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 and it is a principal aromatic component of star anise possessed several therapeutic properties. Results showed that CCl_4 treatment elevated the levels of different serum markers like serum glutamate oxaloacetate transaminase (SGOT) by 4.74 fold, serum glutamate pyruvate transaminase (SGPT) 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 with control. It was observed that decreased levels of various liver antioxidant enzymes *viz.* CAT, GR, and GSH were significantly ameliorated by the pre-administration of rats with different doses (40, 80, and 160 mg kg^{-1} bw) of *trans*-Anethole. Furthermore, pre-treatment of *trans*-Anethole reduced phase I enzymes level, whereas elevated level of phase II detoxifying enzymes. Furthermore, histopathological examination showed that the treatment with *trans*-Anethole was potent in defending the liver from CCl_4 toxic injury and restored normal hepatic architecture. Also, *trans*-Anethole regulated p53 and Cyclin D1 expressions in liver tissue relative to group II treated with CCl_4. Collectively, the findings of the study showed a strong efficiency of *trans*-Anethole in ameliorating the effects caused by CCl_4 through modulation of antioxidants and xenobiotic-metabolizing enzymes.

1. Introduction

Liver is a crucial metabolic organ and displays an essential role in safeguarding several physiological procedures within the body. This organ performs multidimensional functions including nutrients metabolism, maintaining body homeostasis, and involved in the detoxification of xenobiotics, drugs, pollutants and chemotherapeutics (1, 2). Liver diseases have become a worldwide problem and fatalities related to hepatic diseases are rising to an alarming rate (3). Liver is extremely vulnerable to damage from particular toxins and free radicals, regardless of its physiological role (4). Such continuous exposure to these metabolic products and agents may leads to dreadful hepatic conditions such as hepatic fibrosis, liver cirrhosis and even liver cancer.

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin and potent industrial solvent widely used in laboratory to induce liver injury in animals (5). CCl_4 itself has no cytotoxic effect, but its rapid biotransformation to trichloromethyl and Cl^- radicals with reacting oxygen catalyzed by cytochrome P450 in microsomes induced hepatotoxicity (6, 7). It has been proved that several manifestations of liver damage are associated with oxidative stress and redox imbalance. Oxidative stress reflects an imbalance in ROS production and metabolism. Oxidative stress is thus a crucial driving factor in hepatic damage caused by CCl_4 (8). Administration of CCl_4 to rats also enhances lipid oxidation of the cell membranes and changes the activity of the enzymes causing liver dysfunction leading to hepatic necrosis (9).

Many enzymatic and non-enzymatic compounds help in protection of liver from oxidative stress and ROS. Antioxidant enzymes like superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) prevents hepatic damage (10). Reduced Glutathione (GSH) is a highly efficient low-

molecular-weight non-protein thiol and major antioxidant enzyme in mammalian cells present in an ample amount. It provides defence against oxidative stress as ROS and electrophile scavenger. GSH also scavenges lipid peroxides, maintains cellular redox homeostasis, and detoxifies reactive intermediates of xenobiotics (11). Similarly, Phase I (cytochrome P450 oxidases) and Phase II (Glutathione-S-transferase) biotransformation enzymes also assist in the metabolic activation and detoxification of xenobiotics respectively. Phase I enzymes transform such xenobiotics into functional intermediates whereas phase II enzymes catalyze the conjugation of these functional intermediates with endogenous cofactors which turn them into hydrophilic, inert compounds which can be removed from the body in the form of the urine, bile or faeces (12).

Dysregulation of various signalling pathways plays a critical role in hepatocarcinogenesis. p53 and Cyclin D are central to the liver cancer etiology. p53 is a nuclear phosphoprotein that performs a vital role in apoptosis, autophagy, cell division, and growth regulation (13). p53 gene regulates a crucial mechanism for cell cycle arrest, initiation of which results in programmed cell death. When tissues experience a serious injury, p53 starts the process of apoptosis (14, 15). Cyclin D is a proto-oncogene playing vital function in cell cycle progression and is also involved in DNA repair. Several types of cancer, including hepatocellular carcinoma (HCC) have documented overexpression of Cyclin D protein (16, 17).

Despite the discovery of new treatment therapies, liver damage is still considered to be a major public health concern. In this regard, there is an intense need to explore more alternative therapeutic drugs without serious side effects. Therefore, a worldwide tendency towards the use of natural products and nutritional components as possible hepatoprotective agents has been emerging recently (18). Anethole, the phenylpropanoid family member has two isomers, *trans*-Anethole, and *cis*-Anethole. The most abundant isomer of anethole in natural oils is *trans*-Anethole (> 99%) (19). *trans*-Anethole, a naturally occurring major aromatic phytoconstituent of star anise essential oil (*Illicium verum* Hook.), well-known for its culinary uses and has been used as a widely known flavoring enhancer in a wide range of alcoholic and non-alcoholic beverages and confectioneries, other seasonings and is being used as an efficient herbal medicinal ingredient for centuries (20). Anethole has proven to be an effective anticancer agent against breast cancer (MCF-7 and MDA-MB-231) cell lines through the cell death regulation, survival, and proliferation (21). The compound anethole exerted antimetastatic 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 ERK, p38, Akt, and NF-κB signaling pathways in human fibrosarcoma (HT-1080) cells (22). Jana and colleagues (23) 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 the treatment of 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 CCl_4- induced liver damage by analyzing serum marker enzymes (serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase

(ALP), total bilirubin, direct bilirubin, total protein, albumin, urea, creatinine, cholesterol, and triglycerides), biochemical parameters, histopathological and immunohistochemical analysis in male Wistar rats.

2. Materials And Methods

2.1. Chemicals

Silymarin, *trans*-Anethole, 5,5-dithiobisnitrobenzoic acid (DTNB), 2-aminofluorene (2-AF) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma (St. Louis, MO, USA). Reduced glutathione (GSH), bovine serum albumin (BSA), glycylglycine, Triton-X, oxidized glutathione (GSSG), 2-thiobarbituric acid (TBA), sodium pyruvate, trichloroacetic acid (TCA), malondialdehyde, L-histidine, D-biotin and nicotinamide adenine dinucleotide (NADH) were procured from Hi-Media Pvt. Ltd., Mumbai, India. CCl_4 was obtained from Spectro Chem Pvt. Ltd., Mumbai, India. Antibodies used in the present study were acquired from Cell Signaling Technology (Danvers, MA, USA).

2.2. *In vivo* study

2.2.1. 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 animal house of Guru Nanak Dev University, Amritsar following the guidelines of 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 fed with regular pellet diet and tap water *ad libitum* in polypropylene cages with paddy husk bedding, at a temperature of 25 ± 2ºC with a 12 h light/dark cycle. Animals were acclimatized for two weeks until the start of the experiment.

2.2.2. Experimental design

The hepatic damage model caused by carbon tetrachloride (CCl_4) was used to determine hepatotoxicity. A total of 42 rats were divided into 7 groups at random, each group containing 6 rats. The procedure of experimentation was of 28 days. **Group I** animals which served as control were given regular pellet diet and tap water *ad libitum.***Group II** animals served as the positive control group, treated with carbon tetrachloride (CCl_4), 3 days in a week, a known hepatotoxin (1ml kg^{-1}b.wt. in1:1 ratio; CCl_4 and olive oil *via* intraperitoneal doses), **Group III** animals were pre-treated with Silymarin (50 mg kg^{-1}b.wt.) during the experiment using an oral route, used as a typical hepatoprotective medication, followed by CCl_4 intraperitoneal doses, 3 days in a week; **Group IV** animals were treated with *trans*-Anethole (160 mg kg^{-1} b.wt.) for 28 days as negative control; **Group V** animals *trans*-Anethole pre-treatment (40 mg kg^{-1} b.wt.) for 28 days followed by intraperitoneal CCl_4 doses were given to animals; **Group VI** animals received

trans-Anethole pre-treatment (80 mg kg^{-1} b.wt.) for 28 days followed by CCl_4 (i.p); **Group VII** animals received the pre-treatment of *trans*-Anethole (160 mg kg^{-1} b.wt.) for 28 days followed by CCl_4 (i.p).

The various doses were given according to the body weight of the animals.

The concentrations of *trans*-Anethole for present study have been decided from the literature of various *in vivo* studies (24, 25). *trans*-Anethole was given orally in the form of emulsion which was prepared by mixing 500 mg of Xanthine gum and 50 mg of Acacia gum in 100 ml of warm distilled water. After mixing this solution thoroughly, *trans*-Anethole was added drop-wise and further mixed the solution for half an hour, followed by cooling and then given to rats.

2.2.3. Assessment of serum marker enzymes

Blood was collected from each group by puncturing the retro-orbital venous sinus after the completion of treatment period (28 days) using moderate amount of diethyl-ether for anesthetizing the animals. The blood samples were allowed to stand for about 30 min at 37°C followed by centrifugation at 2400 rpm for 20 min. The clear and transparent supernatant obtained was designated as serum. The serum was further used for the estimation of various serum parameters like serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (ALP), total bilirubin, direct bilirubin, total protein, albumin, urea, creatinine, cholesterol, and triglycerides using kits of Erba diagnostics Mannheim GmbH, Germany with BeneSphera autoanalyzer.

2.2.4. Preparation of liver homogenate

The animals were sacrificed using cervical dislocation procedure 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 and weighed for preparation of 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.

2.2.5. Biochemical analysis of liver homogenate

2.2.5.1. Protein estimation

The protein content was determined by the method given by Smith (26). 0.1 ml of liver homogenate was mixed in a ratio of 100:1 with 2 ml of reagent A which comprised of sodium bicinchonate, sodium tartarate, sodium carbonate, sodium bicarbonate, sodium hydroxide and reagent B having copper sulphate. Then, the reaction mixture was incubated at 60°C for 30 min and the absorbance was read at 562 nm using ELISA plate reader. 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.

