Amelioration of oxidative stress by trans-Anethole via modulating phase I and phase II enzymes against hepatic damage induced by CCl₄ in male Wistar rats

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Abstract
The current study was designed to assess the in vivo hepatoprotective properties of trans-Anethole, which is a principal aromatic component of star anise. The hepatoprotective effects of trans-Anethole were evaluated at three doses [40, 80, and 160 mg/kg body weight (b.wt.)] against carbon tetrachloride (CCl₄)-induced hepatic damage in male Wistar rats for 4 weeks. Forty-two male Wistar rats were equally divided into seven groups; the control (group I) received only distilled water. Rats of group II received CCl₄ (1 ml/kg b.wt.) in a 1:1 ratio of CCl₄ and olive oil via intraperitoneal doses, while rats of group III received silymarin (50 mg/kg b.wt.), followed by CCl₄ intraperitoneal doses, 3 days in a week. Rats of group IV received trans-anethole (160 mg/kg b.wt.) for 28 days as a negative control. Trans-anethole at the doses of 40, 80, and 160 mg/kg b.wt. was administered to groups V, VI, and VII, respectively, for 28 days, followed by CCl₄ (i.p.). Results showed that CCl₄ treatment (group II) elevated the levels of different serum markers like aspartate aminotransferase (AST) by 4.74 fold, alanine aminotransferase (ALT) by 3.47 fold, aspartate alkaline phosphatase (ALP) by 3.55 fold, direct bilirubin by 3.48 fold, and total bilirubin by 2.38 fold in contrast to control. Furthermore, it was found that the decreased levels of liver antioxidant enzymes viz. catalase (CAT) and glutathione reductase (GR) were significantly modulated by the pre-administration of rats with different doses (40, 80, and 160 mg/kg b.wt.) of trans-anethole. Furthermore, pre-treatment of trans-anethole reduced the level of phase I enzymes and elevated the level of phase II detoxifying enzymes. Histopathological investigations showed that the treatment with trans-anethole was effective in ameliorating CCl₄-induced liver injury and restored the normal hepatic architecture. Moreover, trans-anethole restored p53 and cyclin D levels in liver tissue relative to group II. Western blot analysis revealed that the trans-anethole treatment downregulated the expression of Bax and caspase-3 while upregulated the expression of Bcl-xL. Collectively, the findings of the study showed the strong efficacy of trans-anethole in ameliorating the hepatic damage caused by CCl₄ through the modulation of antioxidants and xenobiotic-metabolizing enzymes.

Keywords  Bax · Bcl-xL · Carbon tetrachloride · Caspase-3 · Cyclin D · Hepatoprotective properties · p53 · trans-Anethole

Introduction
The liver is a crucial metabolic organ that performs multidimensional functions, including nutrients metabolism, maintaining body homeostasis and being involved in the detoxification of xenobiotics, drugs, pollutants, and chemotherapeutics (El-Hadary and Ramadan Hassanien 2016; Reza et al. 2020). Liver diseases have become a global problem, and fatalities related to hepatic diseases are rising rapidly (Khan et al. 2020). The liver is highly vulnerable to damage from particular toxins and free radicals, regardless of its physiological role (Sobeh et al. 2018).

Carbon tetrachloride (CCl₄) is a well-known hepatotoxin and potent industrial solvent widely used in the laboratory to
induce liver injury in animals (Lu et al. 2016). CCl₄ itself has no cytotoxic effect, but its rapid biotransformation to trichloromethyl and Cl⁻ radicals with reacting oxygen catalyzed by cytochrome P450 in microsomes induces hepatotoxicity (Hussain et al. 2017; Taamalli et al. 2020). It has been proven that several manifestations of liver damage are associated with oxidative stress and redox imbalance. Oxidative stress is thus a crucial driving factor in hepatic damage caused by CCl₄ (Huo et al. 2020).

Many enzymatic and non-enzymatic compounds help in the protection of the liver from oxidative stress. Antioxidant enzymes like glutathione peroxidase (GSH-Px) and catalase (CAT) prevent hepatic damage (El-Hadary et al. 2019). Reduced glutathione is a major antioxidant enzyme in mamalian cells present in ample amounts and provides defense against oxidative stress (Sies 1999). Similarly, phase I (cytochrome P450 oxidases) and phase II (glutathione S-transferase) biotransformation enzymes assist in the metabolic activation and detoxification of xenobiotics, respectively (Chang et al. 2018).

Dysregulation of various signaling pathways plays a critical role in hepatocarcinogenesis. p53, Bax, caspase-3, Bcl-xl, and cyclin D are central to the liver cancer etiology. p53 gene regulates a crucial mechanism for cell cycle arrest, which results in programmed cell death. When tissues experience a severe injury, p53 starts the process of apoptosis (Yee and Vouiden 2005; Buitrago-Molina et al. 2021). Cyclin D is a proto-oncogene playing a vital function in cell cycle progression and is involved in DNA repair. Several types of cancer, including hepatocellular carcinoma (HCC), have documented overexpression of cyclin D protein (Joo et al. 2001; Núñez et al. 2017).

*Trans*-anethole, a naturally occurring major aromatic phytoconstituent of star anise essential oil (*Illicium verum* Hook.), is well known for its culinary uses. It has proven to be an effective anticancer agent against breast cancer (MCF-7 and MDA-MB-231) cell lines through the regulation of cell death, survival, and proliferation (Chen and DeGraffenried, 2012). The compound anethole exerted anti-metastatic and apoptotic effects through the upregulation of tissue inhibitor of metalloproteinase (TIMP)-1 expressions and downregulation of matrix metalloproteinase (MMP)-2 and (MMP)-9 along with suppression of the phosphorylation of extracellular signal-regulated kinase (ERK), p38, Akt, and nuclear factor-κB (NF-κB) signaling pathways in human fibrosarcoma (HT-1080) cells (Choo et al. 2011). Jana et al. (2015) reported that anethole was an effective antitumor agent by preventing liver damage and myelosuppression when administered individually or along with cyclophosphamide in the tumor model.

Although *trans*-anethole has been widely used in treating several diseases, the hepatoprotective potential of *trans*-anethole has not been well studied. Keeping this in view, the current research was designed to assess the in vivo hepatoprotective potential of *trans*-anethole in an experimental model of CCl₄-induced liver damage and its mechanism of action.

**Materials and methods**

**Chemicals**

Silymarin (S0292), *trans*-anethole (117870), 5,5-dithiobisnitrobenzoic acid (DTNB) (D8130), 2-aminofluorene (2-AF) (A55500), and 1-chloro-2,4-dinitrobenzene (CDNB) (237329) were obtained from Sigma (St. Louis, MO, USA). Reduced glutathione (GSH) (TC134), bovine serum albumin (BSA) (TC194), glycylglycine (TC061), Triton-X (M1642), oxidized glutathione (GSSG) (TC282), 2-thiobarbituric acid (TBA) (RM1594), trichloroacetic acid (TCA) (GRM7570), L-histidine (PCT0311), D-biotin (CMS095), and nicotinamide adenine dinucleotide (RM391) were procured from Hi-Media Pvt. Ltd., Mumbai, India. CCl₄ (070301) was obtained from Spectro Chem Pvt. Ltd., Mumbai, India. ECL kit (#1705061) was procured from (Bio-Rad, USA). Antibodies p53 (#2527) caspase-3 (#9662), Bax (#2772), Bcl-xL (#2764), and cyclin D (#55506) used in the present study were acquired from Cell Signaling Technology (Danvers, MA, USA).

