SEMARAK ILMU PUBLISHING

Semarak Current Biomedical Technology Research Journal

Journal homepage:
https://semarakilmu.my/index.php/scbtrj/index
ISSN: 3030-5616

Metabolite Profile, Antioxidant Activity and Ameliorative Efficacy of *Diospyros Mespiliformis* on Carbon Tetrachloride (CCL_4) Induced Oxidative-Stress in Albino Mice

Muhammad Bappah Sani[1], Hamza Ahmed Pantami[2], Nura Abubakar[1], Aliyu Ibrahim Lawan[3], Jamila Aminu[1], Maryam Usman Abdulkadir[1], Hajjagana Hamza[1,*]

[1] Department of Biochemistry, Faculty of Science, Gombe State University, PMB 127 Tudunwada City Gombe Nigeria
[2] Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, Gombe State University, PMB 127 Tudunwada City Gombe Nigeria
[3] Department of Histopathology, College of Medical Sciences, Gombe State University, PMB 127 Tudunwada City Gombe Nigeria

ARTICLE INFO

Article history:
Received 23 November 2024
Received in revised form 28 February 2025
Accepted 5 March 2025
Available online 15 March 2025

Keywords:
Antioxidant activity; *Diospyros mespiliformis;* albino mice; phytochemicals; metabolite

ABSTRACT

Liver diseases are deadly and requires continuous medication, conventional treatment is harmful and expensive, so there need for hepatoprotective remedy from plant sources. Bioactive phytochemicals were profiled and the efficacy of *Diospyros mespiliformis (DM)* stem-bark extracts was evaluated against carbon tetrachloride (CCL_4) induced oxidative stress in mice. Diospyros mesopiliformis have been reported for various activities such as antioxidant, antimicrobial, antiiflammatory. Based on the previous reports it was anticipated *D. mesopiliformis* will ameliorate CCL4 induced liver damage in murine model. Metabolite profile of the extract was obtained using LC-MS/MS. Liver marker enzymes and oxidative stress biomarkers in serum and liver tissue were assessed. Liver tissues and homogenates were assessed for histopathology and estimation of MDA, catalase, superoxide dismutase, and Glutathione S-Transferase activities, qRTPCR mRNA expression of hepatic tissue Glutathione S-Transferase was performed. Free radical scavenging activity of the extract were assessed using DPPH quenching assay, FRAP and peroxide test. *In-silico* molecular modelling was also conducted. Alkaloids, amino acids, anthraquinone, nucleoside derivative, phenolic acid derivatives, fatty amide, flavonoid, and vitamin B12 were detected The CCL_4 caused drastic weight loss in mice, induced liver damage through elevated marker enzymes and bilirubin, reduction in total protein and albumin as well as significant decrease in tissue catalase, GST and superoxide dismutase activities and also marked increase in serum MDA level. Glutathione-S transferase mRNA expression of hepatic tissue and histopathological results also supported the biochemical findings. Extract exhibited concentration dependent free radical scavenging activity. Molecular modelling data corroborates that some of the bioactive compounds may possess the potential to enhance the activities of the antioxidant enzymes. Conclusion: The *DM*stem-bark extract was beneficial in modulating the alterations induced in liver and serum variables of mice under the effect of CCL_4.

* *Corresponding author.*
E-mail address: hghamzah@gsu.edu.ng

https://doi.org/10.37934/scbtrj.4.1.4167b

1. Background

Liver is the main organ responsible for the biotransformation, detoxification, secretions and excretions of various exogenous and endogenous metabolites including xenobiotics [1]. It plays a vital role in maintaining cellular homeostasis and protects the body against deleterious effect of drugs and toxins. Thus, the healthy functioning of the organs defines the health status of the individual [2]. However, during these protective roles the liver is prone to numerous diseases and disorders from toxic chemicals, drugs and other agents due to its unique metabolic roles and the proximal affiliation with the gastrointestinal tract [3].

Hepatic diseases are all-inclusive predicament often involving free radicals induced oxidative stress, if left untreated may advance from steatosis to chronic hepatitis, fibrosis and hepatocellular carcinoma [4]. The conventional drugs commonly used in treatment and management of hepatic diseases are associated with harmful effects [5]. Thus, considerable attentions have been given towards finding alternative, less toxic and effective antioxidants and hepato-curative agents from natural product for treatment and management of diseases affect the liver [6].

Carbon tetrachloride (CCl_4) is a xenobiotic used to induce chemical hepatitis and liver injuries in experimental animals. Carbon tetrachloride-induced liver injuries are the most common experimental model for monitoring the hepatoprotective activity of some drugs. A single exposure to CCl_4, being a strong hepatotoxic could directly leads to severe liver necrosis and steatosis [7]. The major metabolites of CCL_4 are trichloromethyl (CH_3CCl_3·) and trichloromethyl peroxyl ($CH_3CCl_3O_2$·) free radicals, besides they are extremely reactive and are capable of covalently binding to cellular macromolecules such as DNA, protein and fatty acids of the membrane phospholipids. The free radicals induce cell membrane's lipid peroxidation via disrupting polyunsaturated fatty acids within these membranes, initiating a sequential free radical chain reaction [8].

Diospyros mespiliformis (DM), commonly called Jackal berry or African ebony, belongs to the plant family, Ebenaceae. It is one of the most widely distributed African trees and it grows in Nigeria under wider range of conditions. Some native names of *DM* include; Hausa: Kanya, Yoruba: Igidudu, Fulani: Nelbi, Kanuri: Bergem, Arabic: Jukham. The fruit of this plant is a traditional food of high nutritive value in Africa [9].

Ethno-pharmacological applications of different parts of the plant has been reported, the roots and stem-barks are utilized as remedy for inflammation and swelling, pneumonia, leprosy syphilis and diarrhoea [10]. The leaf extract is used against fever and syphilis, as an antidote to variety of poisonous substances. The stem extract was reported to exhibits anti-plasmodial and immunomodulatory effects [11]. Also, the antimicrobial and anti-inflammatory activities of DM leaves have been documented [12]. *Diopyrus mespiliformis* stem bark was shown to have a significant antioxidant and antidiabetic potential, due to the presence of compounds that might be a source of novel therapeutics against oxidative stress and diabetes [13]. In another studies *DM* stem-bark extract ameliorated CCl_4 induced anaemia and hyperlipidemia in mice [14].