2.2.5.2. Lipid peroxidation

The formation of TBARS has been used to evaluate lipid peroxidation by microsomes in liver homogenate (27). In this process, 2 ml of TBA reagent (20% TCA, 0.5% TBA, and 0.25 N HCl) was applied to 500 µl of liver homogenate and properly combined. The reaction was incubated for 30 min at 80°C accompanied by centrifugation at 2000 rpm for 10 min, then the reaction was permitted to cool at ambient temperature and absorbance was recorded at 532 nm and 600 nm. The total amount of TBARS was measured as 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 compound.

2.2.5.3. Reduced glutathione content

The content of GSH was estimated according to the procedure of Anderson (28). In this experiment, potassium phosphate buffer (0.2 M) and DTNB solution (0.6 mM) were added in 0.1 ml supernatant collected by precipitating homogenate with TCA (5%) followed by incubating the reaction mixture for 10 min and reading the absorbance at 412 nm. Glutathione was used as a standard for generating the standard calibration curve.

2.2.6. Antioxidative enzymes

2.2.6.1. Catalase

The CAT activity was determined according to the method of Aebi (29). The rate of elimination of H_2O_2 from the mixture was calculated at 240 nm upon addition of catalase enzyme. The reaction mixture comprised of 0.3 ml of hydrogen peroxide and 2.6 ml of potassium phosphate buffer (pH 7.0), and 0.1 ml of homogenate was added to initiate the reaction enzymes. Observations were recorded at 240 nm for 2 min with an interval of 15 seconds. The extinction coefficient used was 6.93×10^{-3} $mM^{-1}cm^{-1}$.

2.2.6.2. Lactate dehydrogenase

The LDH activity was determined by the method of Kaur et al. (30). The reaction mixture in this assay comprises of 2.2 ml of Tris-HCl buffer (pH 7.1), Triton X (100 µl), sodium pyruvate (300 µl) as well as NADH (300 µl), then allowed to stand the mixture for 5–10 min at 30°C. The oxidation of NADH was started accompanied by the introduction of homogenate (100 µl) and then calculated the rate of oxidation for 2 min at 15 seconds interval at 340 nm. The specific activity of LDH was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$.

2.2.6.3. Glutathione reductase

The method suggested by Carlberg and Mannervik (31) was used to estimate the function of glutathione reductase (GR). The mixture comprised of 1.2 ml of sodium phosphate buffer, 200 µl of EDTA, 200 µl of

oxidized glutathione, and 200 µl of liver homogenate. Eventually, in a reaction mixture, 1mM NADPH was added and the OD was recorded at 340 nm for 3 minutes at 15 seconds intervals. Extinction co-efficient used was 6.22 $mM^{-1}cm^{-1}$.

2.2.7. Phase-I enzyme

2.2.7.1. Estimation of cytochrome P450 and cytochrome P420 contents

The contents of cytochrome P450 and cytochrome P420 was calculated using the procedure as suggested by (32) using the absorption coefficient 91 $mM^{-1}cm^{-1}$ and 111 $mM^{-1}cm^{-1}$ respectively. Briefly, the homogenate was split into two (200µl) distinct 96 well plates. One plate is saturated with around 35–40 CO bubbles (created in an especially built apparatus through the mingling of formic acid and sulphuric acid in the ratio of 1:3) at a rate of one bubble per second and wells were shaken for 1–2 min. Then, freshly prepared sodium hydrofluoride (5–10 µl) was applied in each well and the spectrum was recorded at 420, 450, and 490 nm.

2.2.7.2. Cytochrome b5 content

The cytochrome b5 content was assayed by following the protocol (33). 50 mM Tris-HCl buffer (4.3 ml) has been mixed with tissue homogenate (0.5 ml). 50 µl of this reaction mixture has been dispersed into two separate 96-well plates. 0.5 µM of NADPH (50µl) was added in one plate and Tris-HCl (50µl) was added in the other. Finally, the reaction mixture was incubated for about 15–20 mins and the OD was recorded at 409 nm and 424 nm.

2.2.7.3. NADPH cytochrome P450 reductase and NADPH cytochrome b5 reductase

The NADPH cytochrome P450 reductase activity was measured by using the protocol of Omura and Takasue (34) and NADPH cytochrome b5 reductase activity was measured following the protocol of Mihara and Sato (35). In NADPH cytochrome P450 reductase assay, reaction mixture consists of 0.3 M phosphate buffer (0.5 ml), 0.1 mM NADPH (0.2 ml), and 0.2 mM potassium ferricyanide (0.2 ml). The experiment was started by adding tissue homogenate to make the ultimate volume up to 1 ml. The NADPH oxidation intensity was measured at 340 nm with 6.22 mM^{-1} cm^{-1} extinction coefficient. While estimating NADPH cytochrome b5 reductase activity, the activity of the enzyme was estimated using 1.02 mM^{-1} cm^{-1} as extinction co-efficient and the NADH oxidation intensity was determined at 420 nm. In brief, 0.3 M phosphate buffer (0.5 ml), 0.1 mM NADPH (0.2ml), and 0.2 mM potassium ferricyanide (0.2 ml) and reaction was triggered by the addition 0.1 ml of tissue homogenate.

2.2.8. Phase-II enzyme

2.2.8.1. Glutathione-S-transferase

GST activity was calculated at 37ºC (36). Briefly, 2.7 ml of sodium phosphate buffer was added to 0.1 ml each of cDNB and reduced glutathione, and the reaction mixture was allowed to stand for 3 minutes at 37°C. The activity of the enzyme was recorded for 3 mins at 340 nm by adding 0.1 ml of liver homogenate. The specific activity of GST was determined by using 9.6 $mM^{-1}cm^{-1}$extinction coefficient.

2.2.9. 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 haematoxylin solution, followed by 5 min with eosin Y stain, and finally examined under a light microscope.

2.2.10. Immunohistochemical (IHC) analysis

Using immunohistochemistry, the expression pattern of proteins *viz.* p53 and cyclin D in liver hepatocytes was determined. The samples of liver tissue were instantly fixed in the solution of neutral buffered formalin, dehydrated by graded series of ethanol, and infiltrated with paraffin wax and tissues were sectioned of 4 µm thickness using a rotary microtome. Slides with anti-p53 and anti-cyclin D antibodies were then subjected to incubation. Anti-rabbit IgG HRP-conjugated secondary antibodies were used and samples were counterstained with H&E for visualization. The slides were analyzed using a light microscope, and the extent of expression was shown by the presence of nuclei and cytoplasm which are brown in color.

2.3. Statistical analysis

The experiments were performed in triplicates and represented as mean ± standard error. Significance of the data was checked by using one-way and two-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 $*p < 0.05$ (37). Contour plots were made by using MINITAB software. Heat map and Pearson's Correlation analysis was performed using R-Software V.3.5.1 (Statistical Computing, Vienna, Austria).

3. Results

3.1. *In vivo* hepatoprotective activity

3.1.1. Response of body weight

trans-Anethole effect on the percentage change in male Wistar rats body weight after four weeks of treatment given in Table 1. After7 days, the percentage difference in an animal's body weight was observed. Four 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. In fact, pre-administration of *trans*-Anethole followed by CCl_4 normalize the change in the bodyweight of animals in a concentration-dependent way.

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

Groups	Treatments	Change in body weight of animals (Mean) ± S.E.	Percentage change in body weight of animal relative to control (fold increase)
Group I	**Control (tap water**ad libitum**)**	3.22 ± 0.51[b]	1
Group II	**CCl_4 : Olive oil (1:1; 1ml kg^{-1}b.wt.)**	11.6 ± 1.31[a]	3.63
Group III	**Silymarin (50 mg kg^{-1}b.wt.) + CCl_4**	3.38 ± 0.83[b]	1.05
Group IV	trans**-Anethole (160 mg kg^{-1}b.wt.)**	3.41 ± 0.58[b]	1.06
Group V	trans**-Anethole (40 mg kg^{-1}b.wt.) + CCl_4**	5.99 ± 0.33[b]	1.86
Group VI	trans**-Anethole (80 mg kg^{-1}b.wt.) + CCl_4**	3.85 ± 0.28[b]	1.19
Group VII	trans**-Anethole (160 mg kg^{-1}b.wt.) + CCl_4**	3.68 ± 0.43[b]	1.15

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

3.1.2. Serum markers enzymes

3.3.2.1. Enzymatic Serum markers

The carbon tetrachloride (CCl_4) group exhibited a significant rise in the levels of serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and aspartate alkaline phosphatase (ALP) by 374.3 %, 246.8 %, and 255.3 % respectively with respect to control (Table 2). CCl_4 treatment contributes to serious hepatic injury in male Wistar rats. However, the effect of pre-administration of animals with *trans*-Anethole decreased the level of serum enzymes in groups V, VI, and VII. In binary combination with CCl_4 and *trans*-Anethole at 160 mg kg^{-1}b.wt., the activity of SGOT, SGPT, and ALP reduces by 58.2%, 67.6 %, and 67.9 % respectively with respect to CCl_4 group II animals. The data indicated that *trans*-Anethole alleviate liver injury caused by CCl_4.

Table 2
Effect of *trans*-Anethole on the activities of hepatotoxicity markers SGOT, SGPT and ALP in male Wistar rats treated with CCl_4.