**Procurement and maintenance of animals**

For the present experiment, male Wistar rats weighing 150–240 g were selected and acquired from the National Institute of Pharmaceutical Education and Research (NIPER), Mohali, Punjab (India). On procurement, animals were kept in the well-aerated Central Animal House facility of Guru Nanak Dev University, Amritsar, following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and the research was approved by the Animal Ethical Committee, GNDU, Amritsar (226/CPCSEA/2017/05). Male Wistar rats were given free access to a standard pellet diet and tap water *ad libitum*. Animals were housed in polypropylene cages with paddy husk bedding in a quiet and temperature-controlled room (25 ± 2°C) with a 12-h light/dark cycle and relative humidity of 50 ± 5%. Animals were kept for 2 weeks to be acclimatized before the start of the experiment.

**Experimental design**

The hepatic damage model caused by CCl₄ was used to determine hepatotoxicity. A total of 42 rats were divided into seven groups at random, each group containing six rats. The procedure of experimentation was of 28 days. Group 1
animals that served as control were given a regular pellet diet and tap water ad libitum. Group II animals served as the positive control group, treated with carbon tetrachloride (CCl₄), 3 days in a week, a known hepatotoxin (1 ml kg/b.wt.) in 1:1 ratio; CCl₄ and olive oil via intraperitoneal doses. Group III animals were pre-treated with silymarin (50 mg/kg b.wt.) during the experiment using an oral route, used as a typical hepatoprotective medication (Sahin et al. 2020; Mukhtar et al. 2021), followed by CCl₄ intraperitoneal doses, 3 days in a week. Group IV animals were treated with trans-anethole (160 mg/kg b.wt.) for 28 days as the negative control. Group V animals were pre-treated with trans-anethole (40 mg/kg b.wt.) for 28 days, followed by intraperitoneal CCl₄ doses was given to animals. Group VI animals received trans-anethole pre-treatment (80 mg/kg b.wt.) for 28 days, followed by CCl₄ (i.p). Group VII animals received the pre-treatment of trans-anethole (160 mg/kg b.wt.) for 28 days, followed by CCl₄ (i.p). The various doses were given according to the body weight of the animals.

The concentrations of trans-anethole for the present study have been decided from the literature (Kim et al. 2017; Zhang Mannheim GmbH, Germany, with Bene Sphera autoanalyzer. (CHOL), and triglycerides (TG) using kits of Erba diagnostics protein, albumin (ALB), urea, creatinine, cholesterol (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), direct bilirubin (DB), total protein, albumin (ALB), urea, creatinine, cholesterol (CHOL), and triglycerides (TG) using kits of Erba diagnostics

Preparation of liver homogenate

After 4 weeks of treatment, the animals were sacrificed by cervical dislocation procedure after overnight fasting to alleviate the suffering and excised to remove the liver after extracting blood. The entire liver was instantly perfused into a chilled NaCl solution (0.9%) to keep it free from any adhered tissues and blood. The liver was again rinsed with cold Tris-KCl buffer (0.15 M) solution, dried by filter paper, and weighed to prepare 10% (w/v) liver homogenate using homogenizer and centrifuged the contents at 2000 rpm for 10 min at 4°C. The homogenate was eventually kept in cryovials at −80°C and then used to determine several biochemical parameters of the liver. Approximately 500 μl of this homogenate was precipitated with TCA (5%) and centrifuged at 4°C for around 10 min at 2500 rpm, and the supernatant so obtained was collected to quantify the content of reduced glutathione. The remaining liver part was immersed in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

Biochemical analysis of liver homogenate

Protein estimation

The protein content was determined as per the protocol of Smith et al. (1985). Bovine serum albumin was used as a standard to estimate the protein content using the BSA regression equation, and the protein values are expressed in milligram per gram of tissue.

Lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARS) has been used to evaluate lipid peroxidation by microsomes in liver homogenate (Devasagayam et al. 2003). The total amount of TBARS was measured as malondialdehyde (MDA) content (µmol MDA equivalent/g of tissue), and the regression equation was obtained from its calibration curve for the measurement of TBARS. MDA was used as a standard control.

Reduced glutathione content

The content of reduced glutathione (GSH) was estimated according to the procedure of Anderson (1985). Glutathione was used as a standard for generating the standard calibration curve.

Lactate dehydrogenase

The lactate dehydrogenase (LDH) activity was determined by the method of Kaur et al. (2019) at 340 nm. The specific activity of LDH was calculated using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

Antioxidative enzymes

Catalase The catalase (CAT) activity was determined according to the method of Aebi (1984) at the absorbance of 240 nm. The extinction coefficient used was 6.93 × 10⁻³ mM⁻¹ cm⁻¹.
**Glutathione reductase** The method suggested by Carlberg and Mannervik (1975) was used to estimate the function of glutathione reductase (GR), and the OD was recorded at 340 nm for 3 min at 15-s intervals. The extinction coefficient used was 6.22 mM$^{-1}$ cm$^{-1}$.

**Phase I enzymes**

**Estimation of cytochrome P450 and cytochrome P420** The amounts of cytochrome P450 and cytochrome P420 were calculated using the procedure as suggested by Choi et al. (2003) using the absorption coefficient of 91 mM$^{-1}$ cm$^{-1}$ and 111 mM$^{-1}$ cm$^{-1}$ respectively spectrum was recorded at 420 nm, 450 nm, and 409 nm.

**Cytochrome b5 content** The cytochrome b5 content was assessed by following the protocol of Omura and Sato (1964). The OD was recorded at 409 nm and 424 nm.

**NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase** The NADPH cytochrome P450 reductase activity was measured by using the protocol of Omura and Takesue (1970), and NADH cytochrome b5 reductase activity was measured following the protocol of Mihara and Sato (1972). The NADPH cytochrome P450 reductase oxidation intensity was measured at 340 nm with a 6.22 mM$^{-1}$ cm$^{-1}$ extinction coefficient, while NADH cytochrome b5 reductase activity was estimated using 1.02 mM$^{-1}$ cm$^{-1}$ as extinction coefficient and the NADH oxidation intensity was determined at 420 nm.

**Phase II enzyme**

**Glutathione S-transferase** GST activity was determined as per the protocol of Habig et al. (1974). The specific activity of GST was determined by using 9.6 mM$^{-1}$ cm$^{-1}$ extinction coefficients.

**Histopathological studies**

The liver tissue sections were carefully separated and fixed in a 10% formalin buffered solution. The liver tissues were then coated in paraffin wax and cut into 4-μm thick sections using an automatic microtome. The sections were then mounted on slides and stained for 10 min with Mayer’s hematoxylin solution, followed by 5 min with eosin Y stain, and finally examined under a light microscope (40× magnification).

