



ORIGINAL RESEARCH ARTICLE

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Pretreatment administration of hydro-ethanolic extract of *Syzygium aromaticum* prevents thioacetamide-induced liver injury in rat

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Abstract

Introduction Liver injury is an important problem in healthcare. Thioacetamide (TAA)-induced liver injury is an established model in experimental research for assessing the impact of various toxins and pharmaceuticals on the liver. TAA induces its harmful effects by the production of oxidative biomolecules. Oxidative stress subsequently alters liver function, resulting in alterations in the enzymatic activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). *Syzygium aromaticum* has significant antioxidant and anti-inflammatory properties and has been utilised in traditional medicine for liver diseases. Therefore, this study evaluated the hepatoprotective effect of the hydro-ethanolic extract of *Syzygium aromaticum* (HESA) against TAA-induced liver injury.

Material and methods Hepatotoxicity was induced with intraperitoneal administration of TAA (150 mg/kg body weight, 3 days per week for 4 weeks) in Wistar rats. The pretreatment with HESA was conducted at three doses of 50, 150, and 300 mg/kg body weight, administered orally, starting 4 weeks before TAA administration and continued for 8 weeks. The activities of serum AST, ALT, and ALP, as well as liver superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and malondialdehyde (MDA), were assessed. Histopathological analyses were conducted using hematoxylin and eosin (H&E) staining.

Results The findings showed that HESA pretreatment significantly lowered TAA-induced oxidative stress. Additionally, TAA significantly increased AST, ALT, and ALP serum levels, whereas HESA significantly recovered the corresponding values. H&E staining also showed that TAA-induced structural liver damage was characterised by central vein dilatation, and necrosis, and apoptosis in adjacent cells. The histopathological findings of the TAA group were partially recovered in the pretreatment extract groups.

Conclusion The results of this study support the antioxidant and anti-inflammatory properties of *Syzygium aromaticum* against TAA-induced liver injury.

Keywords *Syzygium aromaticum*, Clove, Liver injury, Thioacetamide, Oxidative stress

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Introduction

Liver injury is a leading healthcare problem, contributing to 2 million deaths annually [1], primarily due to cirrhosis and hepatocellular carcinoma (HCC). The metabolic regulatory nature of the liver and its exposure to exogenous substances makes it prone to injury [2, 3], which is why the liver has a defence system against oxidative stress [4, 5]. Thioacetamide (TAA) is an organosulfur commonly used in liver injury studies as it can mimic the oxidative stress induced in the liver [6–8]. *Syzygium aromaticum* (clove) is a natural herb containing many antioxidant, anti-inflammatory, and hepatoprotective compounds, including eugenol [9–11] and β -caryophyllene [12–15], making it a strong hepatoprotective choice [16].

Pretreating the liver with a hepatoprotective compound (e.g. *Syzygium aromaticum*) strengthens its defence against potential harm [17]. Wang et al. pretreated the liver with oleanolic acid (OA), which exhibited a hepatoprotective effect against concanavalin A [18]. In a clinical context, pretreatment is also crucial, particularly in the management of a hepatitis C patient, where evaluating previous use of hepatoprotective drugs benefits in optimising subsequent therapy strategies [19].

The main goal of this study is to assess the effects of hydro-ethanolic extract of *Syzygium aromaticum* (HESA) as a pretreatment intervention before TAA-induced liver injury. This investigation builds upon the existing literature that endorses the hepatoprotective characteristics of *Syzygium aromaticum* with its minimal side effects and cost-effectiveness relative to alternative treatments [20, 21]. Ultimately, the study aims to enhance liver therapies and improve the global prognosis of liver diseases.

Materials and methods

Extract preparation

Following the verification of the herb, an ethanolic extract of *Syzygium aromaticum* was prepared by grinding the dried clove buds into a fine powder and subsequently soaking them in 95% ethanol for 3 days at room temperature. The supernatant was filtered and concentrated using a rotary evaporator (65 °C). The finalised extract was refrigerated (4 °C) until used for the experiment.