Based on alleged therapeutic value of *DM* in folklore medicine *and* the scarcity of data regarding hepatoprotective activities of this plant. We postulate DM stem-bark extract could be rich in phytochemicals with therapeutic values and also has the potential to mitigate CCL4-induced oxidative injury in mice liver by modulating the activities of liver marker enzymes and oxidative stress biomarkers. Therefore, this study is an attempt to evaluate, chemical profile as well as the hepatoprotective efficacy and antioxidant potentials of *DM*stem-bark in an *in-vivo* murine model as well as *in-vitro* antioxidant experiments.

2. Methods

2.1 Plant Collection and Extract Preparation

Fresh stem bark of *DM* was collected in Bojude, Gombe State, Nigeria. The plant was identified and authenticated at the herbarium unit of the Department of Botany with a voucher specimen No. 202 and deposited at the herbarium. Sample was air dried at room temperature in the laboratory and pulverised to coarse powder using mortar and pestle. Two hundred (200) grams of the powdered stem bark was stirred in one litre of 70% ethanol and allowed to stay for fourteen days. The mixture was then filtered using Whatman no. 1 filter paper and the resultant decoction was evaporated using rotary evaporator. The powder was kept in the desiccator until use.

2.2 Sample Preparation and UHPLC-MS/MS Analysis

The ethanol extract (2 mg) was dissolved in LCMS-grade methanol (1 mL), and was vortexed for 10 min, centrifuged for 10 min and filtered through a nylon filter (0.22 µm) into a glass vial for LC-MS/MS analysis.

The crude methanol extract was separated using a C18 Reversed-phase Hypersil GOLD Q column (100 × 2.1 mm ~1.9 µm) (Thermo, Waltham, MA, USA) at 30 ◦C on Dionex Ultimate 3000 UHPLC with a diode-array DAD-3000 detector (Thermo Fisher Scientific, Waltham, MA, USA). Gradient elution was performed with LC-MS grade solvent A (0.1% formic acid and 10 mMol ammonium formate in 500 mL methanol (70%) and acetonitrile (30%)) and Solvent B (0.1% formic acid and 10 mMol ammonium formate in 500 mL water) for the following gradient: 20% A in 5 min, and 20–80% A in the next 25 min at a flow rate of 0.2 mL/min. The concentration of sample extract was 1 mg/mL and the injection volume was set to 10 µL and the UV detector was set at 210, 310, 410 and 510 nm. The MS analysis was done on Q-Exactive Focus Orbitrap LC-MS/MS system. The eluent was monitored by ESI-MS under positive and negative switching mode and scanned from *m/z* 100 to 1500 amu. ESI was conducted using a spray voltage of 4.2 kV. High purity nitrogen gas was used as dry gas at a sheath gas flow rate of 40 (arbitrary units) and aux gas flow rate of 10 (arbitrary units).

Capillary temperature and aux gas heater temperature were set at 350°C and 10°C respectively. The MS data analysis and data literature were conducted using Thermo-X calibur 2.2 SP1.48 (Thermo Fisher Inc. Waltham, MA, USA). The mass spectrometry molecular networks were created using the Global Natural Products Social Molecular Networking (GNPS) platform (http://gnps.ucsd.edu) [15].

The MS data were converted into mz XML format using MS Convert. Spectral information generated was uploaded on GNPS using FileZilla and was used to generate an MS/MS molecular network using the GNPS Data Analysis Workflow. The precursor ion mass tolerance and a fragment ion mass tolerance were set to 0.02 Da. The fragment ions below 10 counts were removed from the MS/MS spectra. The MN were generated using 6 minimum matched peaks and a cosine score of 0.7. The resulting data were downloaded and visualized using Cytoscape 3.7.1 software (Institute of Systems Biology Seattle, Washington D.C., USA).

2.3 Animal Maintenance

Healthy albino mice were procured from NITR Jos, Plateau state Nigeria. The animals were housed in clean plastic cages under standard laboratory condition of 12 hours light-dark cycle, temperature 25±2°C, humidity 55±5%. They were fed with standard animal pellets (vital feeds Jos Nigeria) and clean tap water, then kept for one week to acclimatized. Prior to the commencement of the experiment an approval was obtained from animal ethic committee for the study protocol

approved by GSU Research ethic committee. Ethical approval: The ethics of the animal study was approved by the Gombe State University (GSU) Institutional Animal Care and Use Committee (IACUC/GSU-R02/2023).

2.4 In-Vivo Hepatoprotective Activity

Determination of acute toxicity (LD_{50}). Median lethal dose (LD_{50}) was determined for evaluating the safety of *DM*stem-bark using slightly modified procedure reported by [16] and ten albino rats were used for the study. Table 1 shows the experimental design of the study.

At the initial stage four animals were divided into 4 groups of a mouse each. Then different doses (1000-2000mg/kg b.wt) of the extract were administered to the mice and were observed for 1 hours post-administration and then 10 minutes every 2 hrs interval for 24 hrs. The behavioural signs of toxicity and also mortality were observed. No mortality was recorded at this stage, the testing was proceeded to the next stage.

Three animals were divided into three groups of a mouse each. Different doses of the extract (2000-3000mg/kg b.wt) were given orally to the animals and then observed for 1 hour after administration and periodically for 24 hours. Behavioural signs of toxicity were noted and mortality as well. No sign of toxicity or death were observed.

This stage three animals which were distributed into three groups of a mouse each. Doses of extract (3500- 4000 mg/kg as the highest) were administered to the different mice and was observed for 1 hour after administration and then 10 minutes every 2 hours for 24 hours. Behavioural toxicity signs and also mortality was recorded. This was the final stage of testing and no death was perceived, then the LD50 of the extract is greater than 4000 mg/kg. Therefore, *D.mespiliformis* stem bark extract was considered safe up to 4000 mg/kg b.wt.