**Immunohistochemistry analysis**

Using immunohistochemistry (IHC), the expression pattern of proteins viz. p53 and cyclin D in liver hepatocytes was determined. The liver sections of 4-μm thickness were deparaffinized, which were subsequently rehydrated on positively charged glass slides. The liver sections were then treated in hydrogen peroxide (0.3 %) in methanol for 30 min to inactivate the endogenous peroxidase. For about 30 min, the sections were blocked with 5% skimmed milk. Furthermore, slides with anti-p53 and anti-cyclin D antibodies were subjected to incubation overnight at 4°C. The slides were rinsed three times with PBS for 2 min each and incubated for 30 min with HRP-conjugated secondary antibodies. Then, the slides were washed three times with wash buffer (PBS) for 2 min each. Protein expression was developed by adding a 3-diaminobenzidine (DAB) substrate chromogen buffer solution to the tissues and incubated for 2–3 min at room temperature to develop brown color, washed with 1× PBS, and then Mayer’s hematoxylin was employed to counterstain the samples (40× magnification). Finally, the slides were examined under a Nikon Eclipse E200 microscope.

**Western blot analysis**

Western blot analysis was performed to check the expression of proteins. The liver was dissected and immediately kept at −80°C according to respective groups. The liver tissue samples were homogenized in RIPA lysis buffer, then centrifuged for 20 min at 12,000 rpm, and the upper transparent solution was then transferred into fresh tubes. Protein concentrations were determined using the Bradford method. Equal concentration of protein (40 μg) was resolved by SDS-PAGE and transferred onto a polypropylene fluoride (PVDF) membrane through western blotting. The membranes were blocked with BSA (5% in TBST, 0.1% Tween-20) at 25°C for 2 h, followed by probing with primary antibodies (caspase-3, Bax, and Bcl-xL) at 4°C overnight with continuous shaking. Afterward, the membranes were rinsed thrice with TBST before at room temperature for 2 h with HRP-conjugated secondary antibodies. After being washed with TBST, ECL reagent was used to develop blots and imaging was done using Image-Quant LAS 4000, GE Healthcare. The densities of each band were quantified with Alphaease FC software (version 4.0). β-actin was used as an endogenous control for normalizing the expression of the protein of interest.

**Statistical analysis**

The experiments were performed in triplicates and represented as mean ± standard error. The significance of the data was checked by using one-way ANOVA, and the findings were evaluated for multiple comparisons accompanied by HSD determination. Means with the same letters are not significantly different from each other using HSD. Significant differences are presented as *p < 0.05 (Sokal and Rohlf 1981). Contour plots were made by using MINITAB software. Heat map and Pearson’s correlation analysis were performed using R software V.3.5.1 (Statistical Computing, Vienna, Austria).
Results

Response of body weight

Trans-anethole effects on the percentage change in male Wistar rat’s body weight after 4 weeks of treatment are given in Table 1. The percentage difference in an animal’s body weight was observed after 7 days; 4 weeks later, a substantial percentage change in the bodyweight of the animals of group II (3.63) was reported relative to the control group I. Pre-administration of trans-anethole followed by CCl₄ normalized the change in the body weight of animals in a concentration-dependent way.

Serum markers enzymes

Enzymatic serum markers

The CCl₄ group exhibited a significant rise in the levels of AST, ALT, and ALP by 374.3%, 246.8%, and 255.3 %, respectively, as compared to the control (Table 2). CCl₄ treatment contributes to severe hepatic injury in male Wistar rats. However, pre-administration of animals with trans-anethole decreased the level of serum enzymes in groups V, VI, and VII. In binary combination with CCl₄ and trans-anethole at 160 mg/kg b.wt., the activity of AST, ALT, and ALP reduces by 58.2%, 67.6%, and 67.9%, respectively, as compared to the CCl₄-treated animals (group II). The data indicated that trans-anethole alleviates liver injury caused by CCl₄.

Non-enzymatic serum markers

The alterations in liver enzymes caused by CCl₄ resulted in a significant decrease in albumin (ALB) level (50.5%) and total protein level (54.8%); however, antagonistic results were found in the case of direct bilirubin (DB) and total bilirubin (TB) which exhibited increase by 133.3% and 138.3%, respectively, causing hepatic damage in comparison to the control group I. The elevated bilirubin levels are pathophysiological and clinical indicators of hepatic tissue necrosis (Rakib

Table 1 Effect of trans-anethole on the percentage change in body weight (b.wt.) of male Wistar rats after 4 weeks of the treatment period

| Groups | Treatments | Change in body weight of animals (mean) ± SE | Percentage change in body weight of animal relative to control (fold increase) |
|--------|------------|---------------------------------------------|--------------------------------------------------------------------------|
| I      | Control (tap water ad libitum) | 3.22 ± 0.51b | 1 |
| II     | CCl₄: olive oil (1:1; 1 ml/kg b.wt.) | 11.6 ± 1.31a | 3.63 |
| III    | Silymarin (50 mg/kg b.wt.) + CCl₄ | 3.38 ± 0.83b | 1.05 |
| IV     | trans-anethole (160 mg/kg b.wt.) | 3.41 ± 0.58b | 1.06 |
| V      | trans-anethole (40 mg/kg b.wt.) + CCl₄ | 5.99 ± 0.33b | 1.86 |
| VI     | trans-anethole (80 mg/kg b.wt.) + CCl₄ | 3.85 ± 0.28b | 1.19 |
| VII    | trans-anethole (160 mg/kg b.wt.) + CCl₄ | 3.68 ± 0.43b | 1.15 |

Values are represented as mean ± SE for six rats in each group. Mean values with the same alphabets are not significantly different from each other at p ≤ 0.05 in all treatments using Tukey’s test.

Table 2 Effect of trans-anethole on the activities of hepatotoxicity markers AST, ALT, and ALP in male Wistar rats treated with CCl₄

| Groups | Treatments | Serum enzyme activities (IU L⁻¹) (mean ± SE) |
|--------|------------|---------------------------------------------|
|        |            | AST  | ALT  | ALP  |
| I      | Control (tap water ad libitum) | 79 ± 15.2d | 43.4 ± 7.21b | 158.3 ± 14.55d |
| II     | CCl₄: olive oil (1:1; 1 ml/kg b.wt.) | 374.7 ± 17.5a | 150.5 ± 24.8a | 562.5 ± 25.85a |
| III    | Silymarin (50 mg/kg b.wt.) + CCl₄ | 139.9 ± 12.9abcd | 46.6 ± 2.45b | 255.6 ± 4.08abcd |
| IV     | trans-anethole (160 mg/kg b.wt.) | 142.4 ± 14.7abcd | 49.1 ± 2.41b | 264.8 ± 7.03abcd |
| V      | trans-anethole (40 mg/kg b.wt.) + CCl₄ | 271.8 ± 32.2abcd | 95.1 ± 9.67b | 311 ± 15.5abcd |
| VI     | trans-anethole (80 mg/kg b.wt.) + CCl₄ | 201.9 ± 35.4abcd | 73.2 ± 8.35b | 242.8 ± 33.8abcd |
| VII    | trans-anethole (160 mg/kg b.wt.) + CCl₄ | 156.7 ± 19.1abcd | 48.7 ± 5.98b | 180.8 ± 6.97abcd |
| HSD    |             | 111.19 | 54.98 | 90.66 |
| F-ratio|             | 14.41*** | 13.38*** | 39.59*** |

Values are represented as mean ± SE for six rats in each group. Mean values with the same alphabets are not significantly different from each other at p ≤ 0.05 in all treatments using Tukey’s test.

***Significant difference at p ≤ 0.001
et al., 2020). It was further found that pre-administration of \textit{trans}\-anethole followed by CCl\textsubscript{4} administration exhibited substantial hepatoprotective ability, as noticed in groups V, VI, and VII (Table 3). \textit{F}\textsuperscript{-}ratio of one-way ANOVA was also found significant for ALB, total protein, direct bilirubin, and total bilirubin.

