33±0.06[a]	1.27±0.08[d]	1.32±0.06[a]
MDA kidney	1.39±0.02[a]	2.65±0.34[b]	1.33±0.02[a]	1.34±0.11[a]	1.37±0.01[a]	1.31±0.07[a]
GSH liver	91.30±6.75[a]	71.62 ± 6.16[b]	90.66±4.28[a]	96.08±2.45[a]	92.71±6.59[a]	92.01±4.91[a]
GSH kidney	94.42±9.31[a]	78.61±6.83[b]	95.49±10.12[a]	75.93±9.51[c]	79.97±9.48[c]	94.32±4.47[a]
SOD liver	2.22±0.05[a]	1.22±0.05[b]	1.82±0.11[c]	2.13±0.06[a]	2.02±0.15[a]	2.32±0.14[a]
SOD kidney	2.18±0.06[a]	1.37±0.01[b]	1.65±0.06[c]	2.00±0.29[a]	2.01±0.12[a]	2.23±0.13[a]
CAT liver	1.21±0.02[a]	0.62±0.03[b]	1.29±0.07[a]	1.27±0.08[a]	1.32±0.12[a]	1.20±0.11[a]
CAT kidney	1.26±0.03[a]	0.65±0.04[b]	1.28±0.06[a]	1.09±0.25[c]	1.02±0.06[c]	1.29±0.12[a]
NO liver	8.36±0.51[a]	14.35±0.77[b]	10.92±0.49[c]	11.85±0.29[c]	9.19±0.39[c]	7.11±1.32[a]
NO kidney	7.60±2.44[a]	17.39±1.65[b]	12.92±0.32[c]	12.74±2.11[c]	11.97±0.37[c]	8.95±1.76[a]

Values are means of eight replicates ± SEM; Values with different superscripts across the row are significantly (p< 0.05) different from others. Group A: control, group B: administered 60 mg/kg tramadol, group C: treated with 250 mg/kg Vit.C, group D: treated with 250 mg/kg b.wt PNAE, group E: treated with 500 mg/kg b.wt PNAE, group F: treated with 1000 mg/kg b.wt PNAE, MDA: Malondialdehyde; GSH: Glutathione; SOD: Superoxide dismutase; NO: Nitric oxide.

Table 5: Protein Levels of male rats induced oxidative stress by tramadol following administration of PNAE

Parameters (mg/dl)	Group A	Group B	Group C	Group D	Group E	F Group
Protein Serum	20.10±1.38[a]	21.81±1.26[a]	24.42±2.17[a]	21.30±0.48[a]	22.04±1.23[a]	21.10±1.15[a]
Protein liver	32.72±0.12[a]	61.2±2.70[b]	31.30±2.09[a]	32.71±0.44[a]	39.23±3.57[c]	30.19±3.40[a]
Protein kidney	33.82±1.12[a]	53.80±0.68[b]	33.63±2.15[a]	34.17±4.10[a]	32.89±2.41[a]	31.98±1.29[a]

Values are means of eight replicates ± SEM; Values with different superscripts across the row are significantly (p< 0.05) different from others**.** Group A: control, group B: administered 60 mg/kg tramadol, group C: treated with 250 mg/kg Vit.C, group D: treated with 250 mg/kg b.wt PNAE, group E: treated with 500 mg/kg b.wt PNAE, group F: treated with 1000 mg/kg b.wt PNAE.

4. Discussion

Oxidative stress and inflammation are interconnected processes that influence each other in a complex relationship. On one hand, inflammation can induce oxidative stress by activating immune cells, such as neutrophils and macrophages, which produce ROS as part of their defence mechanisms (Soomro, 2019). Excessive production of ROS can overwhelm the body's antioxidant capacity and lead to oxidative damage to cells and tissues (Fischer and Maier, 2015). On the other hand, oxidative stress can also trigger and perpetuate inflammation as ROS can activate redox-sensitive signaling pathways, such as NF-κB and MAPKs, which promote the production of pro-inflammatory cytokines and chemokines (Kaur et al., 2015).