Animals and hepatotoxicity induction

Forty adult male Sprague–Dawley rats weighing 224–245 g were used. All ethical standards were met, and the study received approval from the ethics committee of Lorestan University of Medical Sciences (Approval ID: IR.LUMS.REC.1401.044). All animals were provided unrestricted access to food and water in a controlled environment, with a light–dark cycle of 12 h each. Animals were randomly allocated into five groups: sham, thioacetamide (Thi), and thioacetamide combined with

HESA at dosages of 50, 150, and 300 mg/kg body weight orally (Thi+HESA 50, Thi+HESA 150 and Thi+HESA 300, respectively). Thioacetamide (Sigma-Aldrich) was administered intraperitoneally at a dosage of 150 mg/kg body weight three times per week for 4 weeks to induce hepatotoxicity. HESA's pretreatment started 4 weeks before TAA administration and continued for 8 weeks. Twelve hours after the last injection, blood was drawn via the heart, and the serum was isolated and stored at – 20 °C before measuring the targeted factors.

Preparation of liver extract

Ten percent (w/v) homogenates were produced by freezing and homogenising small liver tissue samples in ice-cold saline. The samples were placed in a benchtop centrifuge (Carlbcam, Germany) and centrifuged for an hour at 4 °C at 10,000 g. Bovine serum albumin was used as a reference for measuring protein in liver supernatants via the Bradford method.

Assessment of liver enzymes and oxidative stress biomarkers

The activities of serum AST (aspartate aminotransferase), ALT (alanine aminotransferase), and ALP (alkaline phosphatase) were measured using Pars Azmun commercial kits (Cat No. 1022004, 1022003, and 1022035, respectively). The activities of liver superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and the concentration of malondialdehyde (MDA) were quantified spectrophotometrically (Hitachi, Japan) using commercial kits (Cayman, Item No. 706002, 703102, 707002, and 10009055 respectively).

Histopathological studies

Histopathological assessment was conducted in a standard procedure. The liver tissues were fixed with 4% paraformaldehyde for at least 3 days. Sections with 5 mm thickness were excised from the fixed tissue, dehydrated, and embedded in paraffin blocks. Subsequently, 5 μ m thick sections were prepared, stained with hematoxylin and eosin (H&E), and analysed microscopically. Liver injury was determined based on parameters including hepatocyte necrosis, lymphocyte infiltration, haemorrhage, and abnormal architecture in the liver parenchyma. A light microscope (Leica TCS SP, Germany) was used to examine ten randomly selected microscopic fields from each section in a blinded manner at a magnification of $\times 400$.

Statistical analysis

Data were presented as mean $\pm$ SEM. Statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) software, version 22. The data were

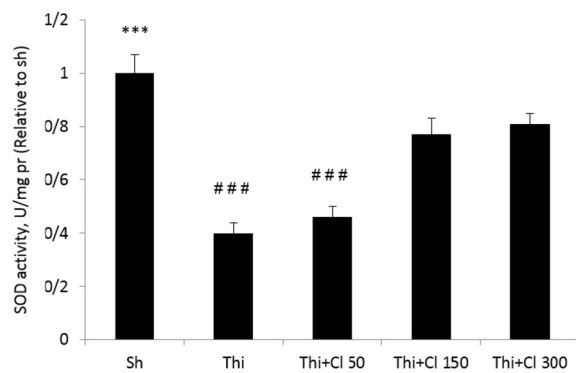


Fig. 1 Comparison of liver superoxide dismutase activity (SOD, mean $\pm$ SEM) in different groups, ***, $P < 0.001$, significant difference to Thi and Thi + HESA 50. ###, $P < 0.001$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups

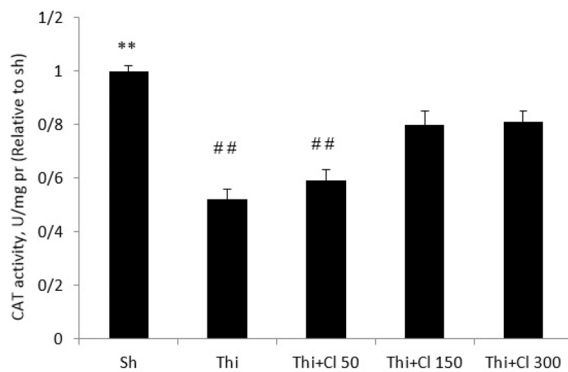


Fig. 2 Comparison of liver catalase activity (CAT, mean $\pm$ SEM) in different groups, **, $P < 0.01$, significant difference to Thi, Thi + HESA 50, Thi + HESA 150, and Thi + HESA 300 groups. ##, $P < 0.01$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups

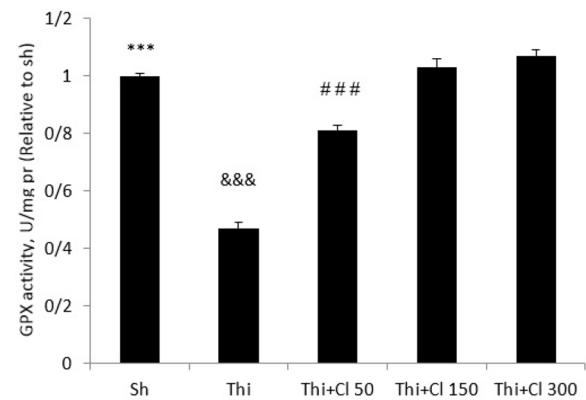


Fig. 3 Comparison of liver glutathione peroxidase (GPx, mean $\pm$ SEM) in different groups. ***, $P < 0.001$, significant difference to Thi and Thi + HESA 50 groups. &&&, $P < 0.001$, significant difference to Thi + HESA 50, Thi + HESA 150 and Thi + HESA 300 groups. ###, $P < 0.001$, significant difference to Thi + HESA 150 and Thi + HESA 300

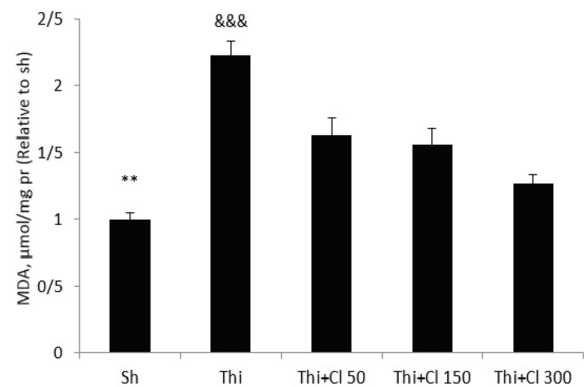


Fig. 4 Comparison of the value of liver malondialdehyde (MDA, mean $\pm$ SEM) in different groups, **, $P < 0.01$, significant difference to Thi, Thi + HESA 50 and Thi + HESA 150 groups. &&&, $P < 0.001$, significant difference to Thi + HESA 50, Thi + HESA 150 and Thi + HESA 300 groups

analysed using one-way ANOVA and Tukey's post hoc test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Effects of HESA on oxidative stress

The results showed that HESA pretreatment significantly reduced TAA-induced oxidative stress. TAA significantly decreased ($P < 0.001$) the activities of liver superoxide dismutase and glutathione peroxidase in comparison to the sham group (Figs. 1 and 3). In the TAA group, liver catalase activities were significantly lower compared to the sham group (Fig. 2). Pre-treatment with the extract (shown as Cl) at doses of 150 and 300 mg/kg significantly increased, the activities of superoxide dismutase, catalase, and glutathione peroxidase in comparison to the TAA groups (Figs. 1, 2, and 3). TAA

significantly increased malondialdehyde levels ($P < 0.01$) in comparison to the sham group (Fig. 4). The pretreatment administration of the extract at dosages of 50, 150, and 300 mg/kg significantly decreased ($P < 0.001$) malondialdehyde levels compared to the TAA group (Fig. 4).