CCl\textsubscript{4} intoxication caused a rise in the levels of urea, creatinine, TG, and CHOL by 112.4\%, 36.9\%, 253.2\%, and 110.5\%, respectively, as compared to group I (control). Pre-treatment with \textit{trans}\-anethole followed by CCl\textsubscript{4} in groups V, VI, and VII attenuated the level of urea, creatinine, TG, and CHOL relative to the CCl\textsubscript{4}-treated group. The \textit{trans}\-anethole-treated group significantly reduced the levels of creatinine and TG effects which were comparable to silymarin (Table 4).

Group VII rats (160 mg/kg b.wt.) completely restored the serum levels of all the tested enzymes and biomarkers to their normal values. HSD was found significant for all biomarkers except creatinine.

**Heat map analysis of serum marker enzymes**

The hierarchical clustering for the effect of \textit{trans}\-anethole on the activities of hepatotoxicity serum markers in male Wistar rats treated with CCl\textsubscript{4} has been analyzed through a heat map (Fig. 1). The dendrogram indicated groups were mainly classified into two main categories. Groups V and II showed little similarities with each other and attributed to the fact that triglycerides, cholesterol, and urea showed little variations in

### Table 3: Effect of pretreatment of \textit{trans}\-anethole on non-enzymatic serum markers in male Wistar rats treated with CCl\textsubscript{4}

| Groups | Treatments                                      | Serum non-enzymatic activities (g dL\textsuperscript{-1}) (mean ± SE) | Serum non-enzymatic activities (mg dL\textsuperscript{-1}) (mean ± SE) |
|--------|------------------------------------------------|-------------------------------------------------------------------|---------------------------------------------------------------------|
|        |                                                | Albumin              | Total protein              | Direct bilirubin              | Total bilirubin              |
| I      | Control (tap water \textit{ad libitum})        | 3.96 ± 0.2\textsuperscript{a}                                  | 8.21 ± 0.23\textsuperscript{a}                                  | 0.12 ± 0.02\textsuperscript{ab} | 0.13 ± 0.02\textsuperscript{b} |
| II     | CCl\textsubscript{4}: olive oil (1:1; 1 ml/kg b.wt.) | 1.96 ± 0.19\textsuperscript{a}                                  | 3.71 ± 0.28\textsuperscript{a}                                  | 0.28 ± 0.04\textsuperscript{a}      | 0.31 ± 0.03\textsuperscript{a} |
| III    | Silymarin (50 mg/kg b.wt.) + CCl\textsubscript{4} | 3.6 ± 0.21\textsuperscript{bc}                                  | 6.96 ± 0.13\textsuperscript{bc}                                  | 0.15 ± 0.03\textsuperscript{ab} | 0.24 ± 0.03\textsuperscript{ab} |
| IV     | \textit{trans}\-anethole (160 mg/kg b.wt.)     | 3.65 ± 0.26\textsuperscript{b}                                 | 6.8 ± 0.13\textsuperscript{b}                                   | 0.13 ± 0.29\textsuperscript{b} | 0.18 ± 0.03\textsuperscript{b} |
| V      | \textit{trans}\-anethole (40 mg/kg b.wt.) + CCl\textsubscript{4} | 3.05 ± 0.19\textsuperscript{d}                                | 5.48 ± 0.15\textsuperscript{d}                                   | 0.14 ± 0.02\textsuperscript{ab} | 0.2 ± 0.03\textsuperscript{b} |
| VI     | \textit{trans}\-anethole (80 mg/kg b.wt.) + CCl\textsubscript{4} | 3.35 ± 0.26\textsuperscript{d}                                | 6.31 ± 0.12\textsuperscript{d}                                   | 0.15 ± 0.02\textsuperscript{ab} | 0.1 ± 0.02\textsuperscript{b} |
| VII    | \textit{trans}\-anethole (160 mg/kg b.wt.) + CCl\textsubscript{4} | 3.68 ± 0.22\textsuperscript{ab}                                | 7.06 ± 0.26\textsuperscript{ab}                                  | 0.11 ± 0.01\textsuperscript{ab} | 0.16 ± 0.03\textsuperscript{b} |

HSD was found significant **p ≤ 0.05** in all treatments using Tukey’s test

### Table 4: Effect of \textit{trans}\-anethole pretreatment on serum markers viz. urea, creatinine, triglycerides, and cholesterol in CCl\textsubscript{4}-treated male Wistar rats

| Groups | Treatment                                      | Serum enzyme activities (g dL\textsuperscript{-1}) (mean ± S.E) |
|--------|------------------------------------------------|-----------------------------------------------|
|        |                                                | Urea                      | Creatinine            | Triglycerides | Cholesterol |
| I      | Control (tap water \textit{ad libitum})        | 47.6 ± 3.05\textsuperscript{c}            | 0.65 ± 0.02\textsuperscript{a}            | 26.5 ± 2.64\textsuperscript{d} | 33.3 ± 2.11\textsuperscript{c} |
| II     | CCl\textsubscript{4}: olive oil (1:1; 1 ml/kg b.wt.) | 101.1 ± 4.37\textsuperscript{a}          | 0.89 ± 0.04\textsuperscript{a}            | 93.6 ± 4.35\textsuperscript{c} | 70.1 ± 3.48\textsuperscript{a} |
| III    | Silymarin (50 mg/kg b.wt.) + CCl\textsubscript{4} | 49.3 ± 1.23\textsuperscript{c}          | 0.72 ± 0.02\textsuperscript{c}            | 36.5 ± 2.79\textsuperscript{c} | 44.3 ± 3.18\textsuperscript{bc} |
| IV     | \textit{trans}\-anethole (160 mg/kg b.wt.)     | 51.6 ± 2.67\textsuperscript{c}          | 0.71 ± 0.02\textsuperscript{c}            | 37.8 ± 2.58\textsuperscript{c} | 45.8 ± 2.20\textsuperscript{bc} |
| V      | \textit{trans}\-anethole (40 mg/kg b.wt.) + CCl\textsubscript{4} | 87.9 ± 3.86\textsuperscript{c}          | 0.77 ± 0.03\textsuperscript{ab}           | 71.3 ± 3.67\textsuperscript{c} | 58.6 ± 4.50\textsuperscript{b} |
| VI     | \textit{trans}\-anethole (80 mg/kg b.wt.) + CCl\textsubscript{4} | 69.4 ± 2.94\textsuperscript{b}          | 0.73 ± 0.02\textsuperscript{c}            | 54.1 ± 4.73\textsuperscript{d} | 49.6 ± 4.50\textsuperscript{bc} |
| VII    | \textit{trans}\-anethole (160 mg/kg b.wt.) + CCl\textsubscript{4} | 55.9 ± 2.69\textsuperscript{bc}         | 0.69 ± 0.04\textsuperscript{bc}           | 40.8 ± 2.54\textsuperscript{d} | 40.0 ± 6.64\textsuperscript{bc} |

HSD was found significant **p ≤ 0.001**; \textit{ns} not significant

Values are represented as mean ± S.E for six rats in each group. Mean values with the same alphabets are not significantly different from each other at \( p \leq 0.05 \) in all treatments using Tukey’s test

\( \ast p \leq 0.05; \ast\ast p \leq 0.01; \ast\ast\ast p \leq 0.001 \)
in groups I and VII, depicting the protective effect of *trans*-anethole at the highest tested dose (160 mg/kg b.wt. + CCl₄).