Groups	Treatments	Serum enzyme activities (IU L^{-1}) (Mean ± S.E)		
		SGOT (AST)	SGPT (ALT)	ALP
I	Control (tap water ad libitum)	79 ± 15.2^{d}	43.4 ± 7.21^{b}	158.3 ± 14.55^{d}
II	CCl_4 : Olive oil (1:1; 1ml kg^{-1} b.wt.)	374.7 ± 17.5^{a}	150.5 ± 24.8^{a}	562.5 ± 25.85^{a}
III	Silymarin (50 mg kg^{-1} b.wt.) + CCl_4	139.9 ± 12.9^{cd}	46.6 ± 2.45^{b}	255.6 ± 4.08^{bc}
IV	trans-Anethole (160 mg kg^{-1} b.wt.)	142.4 ± 14.7^{cd}	49.1 ± 2.41^{b}	264.8 ± 7.03^{bc}
V	trans-Anethole (40 mg kg^{-1} b.wt.) + CCl_4	271.8 ± 32.2^{ab}	95.1 ± 9.67^{b}	311 ± 15.5^{b}
VI	trans-Anethole (80 mg kg^{-1} b.wt.) + CCl_4	201.9 ± 35.4^{bc}	73.2 ± 8.35^{b}	242.8 ± 33.8^{bcd}
VII	trans-Anethole (160 mg kg^{-1} b.wt.) + CCl_4	156.7 ± 19.1^{cd}	48.7 ± 5.98^{b}	180.8 ± 6.97^{cd}
	HSD	111.19	54.98	90.66
	F-ratio	14.41***	13.38***	39.59***

***significant difference at $p \leq 0.001$

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

3.3.2.2. Non-Enzymatic Serum markers

Also, the alterations in liver enzymes caused by CCl_4 resulted in a significant decrease in albumin level (50.5 %) and total protein level (54.8 %), however antagonistic results were found in case of direct bilirubin (DB) and total bilirubin (TB) which exhibited increase by 133.3% and 138.3%, respectively which cause hepatic damage in comparison to control group I. The elevating levels of bilirubin are a pathophysiological and clinical indicator of hepatic tissue necrosis (Rakib et al., 2019). It was further found that pre-administration of *trans*-Anethole followed by CCl_4 administration exhibited substantial hepatoprotective ability as noticed in groups V, VI, and VII (Table 3). F-ratio of one-way ANOVA was also found significant for albumin, total protein, direct bilirubin, and total bilirubin.

Table 3
Effect of pretreatment of *trans*-Anethole on non-enzymatic serum markers in male Wistar rats treated with CCl_4.

Groups	Treatments	Serum non-enzymatic activities ($g\ dL^{-1}$) (Mean ± S.E)		Serum non-enzymatic activities ($mg\ dL^{-1}$) (Mean ± S.E)	
		Albumin	Total Protein	Direct bilirubin	Total bilirubin
I	Control (tap water ad libitum)	3.96 ± 0.2^{a}	8.21 ± 0.23^{a}	0.12 ± 0.02^{ab}	0.13 ± 0.02^{b}
II	CCl_4 : Olive oil (1:1; 1ml kg^{-1} b.wt.)	1.96 ± 0.19^{e}	3.71 ± 0.28^{e}	0.28 ± 0.04^{a}	0.31 ± 0.03^{a}
III	Silymarin (50 mg kg^{-1} b.wt.) + CCl_4	3.6 ± 0.21^{bc}	6.96 ± 0.13^{bc}	0.15 ± 0.03^{ab}	0.24 ± 0.03^{ab}
IV	trans-Anethole (160 mg kg^{-1} b.wt.)	3.65 ± 0.26^{b}	6.8 ± 0.13^{bc}	0.13 ± 0.29^{ab}	0.18 ± 0.03^{b}
V	trans-Anethole (40 mg kg^{-1} b.wt.) + CCl_4	3.05 ± 0.19^{d}	5.48 ± 0.15^{d}	0.14 ± 0.02^{ab}	0.2 ± 0.03^{ab}
VI	trans-Anethole (80 mg kg^{-1} b.wt.) + CCl_4	3.35 ± 0.26^{c}	6.31 ± 0.12^{c}	0.15 ± 0.02^{ab}	0.1 ± 0.02^{b}
VII	trans-Anethole (160 mg kg^{-1} b.wt.) + CCl_4	3.68 ± 0.22^{ab}	7.06 ± 0.26^{b}	0.11 ± 0.01^{ab}	0.16 ± 0.03^{b}
	HSD	0.28	0.68	0.12	0.13
	F-ratio	104.12***	73.14***	3.04*	4.43**
Values are represented as Mean ± S.E for six rats in each group. Mean values with same alphabets are not significantly different from each other at $p \leq 0.05$ in all treatments using Tukey's test.					
(*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$)					

CCl_4 intoxication caused a rise in the levels of urea, creatinine, triglycerides (TG), and cholesterol (CHOL) by 112.4 %, 36.9 %, 253.2%, and 110.5% respectively as compared with group I (control). Pre-treatment with *trans*-Anethole followed by CCl_4 in groups V, VI, and VII attenuated the increase in the level of urea, creatinine, TG, and CHOL relative to CCl_4 treated group. *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^{-1} 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.

Table 4
Effect of *trans*-Anethole pretreatment on serum markers *viz.* urea, creatinine, triglycerides and cholesterol in CCl_4-treated male Wistar rats.

Groups	Treatment	Serum enzyme activities (g dL^{-1}) (Mean ± S.E)			
		Urea	Creatinine	Triglycerides	Cholesterol
I	Control (tap waterad libitum)	47.6 ± 3.05^{c}	0.65 ± 0.02^{c}	26.5 ± 2.64^{d}	33.3 ± 2.11^{c}
II	CCl_4 : Olive oil (1:1; 1ml kg^{-1}b.wt.)	101.1 ± 4.37^{a}	0.89 ± 0.04^{a}	93.6 ± 4.35^{a}	70.1 ± 3.48^{a}
III	Silymarin (50 mg kg^{-1}b.wt.) + CCl_4	49.3 ± 1.23^{c}	0.72 ± 0.02^{c}	36.5 ± 2.79^{d}	44.3 ± 3.18^{bc}
IV	trans-Anethole (160 mg kg^{-1}b.wt.)	51.6 ± 2.67^{c}	0.71 ± 0.02^{c}	37.8 ± 2.58^{cd}	45.8 ± 2.20^{bc}
V	trans-Anethole (40 mg kg^{-1}b.wt.) + CCl_4	87.9 ± 3.86^{a}	0.77 ± 0.03^{ab}	71.3 ± 3.67^{b}	58.6 ± 4.50^{ab}
VI	trans-Anethole (80 mg kg^{-1}b.wt.) + CCl_4	69.4 ± 2.94^{b}	0.73 ± 0.02^{c}	54.1 ± 4.73^{c}	49.6 ± 4.50^{bc}
VII	trans-Anethole (160 mg kg^{-1}b.wt.) + CCl_4	55.9 ± 2.69^{bc}	0.69 ± 0.04^{c}	40.8 ± 2.54^{cd}	40.0 ± 6.64^{bc}
	HSD	15.67	0.153	16.92	18.86
	F-ratio	32.66***	1.88^{ns}	35.16***	11.88***

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

(***$p \leq 0.001$. ns-not significant)

3.3.2.3 Heat map analysis of serum marker enzymes

Heat map was applied to depict the hierarchical clustering for effect of *trans*-Anethole on the activities of hepatotoxicity serum markers in male Wistar rats treated with CCl_4 (Fig. 1). The dendrogram indicated groups were mainly classified into two main categories. Group V and II showed little similarities with each other and attributed to the fact that triglycerides, cholesterol and urea showed little variations in their content. Rest of the groups I, VI, VII, III and IV are included in the same category which is attributed to almost similar content of ALP, and SGOT. Similarly, serum markers showed three clusters: cluster I comprises of ALP and SGOT reflecting little variations of their content in groups VI, VII, III, and IV. Cluster 2 consists of triglycerides, cholesterol, urea and SGPT and showing almost similar content in groups V, and

II. Finally, protein, albumin, creatinine, total bilirubin and direct bilirubin showed quite similarities in their content in groups I and VII depicting the protective effect of *trans*-Anethole at the highest tested dose (160 mg kg^{-1}b.wt.+ CCl_4) as good as control (group I).

3.2. Biochemical parameters

3.2.1. Protein content

The animals of group II that received CCl_4 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 CCl_4 from group V to VII as shown in Fig. 2A.

3.2.2. Lipid Peroxidation

It was observed that the amount of liver peroxidation or MDA content was substantially enhanced in the group II treated with CCl_4 (48.50 ± 4.37 µmol MDA eq/g of tissue) as compared with 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 could not leads to the rise in TBARS. On the other hand, the administration of *trans*-Anethole along with CCl_4 in groups V, VI, and VII showed a concentration-dependent decrease in the content of MDA by 32.2%, 51.9%, and 65.8% respectively. The activity of *trans*-Anethole at a dose of 160 mg kg^{-1} b.wt. showed a prominent effect. (Fig. 2B).

3.2.3. Reduced Glutathione content

The animals treated with CCl_4 group II showed 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 CCl_4 at the highest tested dose of group VII showed significant elevation in the GSH content by 147.7% as compared to group II (Fig. 2C).

3.3. Effects of *trans*-Anethole on Antioxidant enzymes

3.3.1. 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^{-1} b.wt.) of *trans*-Anethole followed by CCl_4 in group 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.

Table 5
Effect of pre-treatment of *trans*-Anethole on the activities of antioxidant enzymes CAT, Glutathione reductase and LDH in the liver of male Wistar rats.

Groups	Treatments	(Mean ± S.E)		
		CAT (IU mg^{-1}protein)	Glutathione reductase (IU $mg^{-1}g^{-1}$protein)	LDH (IU mg^{-1}protein)
I	**Control (**tap water ad libitum**)**	51.5 ± 2.95^{ab}	0.09 ± 0.01^{ab}	0.02 ± 0.01^{ab}
II	**CCl_4 : Olive oil (1:1; 1ml kg^{-1}b.wt.)**	22.9 ± 3.48^{d}	0.04 ± 0.06^{d}	0.06 ± 0.01^{a}
III	**Silymarin (50 mg kg^{-1}b.wt.) + CCl_4**	48.1 ± 4.51^{ab}	0.09 ± 0.09^{ab}	0.03 ± 0.01^{ab}
IV	trans**-Anethole (160 mg kg^{-1}b.wt.)**	45.3 ± 2.45^{bc}	0.09 ± 0.01^{ab}	0.02 ± 0.01^{b}
V	trans**-Anethole (40 mg kg^{-1}b.wt.) + CCl_4**	31.5 ± 3.48^{cd}	0.05 ± 0.08^{cd}	0.01 ± 0.01^{b}
VI	trans**-Anethole (80 mg kg^{-1}b.wt.) + CCl_4**	40.7 ± 2.72^{bc}	0.074 ± 0.07^{abc}	0.05 ± 0.01^{ab}
VII	trans**-Anethole (160 mg kg^{-1}b.wt.) + CCl_4**	62.3 ± 4.96^{a}	0.096 ± 0.01^{a}	0.04 ± 0.01^{ab}
	HSD	16.44	0.034	0.04
	F-ratio	11.69***	7.67***	5.16***
Values are represented as Mean ± S.E for six rats in each group. Mean values with same alphabets are not significantly different from each other at $p \leq 0.05$ in all treatments using Tukey's test.				
(*$p \leq 0.001$**)**				

3.3.2. Glutathione Reductase

The specific activity of glutathione reductase was reduced in the CCl_4 treated group II by 59.6 % relative to the control group. *trans*-Anethole alone (Group IV) and when administrated along with CCl_4 in Groups V, VI, and VII increase the amount of enzyme glutathione reductase, suggesting its protective potential against hepatic damage induced by CCl_4. *trans*-Anethole alone in group IV showed similar results as compared to standard drug silymarin. (Table 5).