Table 1
Experimental design of the study

Group	Treatment
Group IA: Normal control	Administered only distilled water orally three times weekly and 0.5ml/kg bodyweight (BW) olive oil intraperitoneally (IP) twice weekly
Group IB: Extract control	Administered 400 mg/kg BW *DM* stem bark ethanol extracts dissolved in distilled water three times weekly
Group II (CCl_4): Hepatotoxic control	Received IP injection of 0.5ml/kg BW CCl_4 in olive oil (1:1 v/v) twice per week.
Group III (Silymarin): standard control	Administered 100 mg/kg BW silymarin was given intragastrically for 5 days/week and IP injection of 0.5ml/kg BW CCl4 in olive oil (1:1 v/v) twice per week.
Group IV: Test I	The extract at a dose of 200 mg/kg BW, orally was given daily then after 1 hour received IP injection of 0.5ml/kg BW CCl4 in olive oil (1:1 v/v) twice per week
Group V: Test II	The extract at a dose of 400 mg/kg BW, orally was given daily then after 1 hour received IP injection of 0.5ml/kg BW CCl4 in olive oil (1:1 v/v) twice per week

A total of thirty albino mice (n = 36), were randomly divided into six groups (n = 6) a following treatments were completed in four (4) consecutive weeks.

2.5 Biochemical Assays

Serum Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), total protein, total bilirubin and albumin, using Reagent kit by Randox and Agappe according to the manufacturer's instructions.

2.6 Estimation of Oxidative Stress Markers

In-vivo oxidative stress biomarkers Glutathione S- transferase, Catalase, Superoxide dismutase and Lipid peroxidation (MDA) level in liver tissue was assayed using ELISA kit (Beijing Solarbio Science and Technology Co., Ltd.) in accordance with the manufacturer's instructions.

2.7 Estimation of Glutathione S Transferase Mrna Expression

The hepatic tissue GST mRNA expression was assayed using qRT PCR kit. QuickExtract RNA Extraction Kit by PrimeScript™ RT reagent kit; United Kingdom was used to target and extract RNA, and qRTPCR kit SolarbioScience and Technology Co., Ltd Beijing was used for GST mRNA expression. All protocols were performed in accordance with the manufacturer's instructions.

2.8 Histopathological Study of Liver Tissues

The effect of *DM*stem-bark extract was evaluated on the CCL4-induced liver injury using hemotoxylin and eosin (H and E) staining [17]. The liver tissue was isolated, embedded in the paraffin, fixed and dehydrated with the alcohol. The paraffin embedded sample were sectioned in the 3-4µm, deparaffinized, rehydrated and the stained with the H and E stain. The histology images were assessed under light microscope with 10x magnification and were quantified using Image j software 1.8_172 (NIH, USA) as reported [18].

2.9 In-vitro Antioxidant Assays

Determination of Ferric Reducing Antioxidant Power: To 1ml of sample, 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%) were added and mixed. The reaction mixture was incubated at 50˚C for 20 minutes. Then 2.5 ml of trichloroacetic acid (10%) was added and centrifuged for 10 minutes. An aliquot 2.5 ml was mixed with 2.5ml of distilled water and 0.5 ml of $FeCl_3$ (0.1%). The absorbance of all solution was measured at 700 nm and expressed as mg of ascorbic acid equivalent per g of extract (mg AAE/g extract) [19].

DPPH radical method: The free radical scavenging activity was measured by using 2, 2-diphenyl-1-picryl- hydrazyl or 1, 1-diphenyl-2- picrylhydrazyl as previously described by [20]. The reaction mixture consists of 1.0 ml of DPPH in 0.3mM methanol and 1.0ml of the extract. After incubation for 10 min in dark, the absorbance was measured at 517 nm. DPPH scavenging activity was expressed in terms ascorbic acid equivalent. The ability of extract to scavenge the DPPH radical was assessed using the formula;

$$DPPH\ Scavenging\ \text{activity} = \frac{\text{Ao}-\text{A1}}{Ao}\ x\ 100\%$$

where:

Ao is the absorbance before reaction and A1 is the absorbance after reaction has taken place.

Hydrogen Peroxide Radical Scavenging Activity: Hydroxyl radical scavenging activity was determined by the method of Sarma *et al.,* [21]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Different concentrations of the extract (20-60 mg/ml) in phosphate buffer were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing phosphate buffer without H_2O_2 was used.

The percentage $H2O2$ scavenging activity of the extract, Ascorbic acid as standard compound was calculated.

$$\% \text{ H2O2 Scavenged} = \frac{\text{A}o - \text{A1}}{Ao} \; x \; 100$$

where Ao is the absorbance of control A1 is the absorbance of test.

Total Antioxidant Capacity/Phosphomolybdate assay: The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. Three (0.3) ml of the extract at concentration of 25 µg/ml, 50 µg/ml, and 10 µg/ml, 3ml of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was added. For the blank, 0.3ml of methanol was used in place of the extracts. The tubes containing the reaction solution were capped and incubated in a boiling water bath at 95˚C for 90min. after cooling to room temperature; the absorbance of the solution was measured at 695 nm using spectrophotometer. The antioxidant capacity of each sample was expressed as ascorbic acid equivalent [22].

2.10 Molecular Modelling

The molecular docking study was performed to assess the interaction of the phytochemical ligands with the antioxidant protein biomarker as molecular targets. The Swiss Target Prediction version-2019 was used to study the interaction of the phytochemical ligands which are the dominantly active compounds in extracts from *DM* Stem-Bark (e.g 3-amino-1,2,4-triazole Glutathione, etc) with catalase, Glutathione-S transferase and superoxide dismutase. The crystal structures of catalase complexed with 3-amino-1,2,4-triazole (PDB ID: 1DGH) [24], superoxide dismutase (SOD) from ***Aeropyrum pernix*** K1 (PDB ID: 3AK3) [23], and glutathione S-transferase (GT) from ***Schistosoma mansoni*** (PDB ID: 1U3I) were retrieved from the protein data bank (https://www.rcsb.org/). These proteins were prepared using Maestro protein preparation wizard (Schrodinger Inc) in which bonds were optimized, titratable residues were assigned proper protonation states using the predicted pKa values. The three-dimensional structures of all ligands were constructed using Biovia Discover Studio visualize (https://discover.3ds.com/discovery-studio-visualizer-download) and prepared using the Maestro ligprep toolkit. A receptor grid file was generated using the cocrystal ligand in each crystal structure, except for SOD (which lacks a cocrystal ligand). Docking was carried out using the Glide extra precision (XP) docking scoring function [25]. All MD simulations were carried out using Desmond, employing a simulation protocol fully described in our previous study [26]. The root-mean-square displacement (RMSD), root-mean-square fluctuation (RMSF), protein-ligand interaction timeline, and Molecular Mechanism-General Born Surface Area (MM-GBSA) (MMGBSA) ligand binding affinity analyses were carried out on the simulation trajectories [27].