**Protein content**

The animals of group II that received CCl₄ treatment exhibited a significant reduction in the protein content by 56.89% in contrast to group I. Alternatively, with an increase in the concentration of *trans*-anethole, the protein content increased significantly in the animals treated with a binary combination of *trans*-anethole with CCl₄ from groups V to VII, as shown in Fig. 2A.

**Lipid peroxidation**

It was observed that the amount of liver peroxidation or MDA content was substantially enhanced in group II treated with CCl₄ (48.50 ± 4.37 μmol MDA eq/g of tissue) as compared to that of the control group I (24.66 ± 2.87 μmol MDA eq/g of tissue). However, group IV, i.e., *trans*-anethole-treated group (26.58 ± 3.61 μmol MDA eq/g of tissue), had not shown any significant difference relative to the group I, signifying that *trans*-anethole treatment alone does not lead to the rise in TBARS. On the other hand, the administration of *trans*-anethole along with CCl₄ in groups V, VI, and VII showed a concentration-dependent decrease in the content of MDA, i.e., 32.2%, 51.9%, and 65.8%, respectively (Fig. 2B).
Reduced glutathione content

The animals treated with CCl4 group II showed a reduction in GSH content (95.98 ± 10.6 μmol of SH content/g of tissue) as compared to that of control group I (226.14 ± 7.7 μmol of SH content/g of tissue). On the other hand, rats administrated with trans-anethole followed by CCl4 at the highest tested dose in group VII showed significant elevation in the GSH content by 147.7% compared to group II (Fig. 2C).

Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity increased significantly in the liver of male Wistar rats upon CCl4 exposure by 173.9% as compared to control group I. However, in groups III and IV, i.e., silymarin and trans-anethole-treated groups, the specific activity of LDH decreased by 46.03% and 73.01%, respectively, relative to the CCl4 group II. When trans-anethole was administrated in combination with CCl4 in groups V and VII, the activity of LDH has restored relative to the group I, i.e., control (Fig. 2D). On the other hand, group VI showed a decrease in the level of LDH as compared to group VII, which shows significant protection. Besides, the effect of trans-anethole at a dose of 160 mg/kg b.wt. was comparable to silymarin. F-ratio was found significant at p ≤ 0.05 for LDH.

Antioxidant enzymes

Catalase

As shown in Table 5, it was found that the CAT activity in group II demonstrated a significant decrease of 55.5% in comparison to the group I (control). Though pre-administration with all doses (40, 80, and 160 mg/kg b.wt.) of trans-anethole followed by CCl4 in groups V, VI, and VII significantly increased the level of CAT by 37.6%, 77.7%, and 172.1%, respectively, in comparison to group II, which indicates its protective nature. One-way ANOVA and HSD were found significant for catalase.

Glutathione reductase

The specific activity of glutathione reductase was reduced in the CCl4-treated group II by 59.6% relative to the control group. Trans-anethole alone (group IV) and when administrated along with CCl4 in groups V, VI, and VII increased the amount of enzyme glutathione reductase, suggesting its protective potential against hepatic damage induced by CCl4. Trans-anethole alone in group IV showed similar results as compared to standard drug silymarin (Table 5).

Phase I enzymes

The cytochrome P450, cytochrome P420, and cytochrome b5 contents were observed at different doses (40, 80, and 160 mg/kg b.wt.) of trans-anethole. The cytochrome P450 and P420 enzyme levels in group II animals, i.e., the CCl4-treated group, decreased significantly by 59.8% and 40.5%, respectively, as compared to the control group I (Table 6). However, when trans-anethole was administrated along with CCl4, the level of cytochrome P450 and P420 contents was increased, showing substantial defense against liver injury. Groups III and IV that received silymarin and trans-anethole treatment, the cytochrome P450, and P420 contents were found to be almost equal to that of group 1 (control) as proven from HSD. On the other hand, treatment of CCl4 in group II increases the cytochrome b5 content by (225.8 %) with respect

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Table 5 Effect of pre-treatment of trans-anethole on the activities of antioxidant enzymes CAT and glutathione reductase in the liver of male Wistar rats

| Groups | Treatments | CAT (IU mg⁻¹ protein) | Glutathione reductase (IU mg⁻¹ g⁻¹ protein) |
|--------|------------|----------------------|------------------------------------------|
| I      | Control (tap water ad libitum) | 51.5 ± 2.95ab | 0.09 ± 0.01ab |
| II     | CCl4: olive oil (1:1; 1 ml/kg b.wt.) | 22.9 ± 3.48cd | 0.04 ± 0.06cd |
| III    | Silymarin (50 mg/kg b.wt.) + CCl4 | 48.1 ± 4.51ab | 0.09 ± 0.09ab |
| IV     | trans-anethole (160 mg/kg b.wt.) | 45.3 ± 2.45bc | 0.09 ± 0.01ab |
| V      | trans-anethole (40 mg/kg b.wt.) + CCl4 | 31.5 ± 3.48cd | 0.05 ± 0.08cd |
| VI     | trans-anethole (80 mg/kg b.wt.) + CCl4 | 40.7 ± 2.72bc | 0.074 ± 0.07abc |
| VII    | trans-anethole (160 mg/kg b.wt.) + CCl4 | 62.3 ± 4.96c | 0.096 ± 0.01a |
|       | HSD       | 16.44                | 0.034                                     |
| F-ratio |           | 11.69***             | 7.67***                                   |

Values are represented as mean ± SE for six rats in each group. Mean values with the same alphabets are not significantly different from each other at p ≤ 0.05 in all treatments using Tukey’s test

***p ≤ 0.001
to the control group I. This higher content was later normalized with an increase in the doses of trans-anethole. The transformation of CCl\textsubscript{4} into a reactive cancer-causing form was accurately depicted by a massive increase in cytochrome b\textsubscript{5} enzyme in the CCl\textsubscript{4}-treated group II as compared to control group I. All other groups demonstrated considerable safety against the adverse effects of CCl\textsubscript{4} (Table 6). In the CCl\textsubscript{4}-treated group II, the specific activity of NADPH cytochrome P450 reductase decreased by 49.5% relative to control group I. Groups VI and VII showed the same specific activity as that of the control as revealed from HSD except for group V. In the case of NADH cytochrome b\textsubscript{5} reductase, there was a rise in the specific activity of this enzyme in the CCl\textsubscript{4}-treated group II by 127% with respect to the control group I. trans-anethole administered along with CCl\textsubscript{4} showed a reduction in the specific activity of this enzyme. However, the highest tested dose showed a maximum reduction by 58.9% in the NADH cytochrome b\textsubscript{5} reductase activity with respect to CCl\textsubscript{4} group II. Silymarin and trans-anethole showed no significant difference than that of control, as depicted from HSD. F-values for one-way ANOVA were found significant for all the enzymes tabulated in Table 6.

**Phase II enzymes**

**Glutathione S-transferase** The treatment of CCl\textsubscript{4} has been found to lower the activity of GST in the liver homogenate of rats of group II by 35.6% with respect to the control group I animals. Pre-administration of trans-anethole significantly elevated the GST activity, which indicated the enhancement of antioxidant level to counter the high level of toxic electrophiles such as ROS, as shown in Fig. 2E, wherein the maximum increase was noticed at 160 mg/kgb.wt. dose.