These inflammatory mediators further stimulate immune cell activation and recruitment, amplifying the inflammatory response (Soomro, 2019). The current study investigates the potential therapeutic effects of *P. nigrum* whole fruits in male rats induced into oxidative stress and inflammation by tramadol, commonly used as an analgesic. Numerous studies have shown the antioxidant qualities of the tannins and phenolics found in plant extracts. According to the review done by Kurnia et al. (2024), tannins, especially those in avocados (*Persea americana*) have a considerable antioxidant effect via chelating transition metals and scavenging free radicals. The study highlights that these effects are mostly caused by condensed tannin molecules, such as procyanidins and catechins. Hydroethanolic extract of *Blighia sapida* was the subject of another investigation, which found that the plant's high phenolic component concentration is associated with potent anti-inflammatory and antioxidant qualities. Because of its high phenolic content, the extract has a strong potential to scavenge free radicals, as reported in the study which shows a significant decrease in DPPH radicals (Dermane et al., 2024). The concentration of various antioxidant biomarkers in the body, such as MDA, GSH, SOD, CAT, NO and the level of total proteins, can provide valuable insights into different physiological and pathological processes. The increase in total protein levels in the liver and kidney after tramadol treatment indicates a localized physiological reaction to oxidative stress and possible drug-induced cellular damage (Doostmohammadi and Rahimi 2020; Barbosa et al., 2021). As the organs try to counteract oxidative damage supported by the rise in MDA levels, this may include increased activity of enzymes involved in detoxification processes or increased protein synthesis as part of a reparative strategy (Doostmohammadi and Rahimi 2020). The extract may have a protective effect against tramadol-induced oxidative stress by restoring normal protein synthesis and lowering oxidative damage in these essential organs, as evidenced by the reversal of these parameters at all dosages. This is in consistence with the reports of Nurudeen et al. (2024).

Malondialdehyde is a marker of oxidative stress and lipid peroxidation. Increased levels of MDA following the administration of tramadol may indicate the presence of oxidative damage to lipids, which can occur due to various factors such as inflammation, environmental toxins, or excessive production of ROS (Cherian et al., 2019). It is also associated with conditions like cardiovascular disease, neurodegenerative disorders and certain types of cancer (Tsai et al., 2021). The decrease in the levels of MDA in both the liver and kidney following the administration of an aqueous extract of *P. nigrum* might imply a reduction in oxidative stress or lipid peroxidation in these organs (Andrianova et al., 2020). It could also indicate a reduction in free radical generation, an enhancement in antioxidant defence mechanisms, or a combination of both (Andrianova et al., 2020). GSH is a crucial antioxidant that plays a key role in protecting cells from oxidative stress. It helps in neutralising ROS and maintaining cellular redox balance (Valko et al., 2016). The reduction in the levels of GSH after administration of tramadol can indicate an imbalance between oxidative stress and antioxidant defence mechanisms, which is commonly observed in conditions such as chronic diseases, liver dysfunction and exposure to environmental toxins (Valko et al., 2016). The administration of PNAE increases GSH levels in the liver indicating enhanced antioxidant capacity, which may help mitigate the effects of oxidative stress and maintain cellular health. It suggests that the liver is actively responding to oxidative stress and employing protective mechanisms to maintain its function and integrity (Chen et al., 2020).

Superoxide dismutase is an enzyme that catalyses the dismutation of superoxide radicals, converting them into less harmful molecules. It is one of the body's primary defence mechanisms against oxidative stress (Tian et al., 2022). Changes in SOD activity or expression levels can reflect alterations in the body's antioxidant capacity. The decrease in SOD activity following the induction of