2.11 Statistical Analysis

One-way analysis of variance (ANOVA) was used (SPSS, Version 20.0). Data was reported as mean± standard deviation. P value of < 0.05 was chosen for the statistical significance with control group.

3. Results

3.1 Detection of Metabolites by Using LC-MS/MS Molecular Networking Analysis

The extract was analysed using modern LC-MS/MS operated in positive ion mode. Metabolites of the extract were cautiously outlined based on their molecular mass, fragmentation pattern, and with comparison to global natural products molecular social networking (GNPS) database. Fifteen (15) were identified compounds, their structures were apportioned as five alkaloids, two amino acids and peptides, one anthraquinone, one nucleoside derivative, three phenolic acid derivatives, one fatty amide, one flavonoid, one vitamin B12. The identified compounds were listed in Table 2 and the chromatograms of *D. mesiliformis* stem bark extract in positive ion mode were depicted in Figure 1.

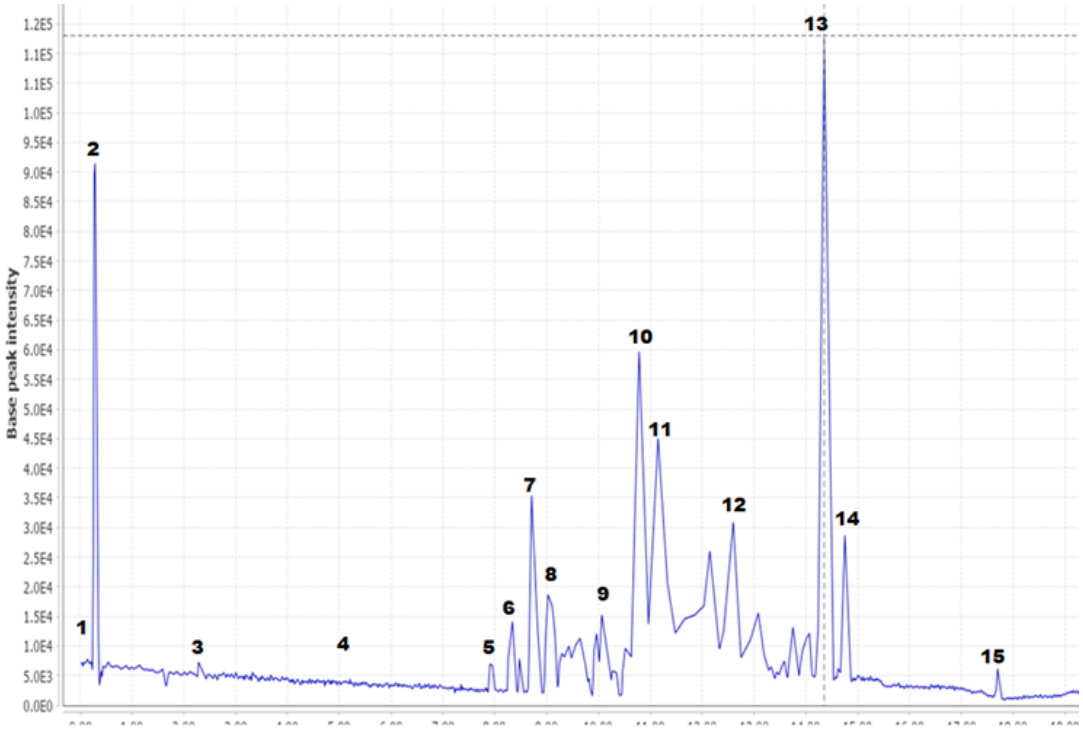

Fig. 1. Total scan PDA chromatogram *DM* stem-bark. The number of peaks are in positive ion mode represent the identified compounds

Table 2 shows the phytochemicals detected in *DM*stem-bark extract. Alkaloids were represented by five compounds from peak 1 to 5. Peak 1 corresponds to 5-Methoxydimethyl-tryptamine identified by ion *m/z* $[M+H]^+$ 219.49 with molecular formula $C_{13}H_{18}N_2O$, Peak 2 was 2,6-diamino-1,7-dihydro-8H-imidazole [4,5-] quinazolin-8-one identified by ion *m/z* (MH^+) 217.081 with molecular formula $C_9H_8N_6O$, peaks 3, 4 and 5 correspond to the following compounds respectively xestomanzamine B, identified by ion *m/z* $[M+H]^+$ 279.124 with molecular formula $C_{16}H_{14}N_4O$, calcimycin, identified by ion *m/z* $[M+H]^+$ 524.275 with molecular formula $C_{29}H_{37}N_3O_6$ and visoltricin identified by ion *m/z* $[M+H]^+$ 235.141 with molecular formula $C_{13}H_{18}N_2O_2$.

Polyphenols were detected and represented by peaks, 6, 7 & 8. These peaks respectively correspond to maniwamycin A identified by ion *m/z* $[M+H]^+$ 199.145 with molecular formula $C_{10}H_{18}N_2O_2$, phomanone A identified with ion *m/z* $[M+H]^+$ 173.062 with molecular formula $C_8H_{12}O_2S$ and Acremofuranone A identified by *m/z* $[M+H]^+$ 421.178 with molecular formula $C_{23}H_{29}ClO_5$.

In present study, one flavonoid was detected, which was annotated peak 9. This peak corresponds to 8-C-Methylvelloquercetin 3,5,3'-trimethyl ether, identified with *m/z* $[M+H]^+$ 425.16 with molecular formula $C_{24}H_{24}O_7$.

Two amino acids were detected which was represented in peaks 11 and 12 as tripeptides and amino acid analogues respectively. Those peaks correspond to Asp-His-Lys identified with *m/z* $[M+H]^+$ 399.199 with formula $C_6H_{26}N_6O_6$ and Ndelta-(N'-sulphodiamino-phosphinyl)-L-ornithine identified with *m/z* $[M+H]^+$ 291.052 with formula $C_5H_{15}N_4O_6PS$. Anthraquinone, Vitamin B precursor and Fatty amide were also detected, which were represented by peaks 13,14 and 15 respectively. Peak 13 corresponds to Penicillanthranin A identified with *m/z* $[M+H]^+$ 537.137 with molecular formula $C_{28}H_{24}O$. Peak 14 corresponds to Percorrin 6B identified with *m/z* $[M+H]^+$ 897.228 having a molecular formula $C_{44}H_{56}N_4O_{16}$. Peak 15 corresponds to Hexadeca-2E,9Z,12Z,14E-Tetranoic acid isobutylamide identified with *m/z* $[M+H]^+$ 304.264 having a molecular formula $C_{20}H_{33}NO$.