**Heat map analysis of biochemical parameters**

The heat map showed the hierarchical clustering for the effect of trans-anethole on the activities of biochemical parameters in male Wistar rats treated with CCl\textsubscript{4}. The dendrogram on the top shows the clustering of groups, and the dendrogram on the side shows the clustering of biochemical parameters. The dendrogram indicated that groups were predominantly categorized into two major categories. Group II and group V showed close resemblance with each other as GST, GR, and LDH levels showed similarity as indicated by the heat map. Similarly, groups I, VII, III, IV, and VI are included in the same category. Biochemical parameters showed four clusters. Cluster I is comprised of GSH and NADH cytochrome b\textsubscript{5} reductase, reflecting slight variations of their content in groups IV, III, VII, and I. Cluster 2 consists of Cyt P450, NADPH cytochrome P450 reductase, catalase, protein, TBARS, and Cyt b\textsubscript{5}. Cluster 3 consists of GST, GR, and LDH. Finally, Cyt P420 showed quite similarities in their content in groups III, VII, and I (Fig. 3).

### Table 6

| Groups | Dose (mg/ kg b.wt.) | Cyt P450 (μ mole mg\textsuperscript{-1} protein) | Cyt P420 (μ mole mg\textsuperscript{-1} protein) | Cyt b5 (μ mole mg\textsuperscript{-1} protein) | NADPH Cyt P450 reductase (mean ± SE) | NADH Cyt b5 reductase (mean ± SE) |
|--------|---------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------|----------------------------------|
| I      | Control (tap water ad libitum) | 48.2±3.22 \textsuperscript{a} | 312.3±4.64 \textsuperscript{a} | 30.6±2.75 \textsuperscript{a} | 46.9±2.91 \textsuperscript{a} | 85.28±3.18 \textsuperscript{d} |
| II     | CCl\textsubscript{4}: olive oil (1:1; 1 ml/kg b.wt.) | 19.4±1.61 \textsuperscript{b} | 185.9±4.51 \textsuperscript{b} | 99.7±5.44 \textsuperscript{d} | 23.7±3.38 \textsuperscript{c} | 193.6±3.87 \textsuperscript{a} |
| III    | Silymarin (50 mg/kg b.wt.) + CCl\textsubscript{4} | 45.5±2.62 \textsuperscript{a} | 303.9±10.65 \textsuperscript{a} | 37.3±3.09 \textsuperscript{ab} | 45.9±3.25 \textsuperscript{a} | 80.17±3.79 \textsuperscript{d} |
| IV     | trans-anethole (160 mg/kg b.wt.) | 49.9±3.29 \textsuperscript{a} | 299.8±11.71 \textsuperscript{a} | 38.2±2.59 \textsuperscript{cd} | 48.2±3.65 \textsuperscript{a} | 81.31±2.60 \textsuperscript{b} |
| V      | trans-anethole (40 mg/kg b.wt.) + CCl\textsubscript{4} | 30.9±4.21 \textsuperscript{b} | 247.7±10.50 \textsuperscript{b} | 72.6±5.84 \textsuperscript{b} | 32.2±4.68 \textsuperscript{ab} | 161.4±4.81 \textsuperscript{b} |
| VI     | trans-anethole (80 mg/kg b.wt.) + CCl\textsubscript{4} | 57.3±3.83 \textsuperscript{a} | 332.4±12.34 \textsuperscript{a} | 52.3±3.28 \textsuperscript{a} | 52.6±2.12 \textsuperscript{a} | 132.9±3.28 \textsuperscript{b} |
| VII    | Silymarin (50 mg/kg b.wt.) + CCl\textsubscript{4} | 48.7±3.50 \textsuperscript{a} | 308.0±5.78 \textsuperscript{a} | 32.5±3.77 \textsuperscript{b} | 43.6±2.99 \textsuperscript{ab} | 79.66±2.37 \textsuperscript{d} |
| F-ratio | | 15.21*** | 30.32*** | 40.91*** | 9.23*** | 178.77*** |

Values are represented as mean ± SE for six rats in each group. Mean values with the same alphabets are not significantly different from each other at \( p \leq 0.05 \) in all treatments using Tukey’s test.

***\( p \leq 0.001 \)
Histopathological analysis

The hepatic histological results are shown in Fig. 4. Liver sections from group I rats revealed a normal hexagonal lobular pattern of the hepatic tissue with normal shape and size of hepatocytes. However, liver tissues from the CCl\textsubscript{4}-treated group exhibited several histopathological alterations viz. hepatic steatosis, necrosis, loss of hepatocytes, enlarged portal area, and hepatocellular ballooning were observed between the central vein portal triad area in hematoxylin and eosin staining. Liver damage decreased with the increase in the concentration of \textit{trans}-anethole (40, 80, and 160 mg/kg b.wt.) administration (Fig. 4D–G), and the histological index of vacuolization and liver necrosis was reduced.

Immunohistochemistry analysis

Overexpression of the levels of p53 and cyclin D markers is indicative of hepatocellular carcinoma. The findings of the IHC analysis indicated that the group treated with CCl\textsubscript{4} showed enhanced expression of p53 and cyclin D compared to group I, while pre-administration of \textit{trans}-anethole at all doses showed downregulation of p53 and cyclin D expression relative to group II, i.e., CCl\textsubscript{4}-treated group (Fig. 5).

Western blot analysis

Western blot results of the liver tissues of CCl\textsubscript{4}-treated group II revealed upregulation of caspase-3, Bax, and downregulation of Bcl-xL. However, there was a dose-dependent reduction in the expression of caspase-3 and Bax proteins and upregulation in the Bcl-xL on the administration of \textit{trans}-anethole along with CCl\textsubscript{4} in groups V, VI, and VII as compared to group II.
Hepatic damage and dysfunction have been considered among the most severe issues impacting human health and have become an epidemic worldwide. Despite the vigorous efforts to establish therapies, there are still significantly less effective hepatoprotective drugs available (Madrigal-Santillán et al. 2014). Thus, the quest for new types of compounds with hepatoprotective and antihepatotoxic properties is essential. One approach for treating liver damage is to find natural compounds with new scaffolds that are distinct from current conventional hepatoprotective agents.