3.3.3. Lactate dehydrogenase

LDH activity increased significantly in the liver of male Wistar rats upon CCl_4 exposure by 173.9 % with respect to control Group I. However, in the group 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 CCl_4 Group II. When *trans*-Anethole administrated in combination with CCl_4 in groups V and VII, the activity of LDH has restored relative to group I i.e. control (Table 5). On the other hand, Group VI showed decrease in the level of LPO as compared to group VII which shows significant protection. Besides, the effect of *trans*-Anethole at a dose of 160 mg kg^{-1} b.wt. was comparable to silymarin. F-ratio was found significant at $p \leq 0.05$ for LDH.

3.4. Phase I enzymes

The cytochrome P450, cytochrome P420, and cytochrome b5 contents were observed at different doses (40, 80, and 160 mg kg^{-1} b.wt.) of *trans*-Anethole. The cytochrome P450 and P420 enzymes levels in group II animals i.e. CCl_4 treated group was decreased significantly by 59.8 % and 40.5 % respectively in comparison to the control Group I (Table 6). However, when *trans*-Anethole administrated along with CCl_4, the level of cytochrome P450 and P420 contents were increased, witnessed substantial defense against liver injury. Group III and IV that received silymarin and *trans*-Anethole treatment, the cytochrome P450 and P420 contents was found to be almost equal to that of Group 1 (control) as proved from HSD. On the other hand, treatment of CCl_4 in Group II increase the cytochrome b5 content by (225.8 %) with respect to control group I. This higher content was later normalized with an increase in the doses of *trans*-Anethole. The transformation of CCl_4 into reactive cancer-causing form was accurately depicted by a massive increase in cytochrome b5 enzyme in the CCl_4 treated Group II when compared with control group I. All other groups demonstrated considerable safety against the adverse effects of CCl_4 (Table 6).

Table 6

Effect of *trans*-Anethole on the activities of hepatic phase I enzymes (cytochrome P450, cytochrome P420, cytochrome b5, NADPH cytochrome P450 reductase and NADH cytochrome b5 reductase) in CCl_4-treated male Wistar rats.

Groups	Dose (mg kg^{-1} bw)	(µ mole mg^{-1} protein) (Mean ± S.E)			(mIUmg^{-1} g^{-1} protein) (Mean ± S.E)	
		Cyt P450	Cyt P420	Cyt b5	NADPH Cyt P450 reductase	NADH Cyt b5 reductase
I	Control (tap water ad libitum)	48.2 ± 3.22[a]	312.3 ± 4.64[a]	30.6 ± 2.75[d]	46.9 ± 2.91[a]	85.28 ± 3.18[d]
II	CCl_4 : Olive oil (1:1; 1ml kg^{-1} bw)	19.4 ± 1.61[b]	185.9 ± 4.51[b]	99.7 ± 5.44[a]	23.7 ± 3.38[c]	193.6 ± 3.87[a]
III	Silymarin (50 mg kg^{-1} bw) + CCl_4	45.5 ± 2.62[a]	303.9 ± 10.65[a]	37.3 ± 3.09[cd]	45.9 ± 3.25[ab]	80.17 ± 3.79[d]
IV	trans-Anethole (160 mg kg^{-1} bw)	49.9 ± 3.29[a]	299.8 ± 11.71[a]	38.2 ± 2.59[cd]	48.2 ± 3.65[a]	81.31 ± 2.60[d]
V	trans-Anethole (40 mg kg^{-1} bw) + CCl_4	30.9 ± 4.21[b]	247.7 ± 10.50[b]	72.6 ± 5.84[b]	32.2 ± 4.68[bc]	161.4 ± 4.81[b]
VI	trans-Anethole (80 mg kg^{-1} bw) + CCl_4	57.3 ± 3.83[a]	332.4 ± 12.34[a]	52.3 ± 3.28[c]	52.6 ± 2.12[a]	132.9 ± 3.28[c]
VII	trans-Anethole (160 mg kg^{-1} bw) + CCl_4	48.7 ± 3.50[a]	308.0 ± 5.78[a]	32.5 ± 3.77[d]	43.6 ± 2.99[ab]	79.66 ± 2.37[d]
	HSD	14.25	39.04	17.06	14.32	14.89
	F-ratio	15.21***	30.32***	40.91***	9.23***	178.77***

Groups	Dose (mg kg^{-1}bw)	(μ mole mg^{-1} protein) (Mean ± S.E)			(mIUmg^{-1} g^{-1} protein) (Mean ± S.E)	
		Cyt P450	Cyt P420	Cyt b5	NADPH Cyt P450 reductase	NADH Cyt b5 reductase

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

(*$p \leq 0.001$)

In the CCl_4 treated Group II, the specific activity of NADPH cytochrome P450 reductase decreased by 49.5 % relative to control Group I. Group VI and VII showed same specific activity than that of the control as revealed from HSD except for Group V. In case of NADH cytochrome b5 reductase, there is a rise in the specific activity of this enzyme in the CCl_4 treated group II by 127 % with respect to the control Group I. When *trans*-Anethole administrated along with CCl_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 b5 reductase activity with respect to CCl_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.

3.5. Phase II enzymes

3.5.1. Glutathione S-transferase (GST)

The treatment of CCl_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 activity of GST which indicated that the enhancement in the consumption of antioxidants to counter the higher level of toxic electrophiles such as ROS as shown in Fig. 2D. Although a maximum increase was noticed at 160 mg kg^{-1} b.wt. dose.

3.6. Heat map analysis of Biochemical parameters

Heat map was applied to depict the hierarchical clustering for effect of trans-Anethole on the activities of biochemical parameters in male Wistar rats treated with CCl_4. The dendrogram on top shows the clustering of Groups, and the dendrogram on the side shows the clustering of biochemical parameters. The dendrogram indicated groups were predominantly categorized into two major categories. Group II and Group V animals showed close resemblance with each other and were due to the fact that GST, GR and LDH showed similarity in their content as indicated by Heat map. Similarly, Group I, VII, III, IV and VI are included in the same category. Biochemical parameters showed four clusters: cluster I comprises of GSH and NADH Cytochrome b5 reductase reflecting little 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 b5. 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).

3.7. Histopathological Analysis

The hepatic histological results are shown in Fig. 4. The CCl_4 treatment to the male Wistar rats has been found to triggered serious harm as exhibited by several histopathological alterations *viz.* hepatic steatosis, fibrosis, necrosis, loss of hepatocytes, mild spotty and confluent necrosis, lobular inflammation, and hepatocellular ballooning. Liver damage decreased with the increase in the concentration of *trans*-Anethole (40, 80, and 160 mg kg^{-1} b.wt.) administration (Fig. 4D–G), and the histological index of vacuolization and liver necrosis were reduced. On the contrary, rats of Groups I, III, and IV have not shown such pathologies.

3.8. Immunohistochemistry (IHC) analysis

Overexpression of the levels of p53 and Cyclin D markers leads to hepatocellular carcinoma. The findings of the IHC analysis indicated that the group treated with CCl_4 showed enhanced expression of p53 and Cyclin D in comparison with group I, while pre-administration with *trans*-Anethole at all doses reduced p53 and Cyclin D immunostaining relative to Group II i.e. CCl_4-treated group (Fig. 5).

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	CCl_4 : Olive oil (1:1; 1ml kg^{-1} bw)	1	3	1	2	7/18
Group III	Silymarin (50 mg kg^{-1} b.wt.) + CCl_4	0	0	0	0	0/18
Group IV	trans-Anethole (160 mg kg^{-1} b.wt.)	0	0	0	0	0/18
Group V	trans-Anethole (40 mg kg^{-1} b.wt.) + CCl_4	0	2	0	1	3/18
Group VI	trans-Anethole (80 mg kg^{-1} b.wt.) + CCl_4	0	1	0	1	2/18
Group VII	trans-Anethole (160 mg kg^{-1} b.wt.) + CCl_4	0	1	0	0	1/18

4. Discussion

Hepatic damage and dysfunction have been considered among the most severe issues impacting human health and have become an epidemic worldwide. Despite of the vigorous efforts to establish therapies, there are still very less effective hepatoprotective drugs available (38). Thus, the quest for new types of compounds with hepatoprotective and antihepatotoxic properties are important. One approach for treating liver damage is to find natural compounds with new scaffolds that are distinct from current conventional hepatoprotective agents.