Table 2
LC-MS analysis of *DM*stem-bark extracts showing the detected peaks and their annotated compounds

Peak	*m/z* (M^+ H)	Molecular formula	Compound name	Systematic name	Compound class
1	219.149	$C_{13}H_{18}N_2O$	5-Methoxydimethyl-tryptamine	[2-(5-methoxy-1H-indol-3-yl)ethyl]dimethylamine	Alkaloid
2	217.081	$C_9H_8N_6O$	2,6-diamino-1,7-dihydro-8H-imidazole [4,5-] quinazolin-8-one	2,6-diamino-1H,7H,8H-imidazo[4,5-g]quinazolin-8-one	Alkaloid
3	279.124	$C_{16}H_{14}N_4O$	Xestomanzamine B	4,9-dihydro-3H-pyrido[3,4-b]indol-1-yl-(3-methylimidazol-4-yl)methanone	Alkaloid
4	524.275	$C_{29}H_{37}N_3O_6$	Calcimycin	5-(methylamino)-2-[[(2S,3R,5R,8S,9S)-3,5,9-trimethyl-2-[1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undecan-8-yl]methyl]-1,3-benzoxazole-4-carboxylic acid	Alkaloid
5	235.141	$C_{13}H_{18}N_2O_2$	Visoltricin	Methyl (E)-3-[3-methyl-5-(3-methylbut-2-enyl)imidazol-4-yl]prop-2-enoate	Alkaloid
6	199.145	$C_{10}H_{18}N_2O_2$	Maniwamycin A	[(E)-hex-1-enyl]-oxido-[(2S)-3-oxobutan-2-yl]iminoazanium	Polyphenol
7	173.062	$C_8H_{12}O_2S$	Phomanone A	(4R)-2-(hydroxymethyl)-3-methyl-4-methylsulfanylcyclopent-2-en-1-one	Polyphenol
8	421.178	$C_{23}H_{29}ClO_5$	Acremofuranone A	3-Chloro-5-[(E)-7-((S)-5,5-dimethyl-4-oxo-tetrahydro-furan-2-yl)-3-methyl-octa-2,6-dienyl]-4,6-dihydroxy-2-methyl-benzaldehyde	Polyphenol
9	425.16	$C_{24}H_{24}O_7$	8-C-Methylvelloquercetin 3,5,3'-trimethyl ether	7-(4-hydroxy-3-methoxyphenyl)-4,6-dimethoxy-9-mrthyl-2-prop-1-en-2-yl-2,3-dihydrofuro[3,2-g]chromen—one	Flavonoid
10	443.044	$C_{10}H_{16}N_6O_1P_2$	Aminophosphonic acid-guanylate ester	{[amino(hydroxy)phosphoryl]oxy}({[2R,3S,4R,5R)-5-(2-amino-6oxo-6,9-dihydro-3H-purin-9-yl)-3,4-dihydroxyoxolan-2-yl]methoxy})phosphinic acid	Nucleosides, nucleotide analogues
11	399.199	$C_6H_{26}N_6O_6$	Asp-His-Lys	L-Aspartyl-L-histidyl-L-lysine	Amino acid, peptide and analogue

12	291.052	$C_5H_{15}N_4O_6PS$	Ndelta-(N'-sulphodiamino-phosphinyl)-L-ornithine	(2S)-2-amino-5-{[(R)-amino(sulfoamino)phosphoryl]amino}pentanoic acid	Amino acid, peptide and analogue
13	537.137	$C_{28}H_{24}O$	Penicillanthranin A	(1S,3R,4S)-6,8-dihydroxy-3,4,5-trimethyl-1-[1,3,8-trihydroxy-6-(hydroxymethyl)-9,10-dioxoanthracen-2-yl]-3,4-dihydro-1H-isochromene-7-carboxylic acid	Anthraquinone
14	897.228	$C_{44}H_{56}N_4O_{16}$	Percorrin 6B	3-[(1R,2S,3S,4Z,7S,11S,17R,18R)-8,13,17-tris(2-carboxyethyl)-2,7,12,18-tetrakis(carboxymethyl)-1,2,7,11,17-pentamethyl-3,10,15,18,19,21-hexahydrocorrin-3-yl]propanoic acid	Vitamin B12 precursor
15	304.264	$C_{20}H_{33}NO$	Hexadeca-2E,9Z,12Z,14E-Tetranoic acid isobutylamide	(2E,9Z,12Z,14E)-N-(2-methylpropyl)hexadeca-2,9,12,14-tetraenamide	Fatty amide

3.2 In-vitro Antioxidant Assays

3.2.1 Ferric reducing antioxidant power assay

FRAP is one of the most significant antioxidant mechanism that reproduces the reducing power of antioxidant molecules. Using this assay, *DM*extract exhibit marked antioxidant activity. The IC_{50} concentration of *DM*stem-bark extracts (0.52mg/ml) showed significant ferric chelating ability paralleled to that of ascorbic acid (0.45mg/ml). The results showed a concentration-dependent significant increase in the reductive ability of the test samples.

3.2.2 Hydrogen Peroxide (H_2O_2) radical scavenging activity

Hydroxyl radicals are extremely reactive free radicals formed in the biological systems. The scavenging ability of *D.mespliformis* stem-bark at a concentration of 0.1 mg/mL, was found to be 79% compared to that of standard ascorbic acid at 82%. In addition, the IC_{50} concentration was found to be 0.49 mg/mL compared to that of standard ascorbic acid 0.45mg/mL.

3.2.3 DPPH radical scavenging activity

The radical scavenging activities of *D.mespliformis* stem-bark extract was assessed by measuring the decrease in absorbance of DPPH in the presence or absence of the extract in the assay mixture in which ascorbic acid solution was used as a standard. The extract showed potent scavenging activity against DPPH radical with IC_{50} concentration of 0.51mg/ml compared to that of ascorbic acid (0.45mg/ml). The percentage scavenging activity of the extract on the DPPH radical was concomitantly increased with increase in extract concentration (Figure 2).