CCl₄ is a well-known hepatotoxin that is extensively utilized as an experimental model to induce hepatocellular damage, which resembles human hepatotoxicity and to screen for hepatoprotective agents (Al-Sayed et al. 2019; Kritika and Verma 2019). The findings revealed that the levels of liver serum transaminases, i.e., AST, ALT, ALP, in rat serum were substantially elevated after CCl₄ exposure, which suggested hepatocellular injury. Damage to liver cells altered the integrity of the membrane changes its functional transition and contributes to enzyme leakage into extracellular spaces. On the other hand, pre-treatment with all the doses (40, 80, and 160 mg/kg b.wt.) of trans-anethole alleviates the activities of AST, ALT, and ALP, in rat serum were substantially elevated after CCl₄ exposure, which suggested hepatocellular injury. Damage to liver cells altered the integrity of the membrane changes its functional transition and contributes to enzyme leakage into extracellular spaces. On the other hand, pre-treatment with all the doses (40, 80, and 160 mg/kg b.wt.) of trans-anethole alleviates the activities of AST, ALT, and ALP, suggesting that trans-anethole had beneficial effects against hepatic injury caused by CCl₄. In a study, eugenol caused a decrease in the AST, ALT, and ALP serum marker enzyme levels triggered by metanil yellow (My1) in albino Wistar rats (Sharma et al. 2019). It was demonstrated that the combination of curcumin with sulfamethoxazole decreased the elevated levels of hepatic markers (AST and ALT) against carbon tetrachloride-induced liver injury in Swiss albino mice (Zahran et al. 2020). Al-Sayed et al. (2014) reported the hepatoprotective potential of ethanol extract of Bauhinia hookeri against CCl₄-induced hepatic damage. The extract significantly reduced the levels of various serum markers viz. ALT, AST, and ALP. The intoxication of rats with CCl₄ showed a substantial rise in TG, CHOL, TB, and DB serum levels relative to the control group. Fahmy et al. (2016) reported the protective effects of Terminalia muelleri against CCl₄-induced hepatic damage in mice. Pre-treatment of Terminalia muelleri significantly decreased the CCl₄-induced enhancement in the levels of creatinine, urea, cholesterol, and uric acid. Trans-anethole pre-treatment at the maximum dose (160 mg/kg b.wt.) substantially decreased the levels of all measured liver markers as compared to the CCl₄-treated group, and the results were as good as those of the standard silymarin. From 3D contour plots, it is clear that ALP is positively correlated with ALT and AST, suggesting that ALP increases with an increase in ALT and AST (Fig. 6A). Similar results have been found by Das et al. (2019) in which means ALT (MALT) is positively associated with ALP and AST. It was evident from the literature that increased levels of AST, ALT, and ALP in animals reflect cellular damage and diminution of cell membrane function (Zeashan et al. 2008). ALT is associated positively with DB and TB, suggesting that with an increase in DB and TB, ALT increases (Fig. 6B). Pearson’s correlation analysis performed on different serum markers showed that AST, ALT, and ALP are positively correlated with DB, TB, urea, creatinine, TG, and CHOL, while these are negatively correlated with total protein and ALB (Fig. 7). In a study, it was reported that the mean ALT is positively related to TB (Das et al. 2019).

Lipid peroxidation contributes to oxidative destruction of cellular membranes and causes excessive production of free radicals and eventually leads to cell death. MDA content can
be used to measure the amount of lipid peroxidation (Lee et al. 2019). Results indicated that there was a significant increase in the lipid peroxidation marker MDA content in the CCl₄-administrated group, and this increase was attenuated by the treatment of trans-anethole at all doses tested with significant effect at 160 mg kg⁻¹ b.wt. Ojeaburu and Oriakhi (2021) demonstrated that gallic acid decreased the levels of malondialdehyde (MDA) that were raised in rats treated with CCl₄. Chebulinic acid significantly decreased the level of MDA in male ICR mice exposed to CCl₄ (Feng et al. 2021). Al-Sayed et al. (2014) evaluated the hepatoprotective potential of cupressuflavone isolated from Cupressus macrocarpa against carbon tetrachloride-induced liver injury in mice. It was found that the pre-treatment of Cupressuflavone significantly reduced the rise in alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, lactate dehydrogenase, cholesterol, creatinine, uric acid, urea, and MDA levels induced by CCl₄ in a dose-dependent manner.

In the animals of group II, the protein and reduced glutathione content were also found to be reduced. A significant lowering of the protein content and the amount of GSH in the liver reflects a decline in the antioxidant levels explicitly. Nonetheless, the pre-treatment of trans-anethole normalized the level of these biochemical parameters at all tested doses. The antioxidant enzymes, viz. GR, CAT, and LDH, are the
main components of the endogenous antioxidant mechanism and perform a vital function in the free radical scavenging activity. The pre-treatment of trans-anethole in the present study moderates the toxic CCl₄ level by regulating antioxidative enzyme activity (GR, CAT, and LDH) as in the control group I. The data indicates that trans-anethole can boost the antioxidant defense ability of the rat liver. The positive effects of p-Coumaric acid were reported against HFD-induced hyperlipidemia in mice models by increasing antioxidative enzymes viz. CAT, SOD, and GSH-Px (Shen et al. 2019). Also, cinnamaldehyde demonstrated restoration of SOD, CAT, and GSH levels in liver tissues of albino Wistar rats exposed to metanil yellow (MY1) (Sharma et al. 2018). Azab et al. (2013) reported the protective effects of Delonix regia leaves extract against CCl4-induced alterations in the antioxidative enzymes viz. superoxide dismutase, catalase, reduced glutathione, and total antioxidant capacity.

The elevation of phase I enzymes (mixed-function oxidases) in the CCl₄-treated group signal a transformation of CCl₄ into hydrophilic electrophiles. The biotransformation of CCl₄ by CYP2E1 generates more toxic and reactive trichloromethyl free radical (CCl₃⁺) and trichloromethyl peroxo radical (CCl₃OO) than the parent compound. These free radicals can bind to biological macromolecules, which ultimately result in lipid peroxidation and cause damage to the liver (Aysun Kepekçi et al. 2017). CCl₄ treatment was shown to increase Cyt b5 and NADH Cyt b5 enzymes, while the pre-treatment of trans-anethole helped to reduce these enzyme levels. Inhibition of CYP2E1 expression is a key pathway for many drugs to combat hepatic injury. However, in all treatment groups, an increase in the NADPH cytochrome P450 content, cytochrome P450, and P420 was found concerning CCl₄-treated rats. Curcumin exhibited a protective effect against chronic alcohol administration by significantly decreasing the CYP2E1 enzyme level (Lee et al. 2019). The experimental results plotted in the 3D graphs showed that Cyt P420 and Cyt P450 are positively associated, suggesting that with an increase in the concentration of Cyt P420, the content of Cyt P450 also increases; on the other hand, the concentration of Cyt P420 decreases with increase in the concentration of Cyt b5 (Fig. 6C). Pearson’s correlations analysis indicated that TBARS is negatively correlated with protein GSH, CAT, GR, Cyt P450, Cyt P420, NADPH cyt P450, and GST. In contrast, it is positively correlated with LDH, Cyt b5, and NADPH Cyt b5 reductase. Protein, GSH, CAT, and GR showed a positive correlation with all studied parameters except LDH, Cyt b5, and NADPH Cyt b5 reductase (Fig. 8).

The decreased amount of GST in rats treated with CCl₄ resulted in the accumulation of free radicals inside the body that are known to cause several deleterious effects. Trans-anethole and silymarin pre-treatment significantly increase the enzyme level that contributed to the removal of toxic metabolites. G-S-T performs a crucial function in detoxifying many endogenous and xenobiotic compounds (Gweshelo et al. 2016). A report revealed that 6-gingerol recovered the activities of GST, SOD, and GSH in diethylnitrosamine (DEN) intoxicated in adult male albino rats (Alsahl et al. 2021).

H&E staining revealed histopathological disorders such as degenerated hepatic parenchyma, central dilated vein, fibrosis, hepatocellular ballooning, and hepatic steatosis in CCl₄-treated group II. Histopathological studies indicated that trans-anethole treatment restored the normal architecture of the liver intoxicated by CCl₄. Groups I, III, and IV showed a zero pathological score indicating its nontoxic effects, while the CCl₄-treated group showed the most severe damage with a score of (7/18) (Table 7) (Ishak et al. 1995). In a study, p-Coumaric acid administration enhanced the appearance of liver histopathology in adult male albino rats (Alsahli et al. 2021).