CCl_4 is a well-known hepatotoxin that results in marked degeneration of centrilobular fat and liver damage induced by extensively utilized as an experimental model for the evaluation of potent hepatoprotective agents (39). The findings revealed that the levels of liver serum transaminases i.e. AST, ALT, ALP in rat serum were substantially elevated after CCl_4 exposure, which suggested hepatocellular injury as these are adopted as non-invasive hepatic markers. 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^{-1} b.wt.) of *trans*-Anethole alleviates the activities of AST, ALT, ALP suggesting that *trans*-Anethole had beneficial effects against hepatic injury caused by CCl_4. In a study, eugenol caused a decrease in the SGOT, SGPT, and ALP serum marker enzyme levels triggered by metanil yellow (My1) in albino Wistar rats (40). It was stated that 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 (41). Also, the intoxication of rats with CCl_4 caused a substantial rise in TG, Chol, TB, and DB serum levels relative to the control group. *trans*-Anethole pre-treatment at the maximum dose (160 mg kg^{-1} b.wt.) substantially decreased the levels of all measured liver markers as compared to CCl_4 treated group, results are as good as those of silymarin. From 3D contour plots it is clear that ALP is positively correlated with SGPT and SGOT, suggesting that ALP increases with increase in SGPT and SGOT (Fig. 6A). Similar results have been found by Das et al., (42) in which MSGPT is positively associated with ALP and SGOT. It was evident from literature that increased levels of SGOT, SGPT, and ALP in animals reflect cellular damage and diminution of cell membrane function (43). SGPT is associated positively with direct bilirubin and total bilirubin, suggesting that with increase in direct bilirubin and total bilirubin, SGPT increases (Fig. 6B). Further, Pearson's Correlation analysis was performed on different serum marker. The SGOT, SGPT and ALP are positively correlated with direct bilirubin, total bilirubin, urea, creatinine, triglycerides and cholesterol, while negatively related with protein and albumin (Fig. 7). In a study, it was reported that the mean SGPT is positively related with TB (42). This indicates that serum marker enzymes correlated with the non-enzymatic serum markers.

Lipid peroxidation is another significant indicator of liver injury. It contributes to oxidative destruction of cellular membranes which causes the excessive production of free radicals and eventually leads to cell death. MDA content is an intracellular end result that is used to measure the amount of lipid peroxidation (44). Results indicated that there was a significant increase in the lipid peroxidation marker MDA content in CCl_4 administrated group and this increase was attenuated due to the application of *trans*-Anethole for all doses tested but the most significant effect was found at 160 mg kg^{-1} b.wt. Ojeaburu and Oriakhi (45) demonstrated that Gallic acid lowered the level of serum markers (AST/ALT) and also decreased the levels of malondialdehyde (MDA) that were raised in rats treated with CCl_4. Chebulinic acid significantly reduced the toxicity of CCl_4, as the application of chebulinic acid decreased the level of MDA in male ICR mice (46). 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 specifically

reflects a decline in the antioxidant levels. 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 CCl_4 level through the regulation of antioxidative enzyme activity (GR, CAT, and LDH) as in control group I. The data indicates that *trans*-Anethole can boost the antioxidant defense ability of rat liver. The positive effects of p-coumaric acid were reported against HFD-induced hyperlipidemia in mice models by increasing the level of antioxidative enzymes *viz.* CAT, SOD, and GSH-Px (47). Also, cinnamaldehyde demonstrated restoration of SOD, CAT, and GSH levels in liver tissues of albino Wistar rats exposed to metanil yellow (MY1) (48).

The elevation of phase I enzymes (mixed-function oxidases) in the CCl_4 treated group is a signal of a transformation of CCl_4 into hydrophilic electrophile. The biotransformation of CCl_4 by CYP2E1 generates more toxic and reactive trichloromethyl free radical (CCl_3.) and trichloromethylperoxy radical (CCl_3OO.) than the parent compound. These free radicals can bind to biological macromolecules which ultimately result in lipid peroxidation and cause damage to the liver (49). CCl_4 treatment was shown to increase the amount of Cyt b5 and NADH Cyt b5 enzymes while the pre-treatment of *trans*-Anethole helped in the reduction of these enzymes level. Inhibition of CYP2E1 expression is a key pathway for many drugs to combat hepatic injury. However, in all treatment groups, increase in the NADPH cytochrome P450 content, cytochrome P450, and P420 was found with respect to CCl_4-treated rats. By significantly decreasing the CYP2E1 enzyme level, curcumin exhibited a protective effect against chronic alcohol administration (50). *trans*-Anethole treatment (160 mg kg^{-1} b.wt.) exerted more protection against CCl_4-induced hepatic injury as compared with treatment with *trans*-Anethole at 40 mg kg^{-1} b.wt. and 80 mg kg^{-1} b.wt. By ploting data into 3D-graphs it was found that Cyt P420 and Cyt P450 are positively associated, suggesting that with 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 was conducted to find the relationship among different liver biochemical parameters in male Wistar rats treated with CCl_4. The results indicated that TBARS is negatively correlated with protein, Glu, CAT, GR, Cyt P450, Cyt P420, NADPH cyt P450 and GST, whereas it is positively correlated with LDH, Cyt b5 and NADH cyt b5 reductase. Protein, Glu, CAT and GR showed positive correlation with all studied parameters except LDH, Cytb5 and NADPH Cyt b5 reductase (Fig. 8).

The decreased amount of GST in rats treated with CCl_4 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 increases the enzyme level that contributed to the removal of toxic metabolites. Glutathione-S-Transferase performs a crucial function in the detoxification of many endogenous and xenobiotic compounds (51). In a report, it was revealed that 6-gingerol recovered the activities of GST, SOD, and GSH in diethylnitrosamine (DEN) intoxicated in adult male albino rats (52).

The influence of *trans*-Anethole was examined on the histopathology, expression level of p53, and cyclin D in liver tissue. Hematoxylin and Eosin (H&E) staining findings revealed the histopathological disorders such as degenerated hepatic parenchyma, central dilated vein, fibrosis, hepatocellular ballooning, and hepatic steatosis in CCl_4-treated group II. Histopathological findings indicated that *trans*-Anethole treatment restored the normal architecture of the liver intoxicated by CCl_4. Group I, III, and IV showed a zero pathological score indicating its non-toxic effects. The highest damage showed in CCl_4 treated group (7/18) (Table 7) (53). In a study p-coumaric acid administration enhanced the appearance of liver histopathology in adult male Wistar rats as indicated by Hematoxylin and Eosin (H&E) staining (54). It was demonstrated that rats treated with eugenol had shown improvement in liver function and structure which was altered by Ischemia/reperfusion (I/R) injury. Eugenol has also inhibited the rise in MDA levels and loss in GSH levels in rats (55).

Pro-apoptotic protein (p53) functions as a tumor-suppressor, nuclear transcription factor, and regulates the transcription of proteins that are 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 which leads to hepatocyte apoptosis (56). In the present study, the CCl_4 treated group II has confirmed a higher expression of p53 as compared to control. With the application of *trans*-Anethole along with CCl_4, the p53 expression level has been significantly reduced in groups V, VI, and VII, confirmed the presence of oxidative DNA damage induced by ROS generated by CCl_4. Oncogene Cyclin D is a crucial regulator for cell cycle progression. Overexpression and magnification of cyclin D have been related to aggressive forms of human hepatocellular carcinoma (HCC). Also, increased levels of Cyclin D cause early-onset and development of tumors (57). The current research showed that the CCl_4-treated group has more expression of Cyclin D with respect to the control group I, while Cyclin D expression was substantially decreased with the application of *trans*-Anethole. Expression of p53 and Cyclin D were low in the controlled liver samples. So, the decreasing expressions of p53 and Cyclin D in the current study reveal the hepatic protection by using *trans*-Anethole treatment. Thus, *trans*-Anethole greatly decreased oxidative stress and tissue injuries caused by CCl_4, and also down-regulated p53 and Cyclin D expression.

5. Conclusion

trans-Anethole has shown significant hepatoprotective ability. The results of present study demonstrated that *trans*-Anethole reduces the pathological consequences caused by CCl_4. The study revealed the normalization of serum marker enzymes (ALT, AST and ALP) and decreased in levels of lipid peroxidation. Further, *trans*-Anethole boost the antioxidant capacity of hepatocytes, attenuates phase I enzymes level and elevated the amount of phase II detoxifying enzymes. The immunohistochemical analysis further authenticated the protective effects of *trans*-Anethole by mitigating the levels of p53 and Cyclin D proteins. Therefore, *trans*-Anethole proves a promising hepatoprotective agent against CCl_4-induced hepatic toxicity *in vivo*.

Abbreviations

ROS, reactive oxygen species; RNS, reactive nitrogen species; MDA, malondialdehyde; CYP, cytochrome P450; GSSG, oxidized glutathione; GSH, reduced glutathione; CAT, catalase; CCl_4, Carbon tetrachloride; b.wt., bodyweight; i.p, intraperitoneal; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; ANOVA, analysis of variance.

Declarations

Data availability

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

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Compliance with ethical standards

Conflict of Interests

The authors declare no known competing financial interests.

Ethical approval

The study was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSE), Ministry of Environment and Forests, Government of India (226/ CPCSEA/2017/05). All animals were human care according to the criteria specified in the guidelines of the Institutional Animal Ethics Committee (IAEC) and written informed consent was obtained.

Consent to participate

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

Consent for publication

The authors certify that this manuscript is original unpublished work, has not been published elsewhere and is not under consideration by another journal. All authors have confirmed the manuscript and agree with its submission.

Plant reproducibility

Not applicable.

