



## T-2 toxin induces dermal inflammation and toxicity in mice: The healing potential of menthol

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### ABSTRACT

According to the World Health Organization and the Food and Agricultural Organization of the United Nations, T-2 is one of the most harmful food-toxic chemicals, penetrates intact skin. The current study examined the protective benefits of menthol topical treatment on T-2 toxin-induced cutaneous toxicity in mice. Lesions were observed on the skin of the T-2 toxin-treated groups at 72 and 120 h. The T-2 toxin (2.97 mg/kg/bw)-treated group developed skin lesions, skin inflammation, erythema, and necrosis of skin tissue in contrast to the control group. Our findings reveal that topical application of 0.25% and 0.5% MN treated groups resulted in no erythema or inflammation, and normal skin was observed with growing hairs. The 0.5% MN administered group demonstrated an 80% blister and erythema healing effect in *in vitro* tests. In addition, MN dose-dependently suppressed ROS and lipid peroxidation mediated by the T-2 toxin up to 120%. Histology discoveries and the immunoblotting investigations with the downregulation of i-NOS gene expression confirmed the validity of menthol activity. Further molecular docking experiments of menthol against the i-NOS protein demonstrated stable binding efficacy with conventional hydrogen bond interactions, indicating compelling evidence of menthol's anti-inflammatory effects on the T-2 toxin-induced skin inflammation.

### 1. Introduction

The T-2 mycotoxin is readily absorbed by various routes, including oral, topical, and inhalational ones. Contrary to most typical biotoxins, which do not affect the skin, T-2 is a potent skin irritant that can be absorbed through *Fusarium* species (*F. poae*, *F. sporotrichioides*, and *F. tricinctum*) are the leading producer of the T-2 mycotoxin, a significant crop and silage contaminant. When stored in wet storage conditions and in the field, these can infect wheat, barley, and corn. Consumption of mycotoxin-contaminated cereal-based food and feed is risky for human and animal health (Kanora and Maes, 2010; Janik et al., 2019). The best

conditions for boosting toxin production include relative humidity of 70% or less, oxygen availability, and temperatures between 0 and 50 °C, depending on the type of fungus (Zhang et al., 2018). The T-2 mycotoxin is non-volatile, insoluble in water, and challenging to degrade despite having a molecular weight of only 466.51 Da. It also exhibits extraordinary resistance to degradation under various environmental conditions, including heat and ultraviolet light. The decontamination procedure nevertheless works well in highly acidic or alkaline conditions, despite problems with toxin deactivation (Adhikari et al., 2017).

Intact skin and causes systemic toxicity. As a skin irritant and blistering agent, it is 400 times more potent than the chemical warfare agent

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sulphur mustard (Mustard gas, Yperite) (Albarenque et al., 1999). In addition, the inhalational toxicity of the T-2 toxin is comparable to that of mustards or lewisite. Therefore, T-2 toxin features more closely mirror those of chemical agents than biological toxins. Rats, rabbits, guinea pigs, and cynomolgus monkeys have been utilized as experimental animals in studies looking at the T-2 toxin's cutaneous irritancy (Hemmati et al., 2012; Dai et al., 2022). Dermatotoxic consequences in the context of chickens include lethal ulceration and necrotizing dermatitis. Several animals exhibit comb cyanosis and depigmented skin on their legs (Yang et al., 2021). The mouse model has shown that T-2-mycotoxin causes cutaneous damage and skin inflammation.

In several studies that successfully investigated the role of medicinal plants in the healing of skin wounds, it was discovered that the extracts or their isolated compounds accelerated the healing process, decreased localized inflammation, and increased cellular antioxidant defence (de-Oliveira et al., 2020; Li et al., 2022). Plant-based treatments have been demonstrated to quicken wound healing while maintaining aesthetics (Shedoeva et al., 2019). Because of this, plants are utilized to make more than 70% of the medications used to treat wounds (Lordani et al., 2018). Menthol, a monocyclic monoterpene, is present in the essential oil of various species of the *Mentha* genus (Rozza et al., 2021). It is an agonist of the transient receptor potential melastatin-8 channels (TRPM8), which are cold receptors in the skin's sensory nerves. As a result, menthol has a cooling effect when applied to the skin or mucous membranes (Silva et al., 2019). TRPM8 channel activation does not impact the speed of cutaneous wound healing (Nguyen et al., 2021). Menthol also has a bimodal action in activating TRPA1 and TRPV1 channels (Koivisto et al., 2022). TRPV3 channel activation enhances skin wound healing, according to some research (Sahu and Goswami, 2023). Menthol is a typical component of cosmetics and drugs used to relieve pain and respiratory problems (Bastaki et al., 2018; Singh et al., 2015; Rachitha et al., 2023). Therefore, the present study aimed to examine the preventive efficacy of menthol against T-2 toxin-induced cutaneous toxicity because there is little information on treating T-2 toxin-induced dermal toxicity.

## 2. Materials and methods

### 2.1. Animals

Committee No. 28/IAEC/CPCSEA and the Institute Animal Ethics Committee approved the permission to employ animals in the experiment. The male Balb/c mice weighing 20–25 g was obtained from Defence Food Research Laboratory, Mysore, India. The mice were housed in acrylic fibre cages at (25 ± 2 °C) with a 12-h light/dark cycle and given a commercial pellet diet (India's Sri Venkateswara Enterprises, Bangalore) and an endless supply of water.