Effects of HESA on liver function tests

TAA significantly increased serum levels of AST, ALT, and ALP ($P < 0.001$) in comparison to the sham group (Figs. 5, 6, and 7). The pretreatment administration of the extract (shown as Cl) at dosages of 150 and 300 mg/kg significantly decreased the serum levels of AST, ALT, and ALP in comparison to the TAA group (Figs. 5, 6, and 7).

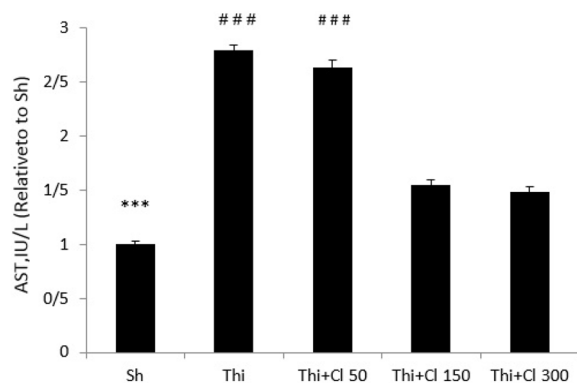


Fig. 5 Comparison of the serum level of aspartate aminotransferase (AST, mean $\pm$ SEM) in different groups, ***, $P < 0.001$, significant difference to Thi, Thi + HESA 50, Thi + HESA 150, and Thi + HESA 300 groups. ###, $P < 0.001$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups

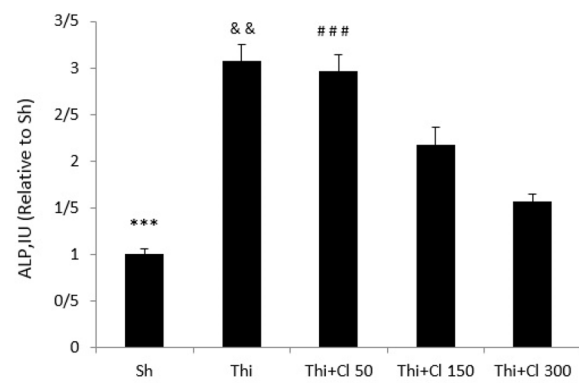


Fig. 7 Comparison of the serum level of alkaline phosphatase (ALP, mean $\pm$ SEM) in different groups, ***, $P < 0.001$, significant difference to Thi, Thi + HESA 50, and Thi + HESA 150 groups. &&, $P < 0.01$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups. ###, $P < 0.001$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups

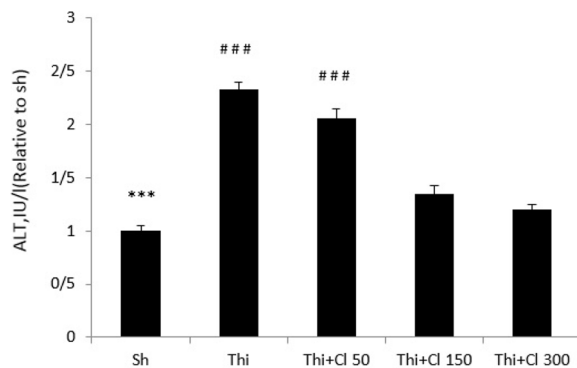


Fig. 6 Comparison of the serum level of alanine aminotransferase (ALT, mean $\pm$ SEM) in different groups, ***, $P < 0.001$, significant difference to Thi, Thi + HESA 50, Thi + HESA 150, and Thi + HESA 300 groups. ###, $P < 0.001$, significant difference to Thi + HESA 150 and Thi + HESA 300 groups

Effects of HESA on liver histopathology

H&E staining showed that TAA administration leads to significant structural liver damage. The histology findings showed that the liver lobules' central vein is dilated and full of blood. Necrosis and apoptosis were seen in the cells around the central vein. In the groups administered HESA, the dilatation of the central vein and the number of necrotic and apoptotic cells decreased in a dose-dependent manner (Fig. 8). In the group that was receiving HESA 300 mg/kg, high-level resolution of the liver histology was seen (Fig. 8D).