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Figures

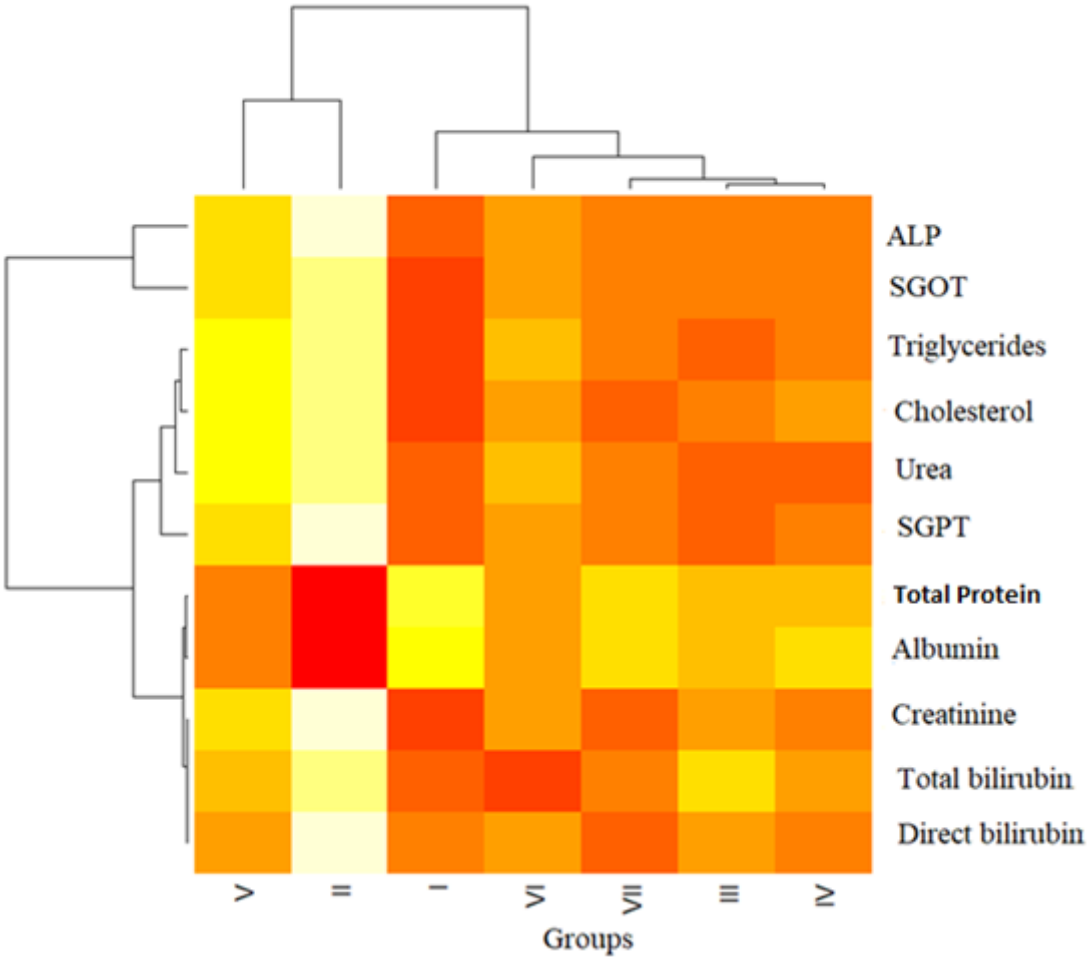

Figure 1

Heat map was applied to depict the hierarchical clustering for effect of trans-Anethole on the activities of hepatotoxicity serum markers in male Wistar rats treated with CCl4.

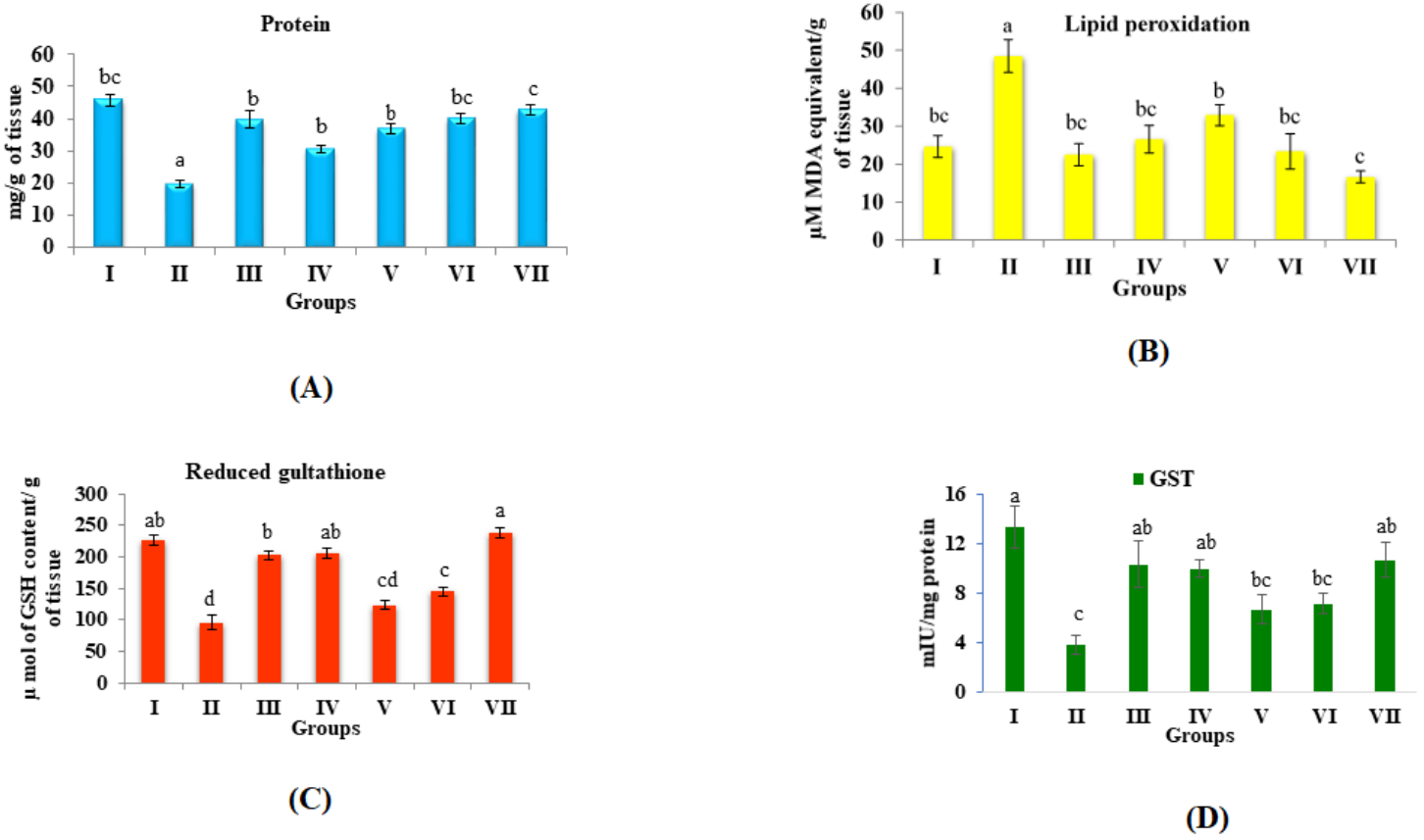

Figure 2

Effect of trans-Anethole on the levels of biochemical parameters (A) Protein, (B) Lipid peroxidation, and (C) Reduced glutathione (D) Activity of hepatic phase-II GST enzyme in CCl4-treated male Wistar rats. The values are expressed as Mean ± S.E for six rats in each group.

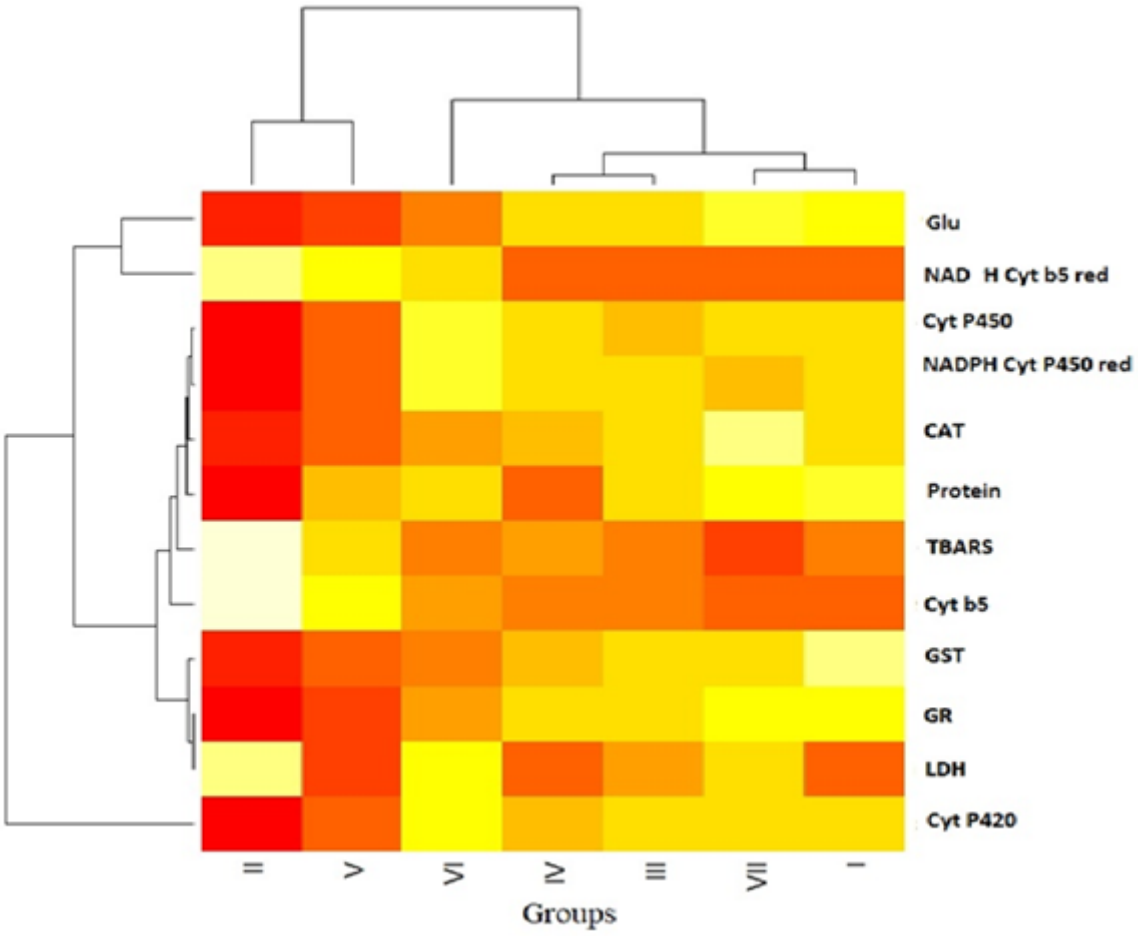

Figure 3

Heat map was applied to depict the hierarchical clustering for effect of trans-Anethole on the activities of biochemical parameters in male Wistar rats treated with CCl4.