### 2.2. Menthol cream development for skin wound treatment

Merck supplied (–)-menthol (99% purity), which was blended with eucerine cream at concentrations of 0.25% (MN 0.25%) and 0.5% (MN 0.5%). Eucerine cream alone used as negative control.

### 2.3. Experimental design

The design and observation of the animal studies have adhered to the standard protocol (Agrawal et al., 2012; Hemmati et al., 2012). In the investigation, six mice from each of the following groups were used: No toxin was given to Group 1 that served as the control group; T-2 toxin was given to group 2 (2.97 mg/kg bwt), group 3 (2.97 mg/kg bwt) with eucerine base, group 4 T-2 (2.97 mg/kg bwt) with 0.25% MN, and group 5 (T-2 2.97 mg/kg bwt) with 0.5% MN. The bottom backs of the mice were made hairless. T-2 solution in ethanol (5 mg/mL) was produced. Using a Hamilton microsyringe, 7 µl of such solution containing 70 µg of T-2 (2.97 mg/kgbw) was administered. Shaved skin (1 cm<sup>2</sup> surface area)

was applied/administered a solution containing 70 µg of the T-2 toxin using a Hamilton microsyringe. The animals were not allowed to move until the toxin was absorbed and dried on the appropriate region. Mice were observed for the onset of erythema, blisters, and inflammation for three days. Following the application of the toxin, animals were given eucerin and different doses of MN (0.25% and 0.5%) twice a day until complete healing was evident, and at that point, the number of days was recorded. To avoid wound infection, the husk bedding in the cages was changed daily, and the cages were maintained tidy. The injured animals were housed in separate cages to avoid infection.

### 2.4. Macroscopic assessments and scoring procedures

A macroscopic evaluation and scoring system was employed, according to Hemmati et al. (2012). The T2 toxin-generated skin damage, such as blisters, inflammation, and erythema, was scored from the 1st to the 4th day, depending on the severity of the damage. Normal skin on the control animal obtained a score of 0. Mice were compared in terms of the time skin lesions took to heal fully.

### 2.5. Histological studies

Skin samples from the experimental area were taken using a scalpel blade and fine forceps. A 10% formalin solution was used to fix the tissues. The tissues were processed, paraffin-embedded, cut into 5 µm pieces using a microtome, and histologically stained with hematoxylin and eosin. A Cool SNAP® 158 Pro colour digital camera was used to take pictures, while a light microscope (Olympus, Japan) was used to examine the morphology of the skin tissue.

### 2.6. Quantification of reactive oxygen species (ROS) and lipid peroxidation in skin tissue

The 2', 7'- dichloro-dihydrofluorescein diacetate (DCFH<sub>2</sub>-DA), a fluorescent dye, was used to measure the production of ROS in the skin (Wang and Joseph, 1999). The skin samples were homogenized in a buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris HCl buffer, pH 7.2). The skin lysates were mixed with 40 µl of 1.25 mM DCFH<sub>2</sub>-DA and incubated for 15 min at 37 °C. The fluorescence was measured at 485 nm excitation and 525 nm emission using a Hidex plate chameleonTM 144 V (Finland).

The lipid peroxidation was assessed by quantifying Malondialdehyde (Buege and Aust, 1978). Skin tissue was homogenized in a lysis buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris HCl buffer, pH 7.2). The procedure involves heating skin homogenate in a boiling water bath for 20 min with TBA reagent. TBA reagent is made up of 20% TCA, 0.5% TBA, and 2.5 N HCl. After cooling, the solution was centrifuged at 8000 rpm for 10 min to remove the precipitate. Absorbance was recorded at 532 nm, and results were expressed as µmol of malondialdehyde (MDA) production per gram of wet tissue.

### 2.7. Western blot experimentation

Western Blotting analytical methodology employing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the specific proteins of interest. The lysis buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris HCl buffer, pH 7.2) was supplemented with a protease inhibitor cocktail and 100 mg skin tissue was homogenized. Afterwards, total protein content was determined using the Bradford method (Bradford, 1976). After separation on an SDS-PAGE, protein was transferred using an electroblotting device onto a nitrocellulose membrane (Cleaver Scientific Ltd, UK). After transfer, the membranes were coated with primary antibodies of i-NOS (sc-651) and tubulin (sc-5286) (Santa Cruz Biotechnology, Santa Cruz, California) and incubated for 3 h at room temperature. The membranes were then washed with TBST and incubated with rabbit anti-goat, goat anti-mouse,