Pro-apoptotic protein (p53) functions as a tumor suppressor, nuclear transcription factor and regulates the transcription of proteins involved in DNA repair, cell differentiation, cell cycle, and apoptosis. p53 is maintained at a low level in
normal cells, but during stress and DNA damage, p53 is triggered and induces fibrosis leading to hepatocyte apoptosis (Ozaki and Nakagawara 2011). In the present study, the CCl₄-treated group II has confirmed a higher expression of p53 as compared to control. With the application of trans-anethole along with CCl₄, the p53 expression level was significantly reduced in groups V, VI, and VII, confirming the oxidative DNA damage induced by ROS generated by CCl₄.

Oncogene cyclin D is a crucial regulator for cell cycle progression. Overexpression and magnification of cyclin D has been related to aggressive forms of human hepatocellular carcinoma (HCC). In addition, increased levels of cyclin D cause early onset and development of tumors (Deane et al. 2001). The current research showed that the CCl₄-treated group upregulated the expression of cyclin D, whereas it substantially decreased with the application of trans-anethole.

Expression of p53 and cyclin D was low in the control liver samples. So, the decreasing expressions of p53 and cyclin D

### Table 7

Modified hepatic activity index (HAI) grading, histopathological scores, i.e., grade of “necro-inflammation” (out of 18), as noted after examination of liver samples (48)

| Groups    | Treatments                                      | Confluent necrosis | Piecemeal necrosis (periportal or interface hepatitis) | Focal lytic necrosis, focal inflammation, and apoptosis | Portal inflammation | Total score |
|-----------|-------------------------------------------------|--------------------|------------------------------------------------------|--------------------------------------------------------|---------------------|-------------|
| Group I   | Control (tap water *ad libitum*)                | 0                  | 0                                                    | 0                                                      | 0                   | 0/18        |
| Group II  | CCl₄: olive oil (1:1; 1 ml/kg b.wt.)             | 1                  | 3                                                    | 1                                                      | 2                   | 7/18        |
| Group III | Silymarin (50 mg/kg b.wt.) + CCl₄               | 0                  | 0                                                    | 0                                                      | 0                   | 0/18        |
| Group IV  | trans-anethole (160 mg/kg b.wt.)                | 0                  | 0                                                    | 0                                                      | 0                   | 0/18        |
| Group V   | trans-anethole (40 mg/kg b.wt.) + CCl₄          | 0                  | 2                                                    | 0                                                      | 1                   | 3/18        |
| Group VI  | trans-anethole (80 mg/kg b.wt.) + CCl₄          | 0                  | 1                                                    | 0                                                      | 1                   | 2/18        |
| Group VII | trans-anethole (160 mg/kg b.wt.) + CCl₄         | 0                  | 1                                                    | 0                                                      | 0                   | 1/18        |

### Fig. 9

(A) Expression level of Bax, Bcl-xL, and caspase-3 protein in the liver homogenate of male Wistar rats detected using western blotting. (I) Control group (tap water *ad libitum*), (II) CCl₄: olive oil (1:1; 1 ml kg/b.wt.), (III) silymarin (50 mg/kg b.wt.) + CCl₄, (IV) trans-anethole (160 mg/kg b.wt.), (V) trans-anethole (40 mg/kg b.wt.) + CCl₄, (VI) trans-anethole (80 mg/kg b.wt.) + CCl₄, (VII) trans-anethole (160 mg/kg b.wt.) + CCl₄. (B) Histograms showing densitometric analysis of Bax, Bcl-xL, and caspase-3 protein bands using western blot analysis. Band density was measured and normalized to that of β-actin. Values are expressed as mean ± SE. *p ≤ 0.05
in the current study reveal the hepatic protection using trans-anethole treatment. Bcl-2 family members act as pro- or anti-apoptotic regulators that are involved in a wide range of cellular activities (Wang et al. 2021). Overexpression of the pro-apoptotic proteins Fas, FasL, and Bax as compared to anti-apoptotic proteins Bcl-2, Bcl-2α, and Bcl-xL were reported in chronic hepatitis (Chen et al. 2004). Caspases and Bcl-2 family members are considered to be important regulators of chemical-induced apoptosis. Caspase-3 is a downstream executioner caspase, which is activated in the apoptotic pathway. Caspase-3 overexpression has been documented in a variety of human cancers, including hepatocellular carcinoma (Persad et al. 2004). In the present study, CCl4 induction upregulated Bax and caspase-3 and downregulated Bcl-xL expression in male Wistar rats. The results exhibited that trans-anethole at different doses significantly alleviated the expression of Bax and caspase-3 but elevated the expression of Bcl-xL in the liver tissue (Fig. 9). Trans-anethole thus plays a significant role as a hepatoprotective agent via modulating the CCl4-induced molecular insults.

Conclusion

The results of the present study demonstrated that trans-anethole reduced the pathological consequences caused by CCl4 and showed significant hepatoprotective ability. The study revealed the normalization of serum marker enzymes (ALT, AST, and ALP) and decreased lipid peroxidation levels. Furthermore, trans-anethole showed a boost in the antioxidant capacity of hepatocytes, attenuated phase I enzymes, and elevated phase II detoxifying enzymes. The immunohistochemical and western blot analysis further corroborated the protective effects of trans-anethole by modulating p53, caspase-3, Bax, Bcl-xL, and cyclin D proteins. Trans-anethole thus has promising hepatoprotective potential against CCl4-induced hepatic injury.

Abbreviations  ALB, Albumin; ALP, Alkaline phosphatase; ALT, Alanine aminotransferase; ANOVA, Analysis of variance; AST, Aspartate aminotransferase; b.wt., Body weight; CAT, Catalase; CCl4, Carbon tetrachloride; CHOL, Cholesterol; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; CYP, Cytochrome P450; DB, Direct bilirubin; df, Degrees of freedom; ECL, Enhanced chemiluminescence; GSH, Reduced glutathione; GSSG, Oxidized glutathione; H&E, Hematoxylin and eosin; HSD, Honestly significant difference; i.p., Intraperitoneal; MDA, Malondialdehyde; PAGE, Polyacrylamide gel electrophoresis; PBS, Phosphate buffer saline; PVDF, Polyvinylidene fluoride; RNS, Reactive nitrogen species; ROS, Reactive oxygen species; rpm, Revolutions per minute; SDS, Sodium dodecyl sulphate; TB, Total bilirubin; TBARS, Thiobarbituric acid reactive substances; TCA, Trichloroacetic acid; TG, Triglycerides

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Author contribution  The author contributed to this paper as follows. Supervision, conceptualization, reviewing, editing the manuscript, and finalizing it: SJK, RB. Project administration and conceptualization: SJ. Performed the experiments, methodology, interpretation of data, writing of the original draft: KP. Formal analysis, methodology, designed the figures and tables: SK, AK, and VK. All authors read and approved the manuscript.

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Data availability  All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval  The protocol for all animal experimentation conducted in the present study was approved by Institutional Animal Ethics Committee (IAEC), Guru Nanak Dev University, Amritsar, according to the regulation of CPCSEA (protocol approval no. 226/CPCSEA/2017/05). All animal experiments were performed as per CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Government of India, New Delhi, guidelines for animal experimentation.

Consent to participate  This research did not involve human subjects, so clinical trial registration is not applicable.

Plant reproducibility  Not applicable.

Consent for publication  All authors agree with the content and give consent to publish.

Conflict of interest  The authors declare no competing interests.

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