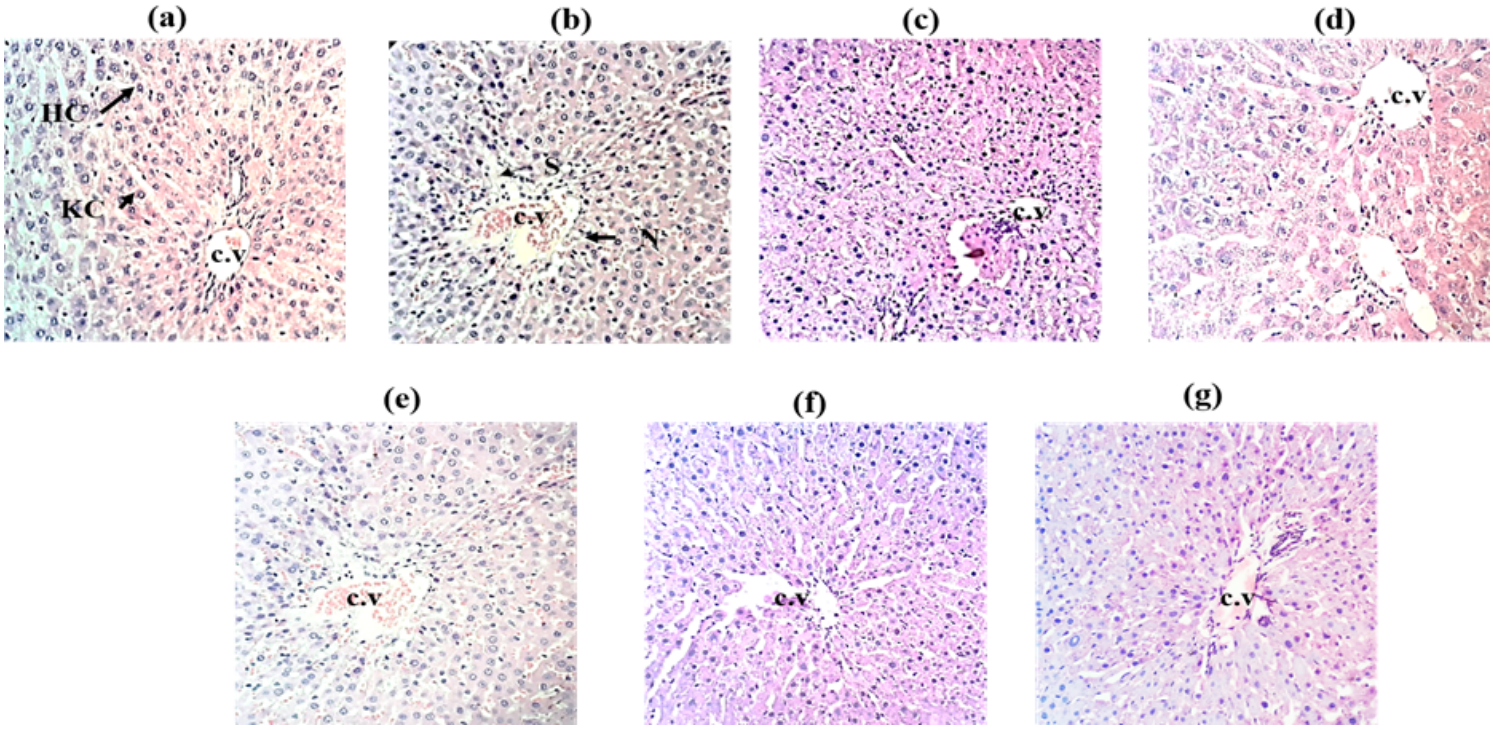

Figure 4

Effect of trans-Anethole on the histopathological changes of liver in CCl4-induced hepatic damage in male Wistar rats. (a) (Group I) Control, (b) (Group II) CCl4-treated, (c) (Group III) Silymarin (50 mg kg-1b.wt.) + CCl4, (d) (Group VI) trans-Anethole (160 mg kg-1b.wt.), (e) (Group V) trans-Anethole (40 mg kg-1b.wt.) + CCl4, (f) (Group VI) trans-Anethole (80 mg kg-1b.wt.) + CCl4, (g) (Group VII) trans-Anethole (160 mg kg-1b.wt.) + CCl4. HC-Hepatocytes, KC-Kupffer cells, c.v-central vein, S-steatosis, N-necrosis.

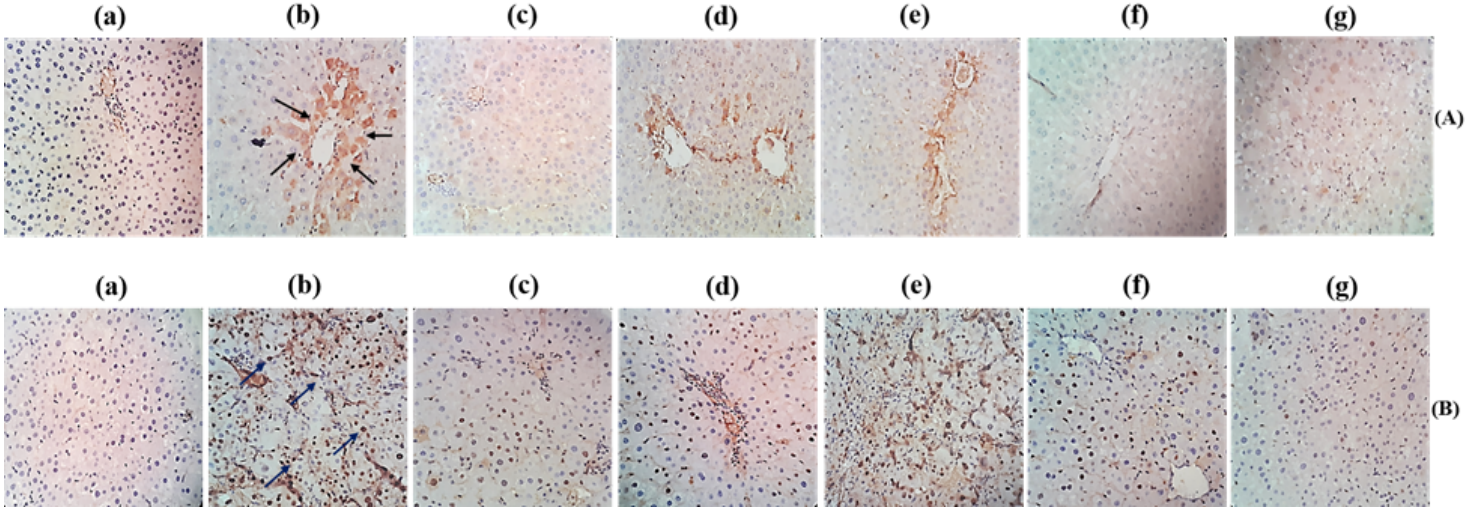

Figure 5

Effect of trans-Anethole on the expression levels of proteins using immunohistochemical analysis. (A) p53 and (B) Cyclin D antibodies. Arrows showed the increase in the expression of p53 and cyclin D proteins in CCl4 treated liver (Original magnification = 40 x). Expression of these proteins was downregulated in liver samples when co-treated with CCl4 and trans-Anethole (160 mg/kg b.wt.). (a) Control group (tap water ad libitum) showing normal lobular architecture with clear portal triad. (b) CCl4: Olive oil (1:1; 1ml kg-1 b.wt.) induced elevation in the expression level of p53 and cyclin D. (c) Silymarin (50 mg kg-1b.wt.) + CCl4, (d) trans-Anethole (160 mg kg-1b.wt.), (e) trans-Anethole (40 mg kg-1b.wt.) + CCl4, (f) trans-Anethole (80 mg kg-1b.wt.) + CCl4, (g) trans-Anethole (160 mg kg-1b.wt.) + CCl4.