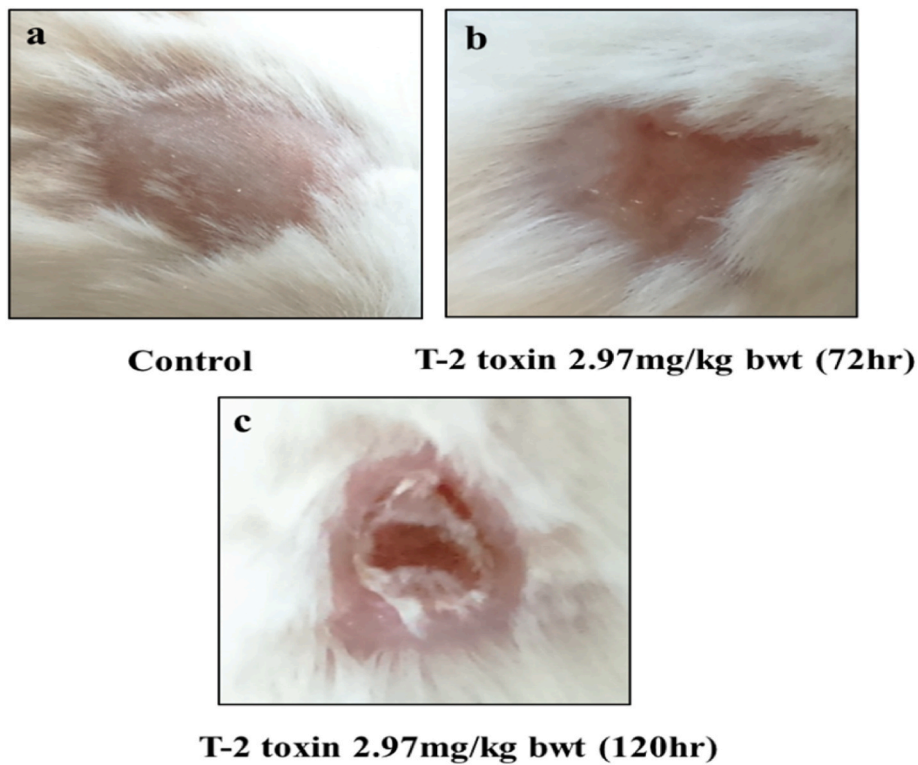


Fig. 1. Skin lesion observed at 72 and 120 h after T-2 toxin administration. The hallmarks of a toxin's effect are erythema, inflammation, and tissue necrosis.

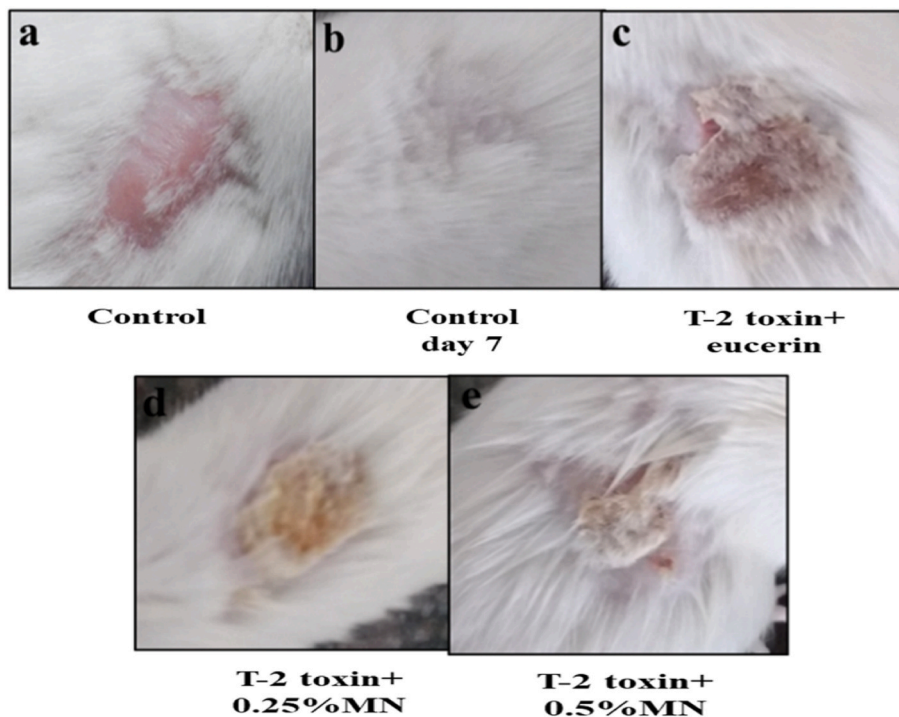


Fig. 2. Skin lesions of the mice after giving MN and T-2 toxin.

and goat anti-rabbit secondary antibodies (DAKO, Denmark) at 1:10,000 dilutions for 2 h at room temperature in the dark. The membranes were developed using a chemiluminescence detecting method after a second round of cleaning (Proteo Qwest1, Sigma). Membranes were exposed to X-ray film, and the resulting band intensity was measured. The NIH Image J programme was used to determine the intensity of Western blot

bands (Krupashree et al.,2022).