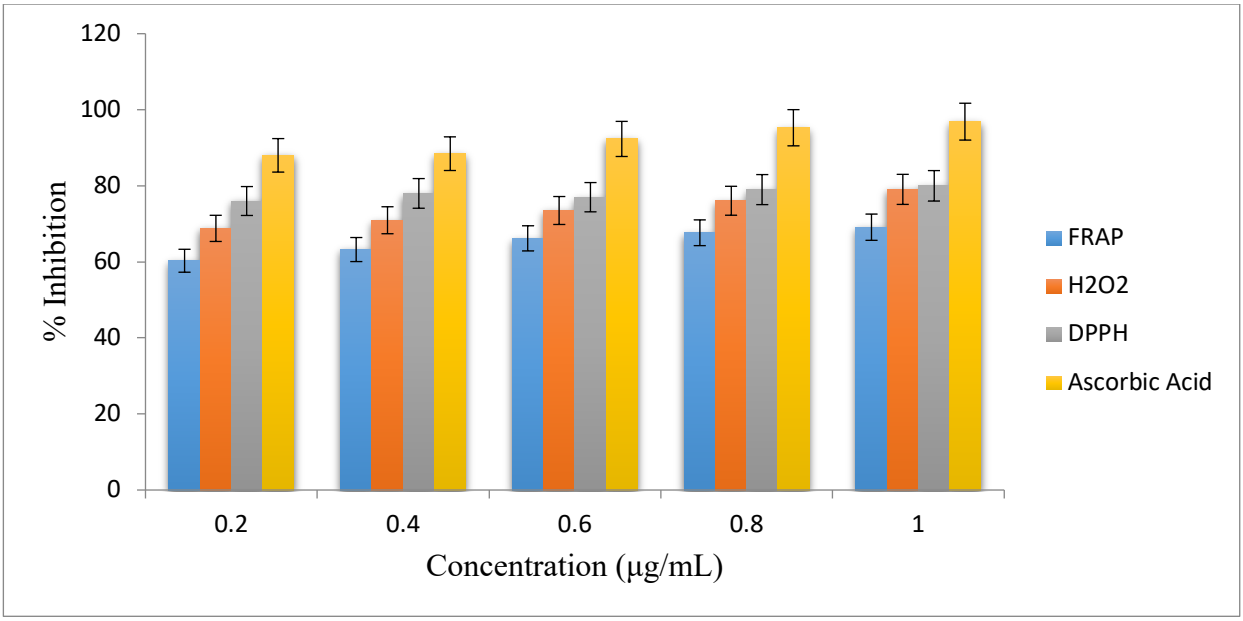

Fig. 2. *In-vitro* antioxidant activity of *DM*extract using different antioxidant models using ascorbic acid as standard. Data obtained from representation of three independent experiments

3.3 In-vivo Assays

3.3.1 Effect of the DMethanol extract on the acute toxicity

The extract did not exhibit any sign of toxicity at the dose of 400 mg/kg body weight (the highest concentration used in present study) and no mortality was observed in all the recruited groups.

3.3.2 Effect of D.mespiliformis stem-bark extract treatment on the body weight and liver weight

The variation in both body and liver weights were examined to assess the impact of the extract treatment on the overall changes following liver injury induction with the CCL_4. The general body weights were assessed on weekly basis, while the liver weight was measured at the end of the experiment. The *D.mespiliformis* treatment markedly improved the body weight in contrast to the negative control.

Similarly, following injection of the CCl_4, a marked increase in liver weight was observed (Figure 3), however, the extract treatment decreased the liver inflammation and hence subsequently the liver weight was strikingly reduced in the extract and silymarin treated groups.

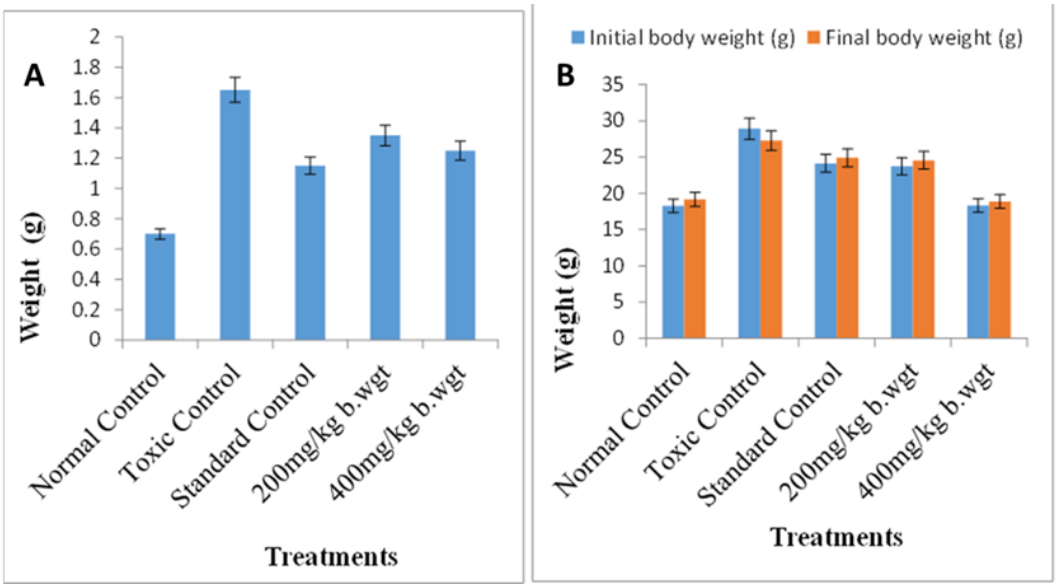

Fig. 3. Histogram plots showing effect of *DM*ethanol extract on (A) liver tissue weight (B) body weight in CCl_4 induced oxidative stressed albino mice

i. *D.mespiliformis* stem-bark extract reduces serum level of ALT and AST in CCL_4 induced oxidative stress in albino mice.

ii. Administration of 0.5ml of CCL_4 in olive oil (1:1v/v) twice weekly for 28 days to albino mice caused an increase ($P < 0.05$) in serum level of ALT and AST activities compared to the control. However, the group that received 100mg/kgBW silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract, a significant reduction ($P < 0.05$) in serum level of ALT and AST was observed compared to CCl_4 treated (Figure 4A&B).

iii. *DM* stem-bark extract ameliorates the CCL_4 induced decrease in serum level of Albumin and Total protein in albino mice.

iv. A significant reduction ($P < 0.05$) in serum level of albumin and total protein were observed in CCl_4 induced group compared to the control. But in groups that received 100mg/kg BW silymarin, 200 mg/kg BW, and 400 mg/kg BW of the extract, a significant increase ($P < 0.05$) in serum level of albumin and total protein were observed compared to CCl_4 induced group (Figure 4C&E).

v. Effect of *DM* stem-bark extract on serum level of total bilirubin in CCL_4 induced oxidative stress mice.

vi. A significant increase ($P < 0.05$) in serum level of total bilirubin was observed in CCl_4 group compared to the normal control. But in groups that received 100mg/kg body weight silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract, a significant reduction ($P < 0.05$) in serum level of total bilirubin was observed compared to CCl_4 induced group (Figure 4D).

vii. A marked decrease in hepatic Glutathione S transferase activity and GST mRNA expression in CCl_4 induced oxidative stress albino mice was reversed by *DM* extract treatment.