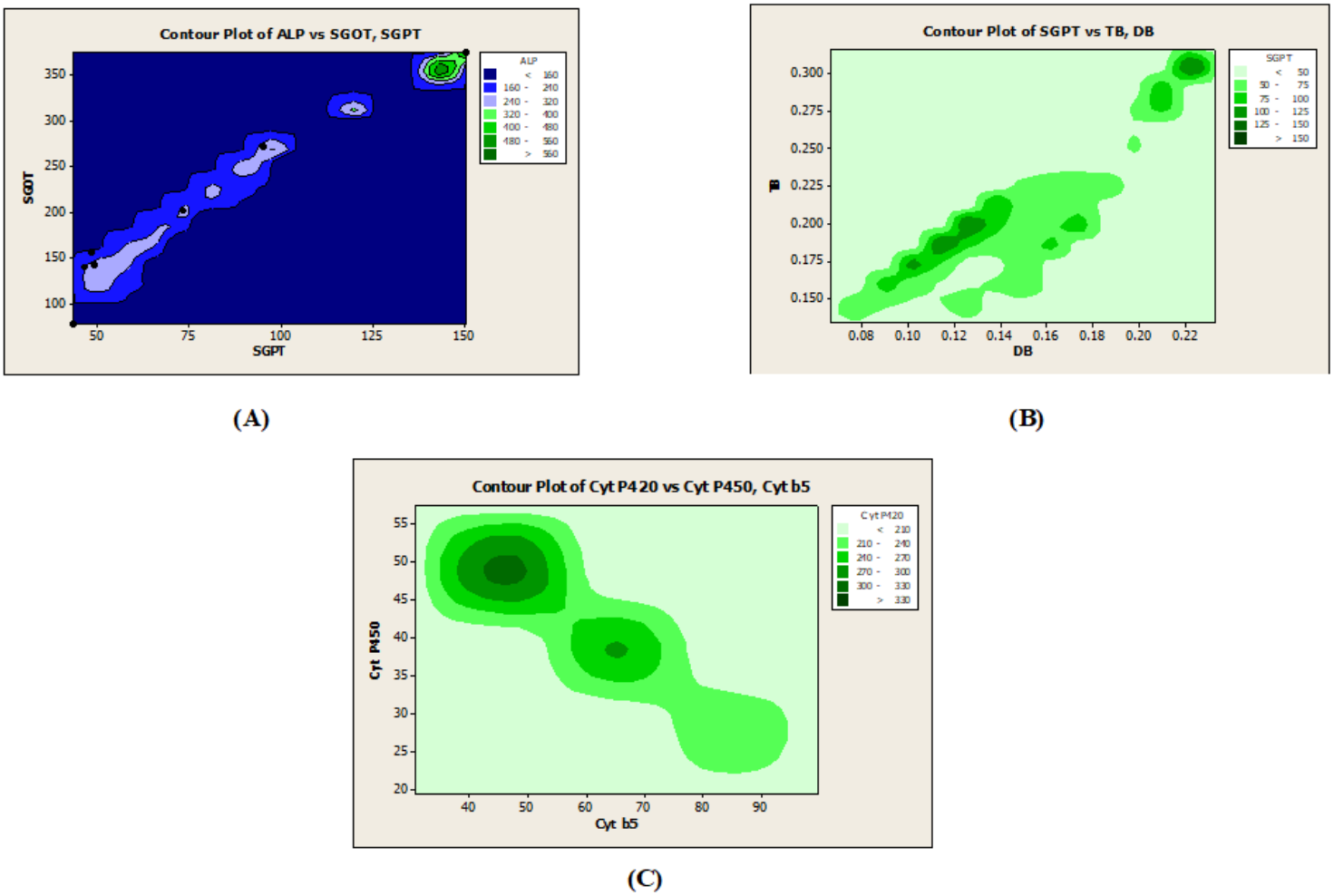

Figure 6

3D contour plot graph showing relationship between: (A) ALP vs. SGOT, SGPT. (B) SGPT vs. TB, DB. (C) Cyt P420 vs. Cyt P540, Cyt b5.

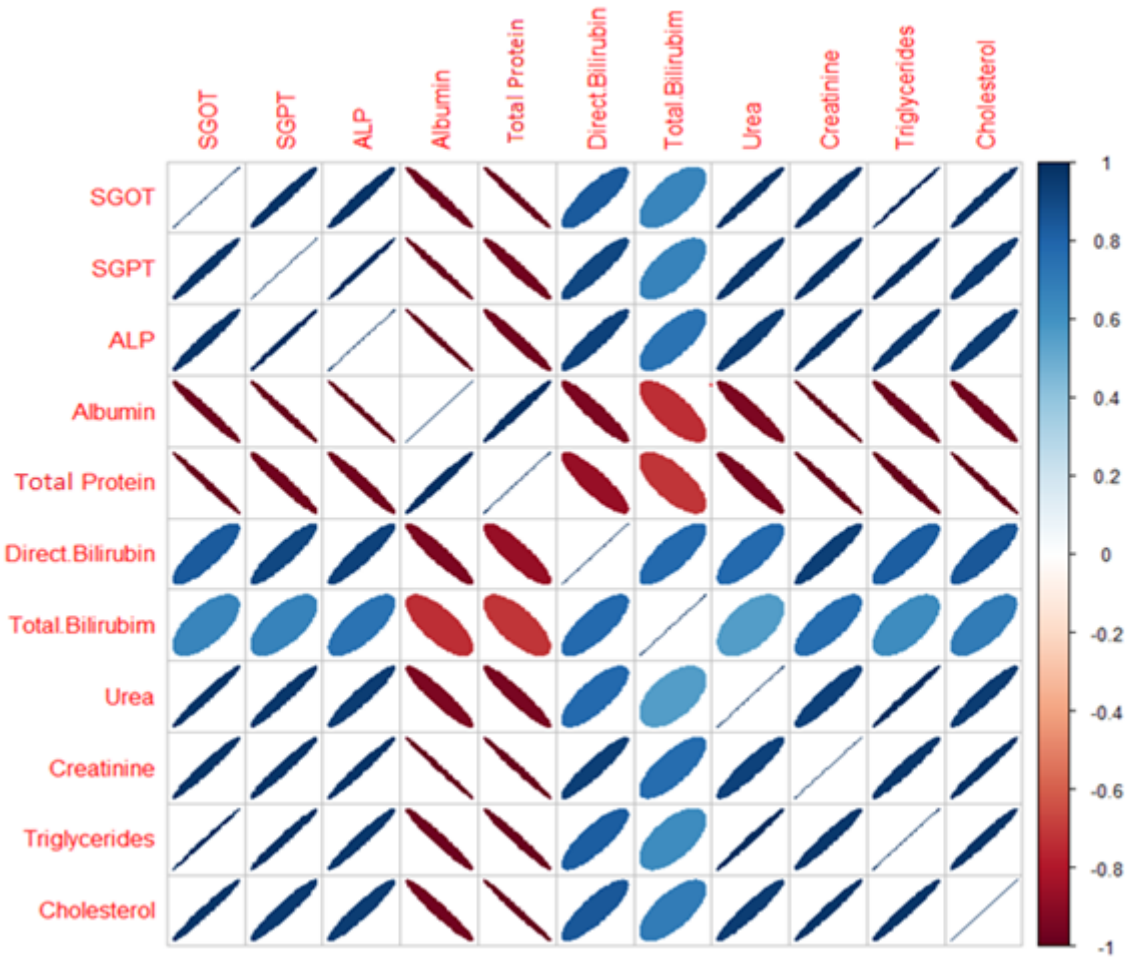

Figure 7

Pearson's correlation analysis of serum markers in male Wistar rats. The blue colour indicates the positive correlation, while red colour signifies the negative correlation. Serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (ALP).

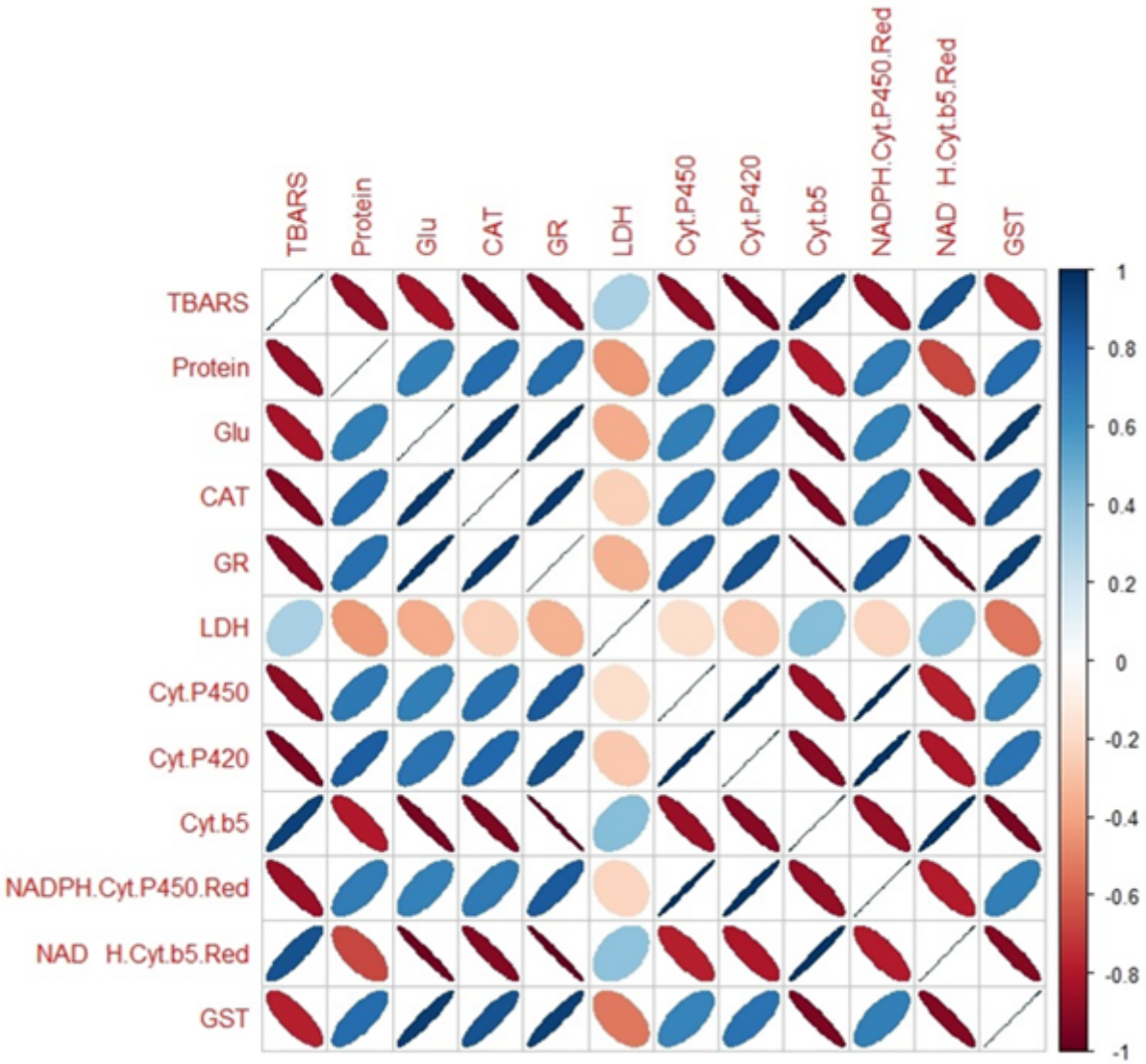

Figure 8

Pearson's correlation analysis of liver biochemical parameters in male Wistar rats. The blue colour indicates the positive correlation, while red colour signifies the negative correlation. GSH (Reduced Glutathione content), CAT (Catalase), GR (Glutathione reductase), LDH (Lactate dehydrogenase), Cyt P450 (Cytochrome P450), Cyt P420 (Cytochrome P420), Cyt b5 (Cytochrome b5), NADPH Cyt P450 Red (NADPH cytochrome P450 reductase), NADH Cyt b5 Red (NADH cytochrome b5 reductase), GST (Glutathione-S-Transferase).

Supplementary Files

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