2.8. Molecular docking studies

2.8.1. Ligand preparation

Ligand was prepared by retrieving the 3D structure of MN from



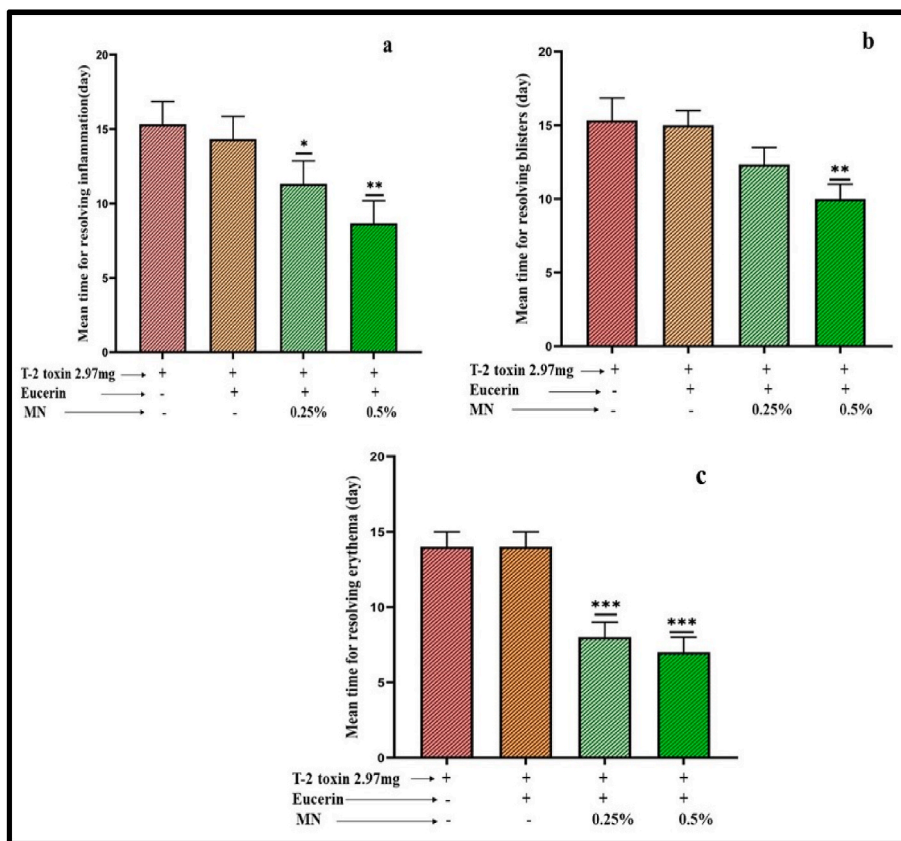


Fig. 3. (a). Effect of MN on skin inflammation and the time required for complete healing were compared. (b). Effect of MN on skin blisters and the time required for complete healing were compared. (c). Effect of MN on skin erythema and the time required for complete healing were compared. \* ( $P < 0.05$ ), \*\* ( $P < 0.01$ ), \*\*\* ( $P < 0.001$ ) denotes significant differences between treatment groups ( $n = 6$ ) with eucerin and no treatment groups.

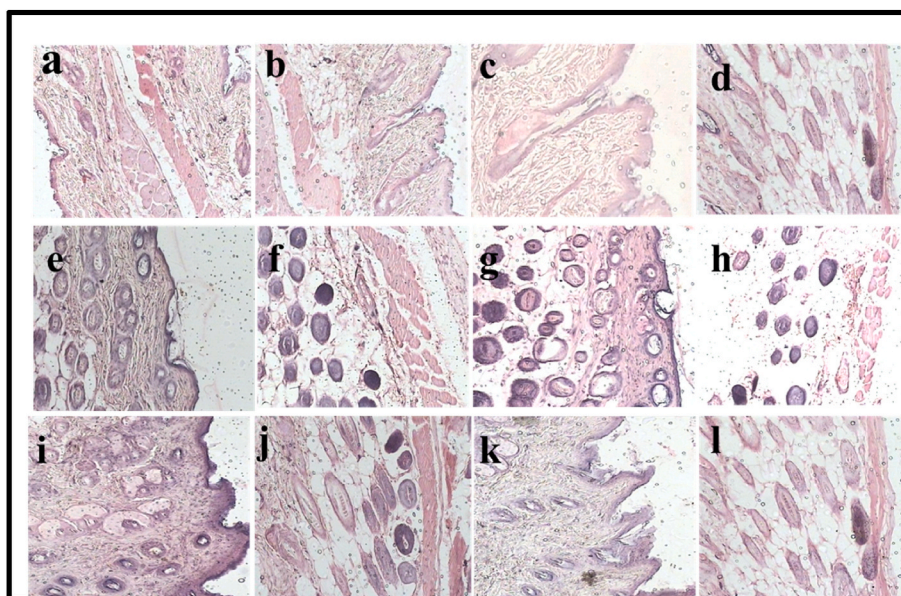


Fig. 4. Histology studies: (a–d) control; (e,f) 2.97 mg/kg bwt T-2 toxin 72 h. There was epidermal necrosis associated with dermal inflammation; (g,h) 2.97 mg/kg bwt T-2 toxin 120 h. It is possible to see incomplete epidermis granulation tissue formation; (i,j) T-2 toxin+ 50 µg MN inflammation and incomplete epidermis reduced; (k,i) T-2 toxin+ 100 µg MN. A new epidermis layer with granulation tissue is forming.

PubChem in sdf file format. This was again converted to pdb format using Open Babel GUI software for the docking studies (Pires et al., 2015).