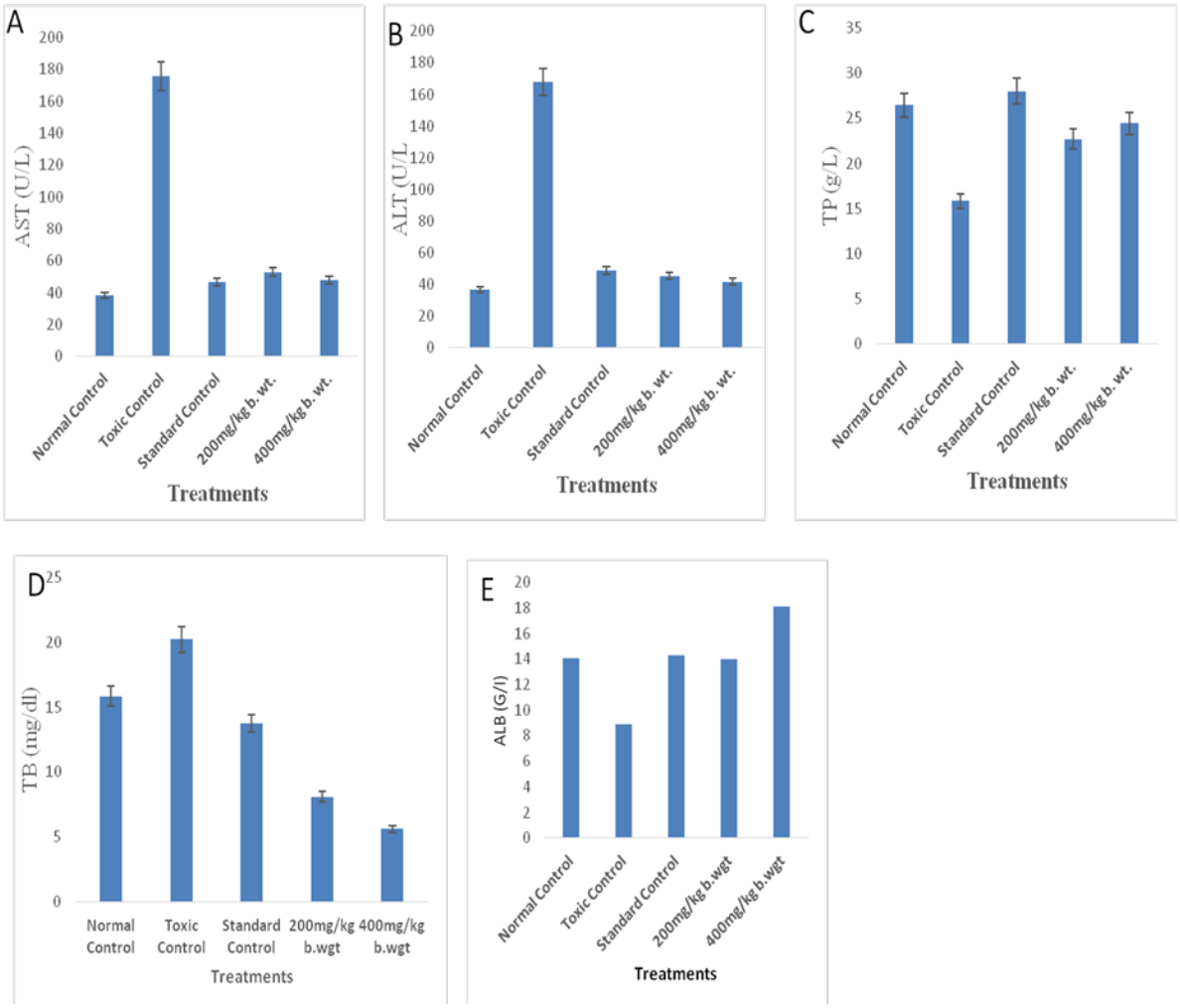

Fig. 4. The effect of DMethanol extract on serum level of (A) AST (B) ALT (C) TP (D) TB (E) ALB in CCl_4 induced oxidative stress in albino mice. All values are represented as mean ± standard error of mean (SEM) of six different replicates

A significant reduction ($P < 0.05$) in liver GST activity was observed in CCl_4 group compared to the normal control. But in groups that received 100mg/kg body weight silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract, a significant increase ($P < 0.05$) in liver GST activity was observed compared to CCl_4 group (Figure 5A). This effect was evident with a significant ($p < 0.05$) increase in GST mRNA expression in groups that received 100mg/kgBW silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract compared to CCl_4 induced group. GST mRNA expression data is representing the relative mRNA level of GST performed in 45 cycle of PCR. A strong positive GST expression was detected in normal control. A significant decrease ($P < 0.05$) in mRNA expression of GST was observed in CCl_4 group compared to the normal control. Also, there is a significant difference ($P < 0.05$) in groups that received 100mg/kg body weight silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract compared to CCl_4 group. No significant difference ($P < 0.05$) was observed between group that received 100mg/kg body weight silymarin, 200 mg/kgBW of extract and the group that received 400 mg/kg body weight of the extract (Table 3).

i. *DM* extract up-regulates hepatic catalase and superoxide dismutase activities in CCl_4 induced oxidative stress mice.
ii. Liver catalase and superoxide dismutase markedly reduced in response to CCl_4. However, administration of 100mg/kg BW silymarin, 200 mg/kg BW, and 400 mg/kg BW of the

extract caused a significant increase ($P < 0.05$) in liver catalase and superoxide dismutase activities compared to CCl_4 induced group (Figure 5B&C).

iii. Effect of *DM*Stem-Bark Extract on Liver MDA in CCl_4 induced oxidative stressed mice.

iv. A significant increase ($P < 0.05$) in liver MDA was observed in CCl_4 treated group compared to the control. But in group that received 100mg/kg body weight silymarin, 200 mg/kgBW, and 400 mg/kgBW of the extract, a significant decreased ($P < 0.05$) in liver MDA was observed compared to CCl_4 intoxicated group (Figure 5D).