Discussion

This study explored the hepatoprotective effects of HESA on TAA-induced liver damage and demonstrates how pretreatment with HESA may contribute to preventing liver damage caused by TAA.

The study revealed a dose-dependent hepatoprotective effect of HESA, particularly at doses of 150 and 300 mg/kg, similar to comparable studies ranging from 100 mg/kg to 800 mg/kg [22–24]. A study by Jose et al. demonstrated the hepatoprotective effects of clove at the dosage of 100 mg/kg against alcohol-induced liver injury [24]. Similarly, Shojaeifard et al. reported the hepatoprotective properties of clove against TAA-induced liver injury at doses of 50, 150, and 300 mg/kg doses [25]. The cirrhosis protective properties of the eugenol-rich fraction (ERF) of clove were shown by Ali et al. at a dose of 80 mg/100 g body weight [26].

In the present study, HESA increased the levels of SOD, GPx, and CAT and decreased MDA levels, indicating HESA's anti-oxidant properties. These findings align with previous studies on the antioxidant properties of clove. Baskaran et al. reported an increase in SOD, CAT, and GPx levels and a decrease in lipid peroxidation in groups pretreated with eugenol [27].

Similar to other studies, liver enzymes (AST, ALT, and ALP) decreased in the treatment groups compared to the TAA group. El-Hadary et al. observed a decrease in liver enzymes in clove oil-treated groups compared to the positive control group over an 8-week period [11].

The histopathological findings showed severe liver damage in the TAA group (central vein dilation, necrosis, and apoptosis in the adjacent cells) compared to the control group. However, HESA-treated groups displayed a decrease in liver injury in a dose-dependent manner compared to the TAA group. Abdelrahman et al. also reported mitigated histopathological liver injury results (reduced portal area inflammation without any