### 2.8.2. Protein preparation

Protein preparation was carried out by retrieving 3D X-ray crystallographic structure from PDB. The target protein selected for the studies was i-NOS (PDBID: 2NSI). The structure was cleaned using UCSF

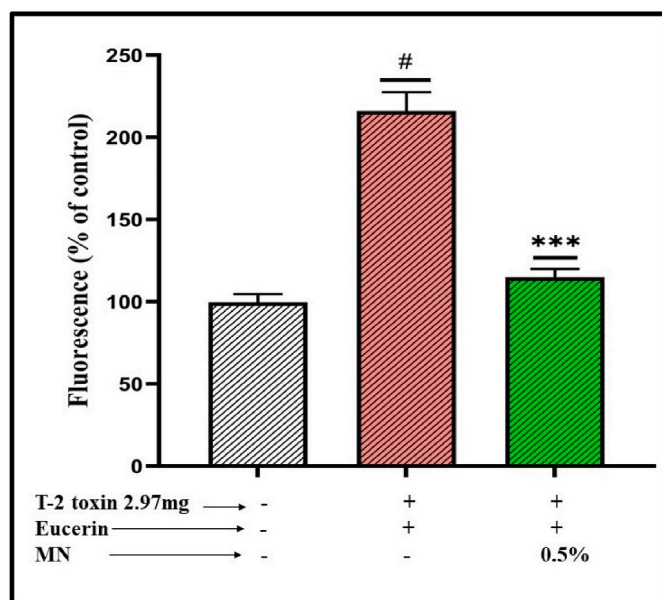


Fig. 5. ROS content in the skin. One-way ANOVA was used to analyze the results. \*\*\* $P < 0.001$  versus T-2 Toxin ( $n = 6$ ). # $(P < 0.05)$  versus control.

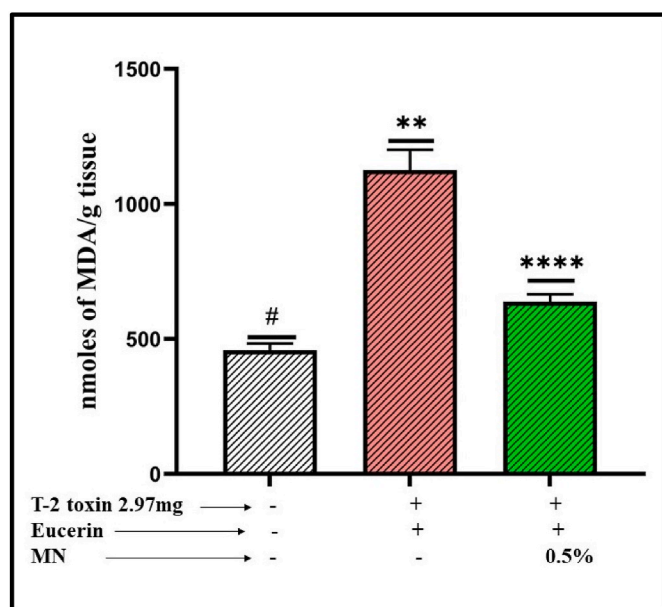


Fig. 6. Malondialdehyde content in the skin. One-way ANOVA was used to analyze the results. \*\*\*\* $(P < 0.0001)$  versus T-2 Toxin ( $n = 6$ ). \*\* $(P < 0.05)$  versus control, # $(P < 0.05)$ .

Chimera software by removing water and ligand molecules (if any) from the protein structure. The protein active pose was generated using CASTp (Jain et al., 2021).

### 2.8.3. Molecular docking

Molecular docking was performed using Autodock Vina embedded into PyRx software. BIOVIA Discovery Studio was used to visualize the docked ligand and generate the 2D image of the ligand interacting with the amino acid residues of the target proteins (Kumar et al., 2021).

### 2.9. Statistical analysis

One-way analysis of variance (ANOVA) was performed to identify

differences between groups, and the mean (M) and standard error (SE) of the mean (S.E.M.) was tested using Tukey's multiple comparison test with a  $P < 0.05$  (95% confidence and significance level) alpha level. Graph Pad Prism software, version 9.04, was used for all analysis.

## 3. Results and discussion

### 3.1. Macroscopic examination

The T-2 toxin is a powerful active skin irritant that causes systemic poisoning and has purportedly been used as a biological weapon (Janik-Karpinska et al., 2022; Ueno, 1980). Numerous eyewitness and patient accounts claim that low-flying aircraft poured the yellow, oily liquid on the victims after dispersing the aerosolized T-2 mycotoxin, known as "yellow rain" (Franz et al., 1997). So, the current study was designed to evaluate the ameliorating effect of MN on T-2 toxin-induced cutaneous toxicity. Lesions were observed on the skin of the T-2 toxin-treated groups at 72 and 120 (Fig. 1a-c). The T-2 toxin (2.97 mg/kg/bw) treated Group developed skin lesions, skin inflammation, erythema, and necrosis of skin tissue in contrast to the control group. Our findings reveal that topical application of 0.25% and 0.5% MN pre-treated groups resulted in no erythema or inflammation and normal skin was observed with grown hairs (Fig. 2a-e). The findings support the study by Hemmati et al. (2012) on the anti-inflammatory effects of quince seed mucilage applied topically against T-2 toxin-induced skin irritation.