oxidative stress is associated with conditions like neurodegenerative diseases, cardiovascular disorders and ageing (He et al., 2022). The significant increase in SOD levels in both liver and the kidney, after the administration of PNAE suggests that the antioxidant capacity and the ability to neutralise ROS are relatively balanced between the kidney and the liver (Miao and Clair, 2009). CAT is an enzyme that breaks down hydrogen peroxide into water and oxygen, thereby reducing oxidative stress. It plays a crucial role in protecting cells from the harmful effects of hydrogen peroxide. Decreased CAT activity in oxidatively stressed rats is linked to conditions such as diabetes, liver diseases and certain genetic disorders (Farzanegi et al., 2019). The administration of PNAE showed a significant increase in catalase activity in the liver. This indicates an increased ability of the liver to eliminate hydrogen peroxide and protect against oxidative stress (Risteen et al., 2018) and also suggests that the liver is better equipped to handle detoxification processes and eliminate toxic compounds and may reflect a healthier liver with enhanced metabolic activity (Giannini et al., 2005). The significant increase in catalase activity in the kidney also suggests that the antioxidant defense mechanism mediated by catalase is maintained at a relatively stable level, thus functioning as a defense against oxidant-mediated tissue injury (Kobayashi et al., 2005). NO is a signaling molecule involved in various physiological processes, including vasodilation, neurotransmission and immune response. It acts as a potent vasodilator and regulates blood pressure (Sheng and Zhu, 2018).

The significant increase in the levels of NO in oxidatively stressed rats can contribute to cardiovascular diseases, inflammation and impaired immune function. The administration of PNAE significantly reduced NO levels in the liver and kidney, which could suggest a balance between these isoforms or that their respective activities are brought to normal (Iwakiri and Kim, 2015). Similarly, this may suggest that the kidneys are in a normal functioning state. The level of NO was also comparable to the control indicating that the kidneys are adequately regulating blood flow and maintaining their essential functions and there may not be any underlying kidney disease affecting NO production or metabolism (Jain and Green, 2016). There is a dose-dependent reversal in the levels of kidney GSH, kidney SOD, and NO (both in the liver and kidney). This occurrence implies that the extract's ability to strengthen antioxidant defenses and lessen oxidative damage rises with dosage.

Inflammatory cytokines play a crucial role in the immune response and are involved in various inflammatory processes (Kany et al., 2019). Cytokines are small, secreted proteins (<40 kDa), which are produced by nearly every cell to regulate and influence immune response (Takeuchi and Akira, 2010). The release of pro-inflammatory cytokines leads to the activation of immune cells and production as well as the release of further cytokines (Schaper and Rose-John, 2015). TNF-α acts as a potent activator of other immune cells and stimulates the production of other pro-inflammatory cytokines, such as IL-1β. It also promotes the recruitment and activation of immune cells at the site of inflammation (Kany et al., 2019), and its excessive activation can lead to chronic inflammation (Jang et al., 2021).

The significant increase in TNF-α following the administration of tramadol suggests that it may have induced cardiac inflammation and increased the relative expression of TNF-α mRNA levels compared to the control group. This can also lead to the activation of cerebral NF-κB which is often associated with significant increase in the level of circulating TNF-α (Jang et al., 2021). The significant reversal of TNF-α level following the administration of PNAE can be attributed to the anti-inflammatory properties of the extract. Dysregulated IL-1β production and inflammation have been observed in autoinflammatory diseases due to the proteotoxic effects of mutant proteins. Harmful mutant proteins can induce dysregulated IL-1β production and inflammation through different pathways

depending on the cell type involved (Carta et al., 2017). The significant decrease in IL-1β following the administration of tramadol suggests that it may have disrupted the balance of IL-1β signalling which may contribute to the pathogenesis of inflammation. The significant reversal of IL-1β level in a dose dependent manner following the administration of PNAE can be attributed to its ability to balance IL-1β levels and decrease the activity of NLRP3, a crucial part of the inflammasome complex that produces IL-1β and other pro-inflammatory cytokines.

Conclusion

The study of the therapeutic potentials of the PNAE on tramadol-induced oxidative stress and inflammation provided valuable insight into its anti-inflammatory and antioxidant properties. The study shows that the dosages of 1000 mg/kg body weight of the extract display the most significant activity in the treatment of oxidative stress and inflammation. The findings from this study may contribute to the development of novel strategies for minimizing the adverse effects associated with tramadol therapy and provide a basis for further research in this field.

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Statements on compliance with ethical standards and standards of research involving animals

All institutional and national guidelines for the care and use of laboratory animals were followed.

Disclosure and conflict of interest

Conflict of Interest: The authors declare that they have no conflicts of interest.

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