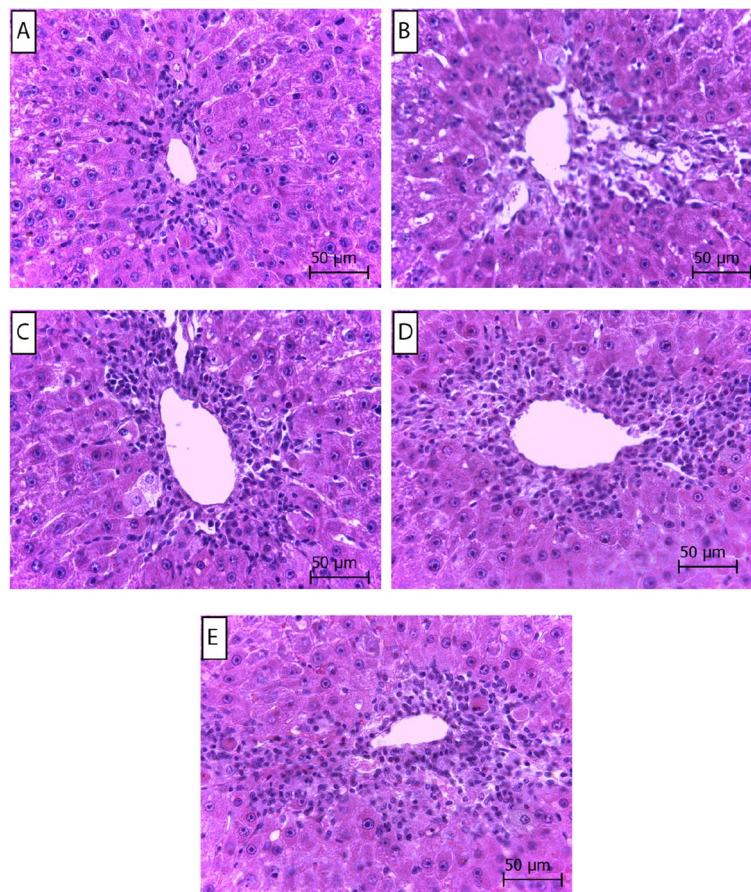


Fig. 8 Representative photomicrographs of liver tissue sections from animals with different experimental groups (H&E staining). **A** Normal histological structure and architecture of the liver from the sham group (sham). **B** Severe structural damage was seen in the liver from the TTA-treated group (Thi). **C** Mild resolution of liver damage of liver from the Thi + HESA50-treated group. **D** Moderate partially preserved hepatocytes and architecture were observed in the liver of the Thi + HESA150-treated group. **E** High-level resolution of liver damage of liver from the Thi + HESA300-treated group

fibroblastic proliferation) compared to the CCl_4 -induced liver injury group [28].

While this study used 95% ethanol, other studies, such as Hussien et al. or Prasad et al., utilised 70–50% ethanol [22, 29]; this could influence the concentration of eugenol in the final extract [30]. The study duration was 8 weeks, while other studies ranged from a few days to 15 weeks [22, 24, 31]. These differences in clove preparation and the duration of studies could also influence the study outcomes.

The main objective of this study was pretreatment, which is a way to fortify the liver before contact with hepatotoxins. This method is relatively rare in liver health research, where the treatment usually begins in parallel or after the hepatotoxin induction. Baskaran et al. pretreated liver with eugenol for 15 days with TAA administration in the last 2 days; all animals were sacrificed on the 16th day. The results showed significantly reduced lipid peroxidation, oxidation, and inflammation

in TAA-induced liver injury model rats pretreated with eugenol [27]. Shojaeifard et al. pretreated the mouse model for 21 days with TAA induction in the last 3 days. All animals were sacrificed 48 h after the last injection; the results showed mitigated liver damage in clove groups compared to the TAA-induced group [25]. Unlike the mentioned studies, this study administered HESA 4 weeks before TAA induction and continued HESA and TAA induction for 4 weeks. This creates a better model for studying the hepatoprotective effects of pretreatment of HESA in chronic liver injury.

Syzygium aromaticum exerts its protective roles through its antioxidant, anti-inflammatory, and hepatoprotective properties. Oxidative stress is one of the main ways to cause liver damage, both in disease and substance-induced liver damage. Studies have shown that *Syzygium aromaticum* reduces MDA—an end product of membrane lipid peroxidation—and increases SOD and GPx [32]. In a study on the antioxidant effects of

Syzygium aromaticum and argan oil, it was shown that *Syzygium aromaticum* not only had a higher antioxidant capacity than argan oil (3235.50 mg Eq/100 g vs. 90.90 total antioxidant capacity (TAC)), but it also protected liver, kidney, and brain against H_2O_2 [33].

Inflammation is another key element in liver damage. In a study by Ali et al., *Syzygium aromaticum* inhibited hepatic cell proliferation, reduced leukocyte migration, and lowered proinflammatory cytokines such as tumour necrosis factor- α (TNF- α), suggesting an anti-inflammatory effect that prevents liver cirrhosis and fibrosis [26]. In addition, clove has also shown its anti-inflammatory effect by regulating nuclear factor- κ B (NF- κ B) and ERK/MAPK pathways, nitric oxide (NO), and pro-inflammatory interleukins [34].

In one study, oral administration of clove in TAA-induced liver damage animal models was shown to reduce liver enzymes such as ALP, and AST, which are indicators of liver damage [25]. In addition, histological findings showed reduced liver necrosis, inflammation, and cirrhosis in treated groups, indicating the hepatoprotective effects of clove extract.