### 3.2. Assessment of the inflammatory damage

In the T-2 toxin-treated Group, skin irritation was noticed on the 3<sup>rd</sup> day. The inflammation brought on by the T-2 toxin did not impact the Group that got eucerine therapy. On the 11<sup>th</sup> day, the 0.25% MN-treated group demonstrated decreased skin inflammation. While at 7<sup>th</sup> day, the Group that got 0.5% MN treatment demonstrated an 80% reduction in inflammation (Fig. 3a). Findings on cutaneous toxicity have showed edema, abnormal single-nucleus cell accumulation, necrosis, inflammation, increased thickness of the malpighian layer, and cellular infiltration (Chaudhary and Rao, 2010; Hemmati et al., 2012). These skin wounds mimic those brought on by a poison utilized as a biological weapon concerning the yellow rain illness (Smith, 1984).

### 3.3. Evaluation of the blister and erythema damage

Observations were recorded on 3<sup>rd</sup> day for evaluation of blister damage where T-2 toxin-treated groups showed symptoms of blistering. Eucerin alone did not affect the T-2 toxin-induced blister. On the other hand, on the 7<sup>th</sup> day, the 0.5% MN administered Group demonstrated an 80% blister healing effect (Fig. 3b).

On 3<sup>rd</sup> day, skin erythema began to develop in the T-2 toxin group, and the Group that topically received 0.25% displayed decreased skin erythema on the 9<sup>th</sup> day. The erythema redness entirely disappeared 7 days after receiving 0.5% MN (Fig. 3c). The phytochemical MN from the *Mentha piperita* plant has a cooling effect by inhibiting the calcium stream that goes along the neurons and feels temperature (Kanezaki et al., 2021). It does not reduce the temperature of the skin or the body. Each nerve ending is employed for temperature sensing since neurons operate as the brain's wiring. TRPM8, a receptor protein that recognizes temperature changes, is present in all cold-sensing nerve cells (Wang, 2021; Yin et al., 2018). According to earlier studies, MN has been used to treat inflammation and skin blemishes (Cheng and An, 2022; Liu et al., 2021; Panahi et al., 2007). MN evidently halted the oxidative stress and inflammatory processes triggered by the T-2 toxin in our investigation.

### 3.4. Histology studies

During the first 72 h after the main loss of the epidermis, lymphocyte

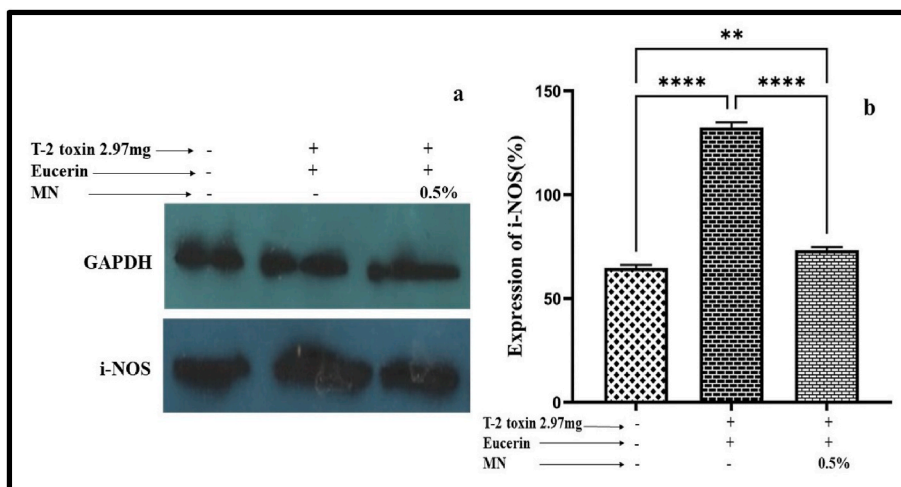


Fig. 7. Western blot analysis of i-NOS expression. The data are presented as means± standard deviations of three independent experiments. \*\*\*\*P < 0.0001 versus the T-2 toxin treated group; \*\*P < 0.01 versus the control group.

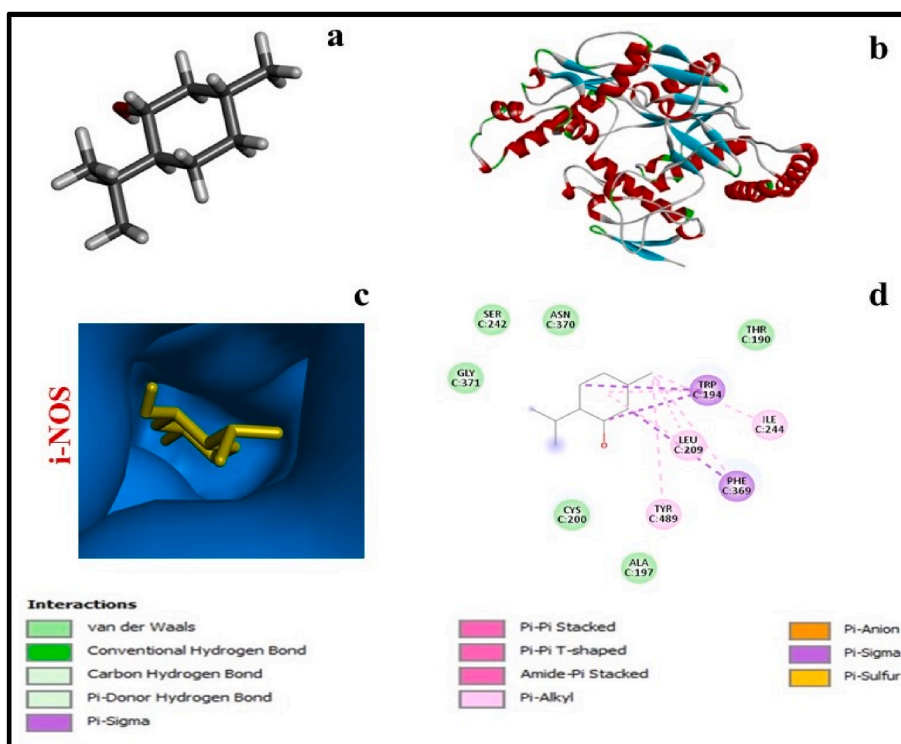


Fig. 8. (a) 3D structure of the ligand A Menthol; (b) 3D X-ray crystallographic image of the native unbound iNOS Protein; (c) 3D structures showing the interaction of the menthol with the amino acid residues of the iNOS protein; (d) 2D structures showing the interaction of the menthol with the amino acid residues of the iNOS protein.

**Table 1**  
Binding affinity (kcal/mol) of the ligand with corresponding protein.

Metabolite Protein	Menthol
2NSI	-6.8

aggregation and vessel dilatation were seen, signalling the start of inflammation. The epidermis demonstrated necrosis after a 120-h T-2 toxin treatment, while the derma displayed significant edema and vascular dilatation. A thin epidermis layer, a little inflammatory

reaction, and dermal edema were observed in the Group receiving 0.25% MN. The Group given 0.5% MN showed normal cutaneous granulation tissue and epidermis (Fig. 4 a–d) (control), e–f (2.97 mg/kg bwt T-2 toxin 72 h), g–h (2.97 mg/kg bwt T-2 toxin 120 h), i–j (T-2 toxin+ 0.25% MN), and k and l (T-2 toxin+ 0.5% MN). Macroscopic and histological studies revealed that the relieving effect was due to MN’s ability to reduce inflammation and promote wound healing. Findings show that MN is a powerful anti-inflammatory medication. Literature suggests that the wound healing process is carried out by modifying the immune molecules involved in the inflammation process (Rozza et al., 2014; Zaia et al., 2016).



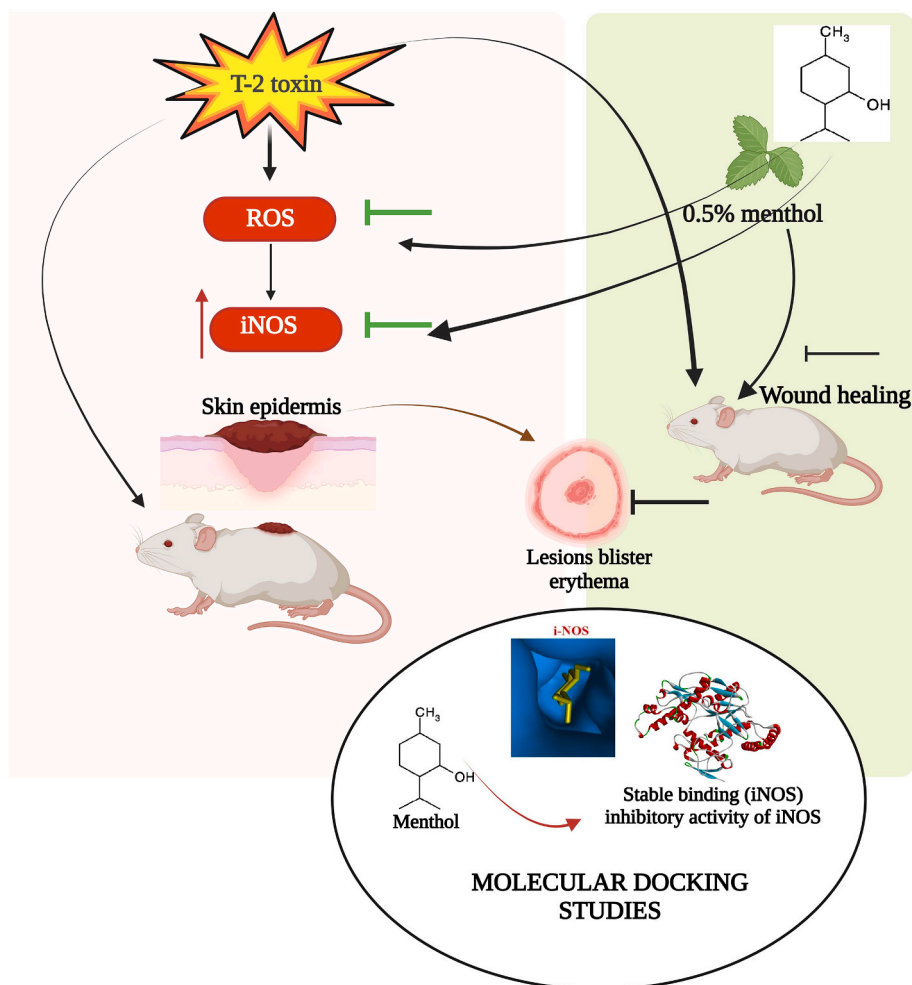


Fig. 9. Schematic diagram of T-2 toxin and molecular docking studies.