Eugenol inhibits COX-2 and 15-LOX as well as the NF- κ B pathway, resulting in reduced levels of prostaglandins, leukotrienes, and pro-inflammatory cytokines such as interleukin-6 (IL-6), respectively [35]. Additionally, it increases the expression of nuclear factor erythroid 2-related factor 2 (NRF2) transcription factor, which subsequently upregulates the glutamate-cysteine ligase regulatory subunit (GCLM) and glutathione S-transferase A1 (GSTA1) genes [36]. The scavenging capabilities of the eugenol phenolic hydroxyl group mitigate reactive oxygen species (ROS) by decreasing Fe^{3+} ions [37]. It also inhibits the activation of hepatic stellate cells (HSCs), preventing liver fibrosis [38].

β -caryophyllene binds to cannabinoid receptor 2 (CB2) and reduces pro-inflammatory mediators such as IL-1 β , IL-6, TNF- α , and NF- κ B in immune cells [39]. It also has an antioxidant property, neutralising ROS and preventing lipid peroxidation [40–42]. These two compounds contribute to the anti-inflammatory, antioxidant, and hepatoprotective effects of *Syzygium aromaticum* [43, 44], alongside other compounds found in clove, including quercetin, gallic acid, kaempferol, and ellagic acid [45].

The combined effect of *Syzygium aromaticum* and conventional pharmaceutical drugs, while not extensively studied, has promising potential due to their synergistic benefits. *Syzygium aromaticum* is a cost-effective and accessible option with few side effects, while pharmaceutical drugs provide more potent outcomes. Beltrán-Villalobos et al. studied the synergistic antinociceptive effect of *Syzygium aromaticum* and ketorolac [46]. While the antinociceptive effect of *Syzygium aromaticum* (10 mg/

kg) and ketorolac (10 mg/paw) alone were 23.3% and 42.6%, the combination resulted in a 61% antinociceptive response with much lower doses (1.70 mg/paw of ketorolac and 1.80 mg/paw of *Syzygium aromaticum*). This approach could maximise the therapeutic efficacy while maintaining affordability, efficacy, and safety.

However, using clove in real-life practices and clinical cases is still in its early stages. The current study had limitations, including financial constraints and the method of TAA injection (intraperitoneal). More clinical and experimental studies, including clinical trials, must be conducted to assess the safety, effectiveness, tolerability, and any possible side effects and drug interactions of *Syzygium aromaticum*.

In conclusion, this study shows *Syzygium aromaticum*'s hepatoprotective effects against TAA-induced liver injury and suggests conducting more clinical studies to better determine its usability in clinical scenarios.

Abbreviations

HESA	Hydro-ethanolic extract of <i>Syzygium Aromaticum</i>
TAA	Thioacetamide
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
CAT	Catalase
MDA	Malondialdehyde
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
H&E	Hematoxylin and eosin
TNF- α	Tumour necrosis factor alpha
IL-1	Interleukin-1
IL-6	Interleukin-6
OA	Oleanolic acid
HCC	hepatocellular carcinoma
SPSS	Statistical Package for the Social Sciences
ERF	eugenol-rich fraction
TAC	total antioxidant capacity
NF- κ B	nuclear factor- κ B
NO	nitric oxide
NRF2	nuclear factor erythroid 2-related factor 2
GCLM	Glutamate-cysteine ligase regulatory subunit
GSTA1	Glutathione S-transferase A1
ROS	reactive oxygen species
HSCs	hepatic stellate cells
CB2	cannabinoid receptor 2

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Authors' contributions

A.P., N.P., R.A., and A.K.R. wrote the main manuscript text. A.P. and A.K.R. reviewed and edited the manuscript. N.P. analysed and curated data. All authors reviewed the manuscript.

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Data availability

Raw data files can be accessed on request.

Declarations

Ethics approval and consent to participate

All ethical criteria were met, and the study was approved by the Lorestan University of Medical Sciences ethics committee (Approval ID: IRLUMS.REC.1401.044).

Competing interests

The authors declare no competing interests.

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