### 3.5. Protective effect of MN on ROS and lipid peroxidation

The ROS generations in skin tissue were estimated using a fluorescent probe. T-2 toxin treatment on skin homogenates led to a 220% increase in fluorescence ( $P < 0.05$ ), which diminished dose-dependently with up to 120% (0.5% MN group) (Fig. 5). Malondialdehyde, a substance formed when free radicals cause oxidative damage to skin tissue, is used to quantify the degree of lipid peroxidation. The T-2 toxin increased lipid peroxidation up to 120%, but 0.5% MN treatment reduced it up to 70% ( $P < 0.05$ ) (Fig. 6). The MN exhibits anti-free radical properties that reduced the lipid damage (Rozza et al., 2014; Joubert and Malan, 2011).

### 3.6. Examination of MN against T-2 toxin-induced i-NOS expression

The expression of numerous inflammatory response-related genes, including i-NOS, contributes to skin inflammation. The T-2 toxin-treated Group in the current investigation showed i-NOS modulation. Inflammation is caused by T-2's upregulation of i-NOS expression, which was shown in an earlier study by Seeboth et al. (2012). In accordance with this result, T-2 also elevated i-NOS expression in our findings. Using 0.5% of MN with T-2 toxin lowered the expression of i-NOS (Fig. 7a & b). According to an *in vitro* investigation by Liu et al. (2021) reported that menthol has a strong inhibitory effect on the expression of i-NOS in mammary epithelial cells. Similar trends were seen in the current *in vivo* study ie, inhibitory action of menthol against T-2 induced i-NOS expression. The i-NOS is elevated in a variety of clinical disorders linked

to inflammation, and it appears that blocking NOS is a valuable strategy for reducing tissue damage brought on by the presence of reactive nitrogen intermediates (Robertson et al., 1996). Our results align with those of the prior work by Liu et al. (2021).

### 3.7. Molecular docking studies

The 3D structure of MN retrieved from PubChem is represented in Fig. 8a and the native, unbound, 3D X-ray crystallographic structure of i-NOS protein is shown in Fig. 8b. Molecular docking studies allow explaining the nature of the interactions between the ligand and the amino acid residues of the protein within its active site. Table 1 displays the binding affinity (kcal/mol) of the MN ligand with the i-NOS target protein (PDBID: 2NSI). Figure (8c&d) depict the interaction in 3D and the matching 2D image, respectively.

The stability of the docked molecule depends on the nature of the interaction made with the amino acid residues of the proteins. The conventional Hydrogen bond, Pi, Sigma bonds, van der Waal are some of the bonds that contribute to the complex's stability. It is possible to infer that the complex established is stable based on the binding affinity and type of contact that MN created with i-NOS. MN reduced inflammation brought on by the T-2 toxin, by interacting with i-NOS hydrogen bonds. The reduction of T-2 toxin induced inflammation in mice skin by upregulation of i-NOS and amelioration activity by MN raises the possibility of new therapeutic avenues for disorders linked to cutaneous inflammatory processes. Our results are consistent with prior studies by Sousa et al., 2021; Loza-Mejía and Salazar, 2015; and Baig et al., 2021,

indicating the anti-inflammatory effect supported by the molecular docking analysis by evaluating the phytochemicals binding efficacy and their inhibitory action against inflammatory genes in *in vivo* models (i-NOS, TNF- $\alpha$ , IL-6, cyclooxygenase-2). Menthol protective efficacy against T-2 toxin induced dermal toxicity is illustrated in Fig. 9.

#### 4. Conclusion

Owing to its capacity to release as aerosols, the T-2 toxin is recognized as a skin irritant and is considered to be more harmful than the other trichothecenes. Herbal sources of polyphenols and flavonoids, which are abundant in antioxidants, are well known for their ability to reduce inflammation. Due to its ability to reduce inflammation and cool the skin, MN is used in the pharmaceutical and cosmetic sectors. Menthol was observed, and its capacity to lessen skin irritation brought on by T-2 toxins was investigated on the topical application in a mouse model. Research at the cellular and molecular levels is further required to confirm the additional validation of menthol.

#### Credit author statement

**Rachitha P:** Conceptualization, Methodology, Formal analysis, Data curation, Software, Investigation, Writing-original draft, Visualization. **Krupashree K:** Writing, Review & Editing. **Jayashree G.V:** Writing, Review & Editing. **Vinay B. Raghavendra:** Review & Editing, Supervision. **Ajay Pal:** Writing, Review & Editing. **Arunachalam Chinnathambi:** Writing, Review & Editing. **Sulaiman Ali Alharbi:** Writing, Review & Editing. **Rajasree Shanmuganathan:** Writing, Review & Editing. **Indira Karuppusamy:** Writing, Review & Editing. **Kathirvel Brindhadevi:** Review & Editing, Supervision.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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