Table 3
Effect of oral administration of *DM*stem bark extract on Glutathione S Transferase mRNA expression in CCl_4 induced oxidative stress in albino mice. All values are represented as mean ± standard error of mean (SEM) of six different replicates. Values with different superscripts down the group are statistically different from normal control at ($P \leq 0.05$)

Groups	GST relative mRNA level
Normal Control	85.730 ± 4.062^{a}
CCl_4 induced	12.165 ± 0.663^{b}
CCl_4 + Silymarin	41.702 ± 5.515^{c}
CCl_4 + 200 mg/kg BW	34.920 ± 3.004^{c}
CCl_4 + 400 mg/kg BW	33.257 ± 6.216^{c}

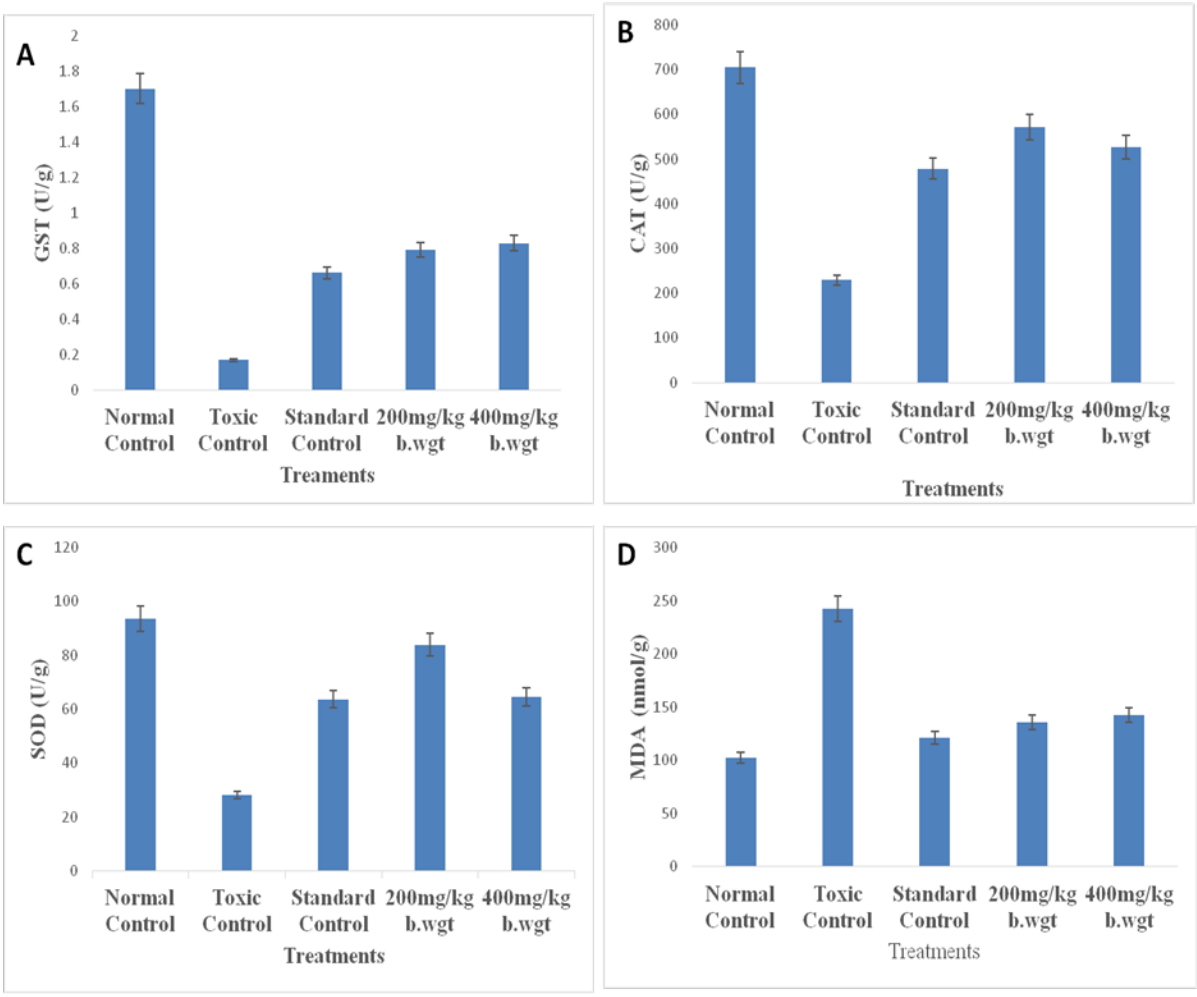

Fig. 5. The effect of extract on liver level of (A) GST (B) CAT (C) SOD (D) MDA in CCl_4 induced oxidative stress in albino mice. All values are represented as mean ± standard error of mean (SEM) of six different replicates

3.3.3 DM stem-bark extract treatment improved histological parameters in CCl_4 treated mice

The Hemotoxylin and Eosin staining was performed to assess the CCL_4 mediated liver damage and determine the effect of the extract treatment on the liver histology. The H and E staining showed that extract treatment significantly improved the histological parameters when compared with the toxic control. Similarly, the positive control also improved the histopathology of the liver (Figure 6C). But, CCl_4-induced histopathological changes in liver were observed in CCl_4 intoxicated group (Figure 6B), including liver steotohepatitis (red arrow) and hepatocyte drop-out and focal necrosis (blue arrow), congestion of the central vein (black arrow) and obvious inflammatory infiltrates in focal areas.

The micrograph from silymarin treated group (Figure 6C) showed slight alteration and restoration of the hepatocytes. The sections from the groups treated with *DM*stem bark extract 200 mg/kg and 400 mg/kg in (Figure 6D&E) and E respectively showed slight alteration and regeneration of the hepatocytes. Focal area of hepatic degeneration was also observed in the liver of the treated group.

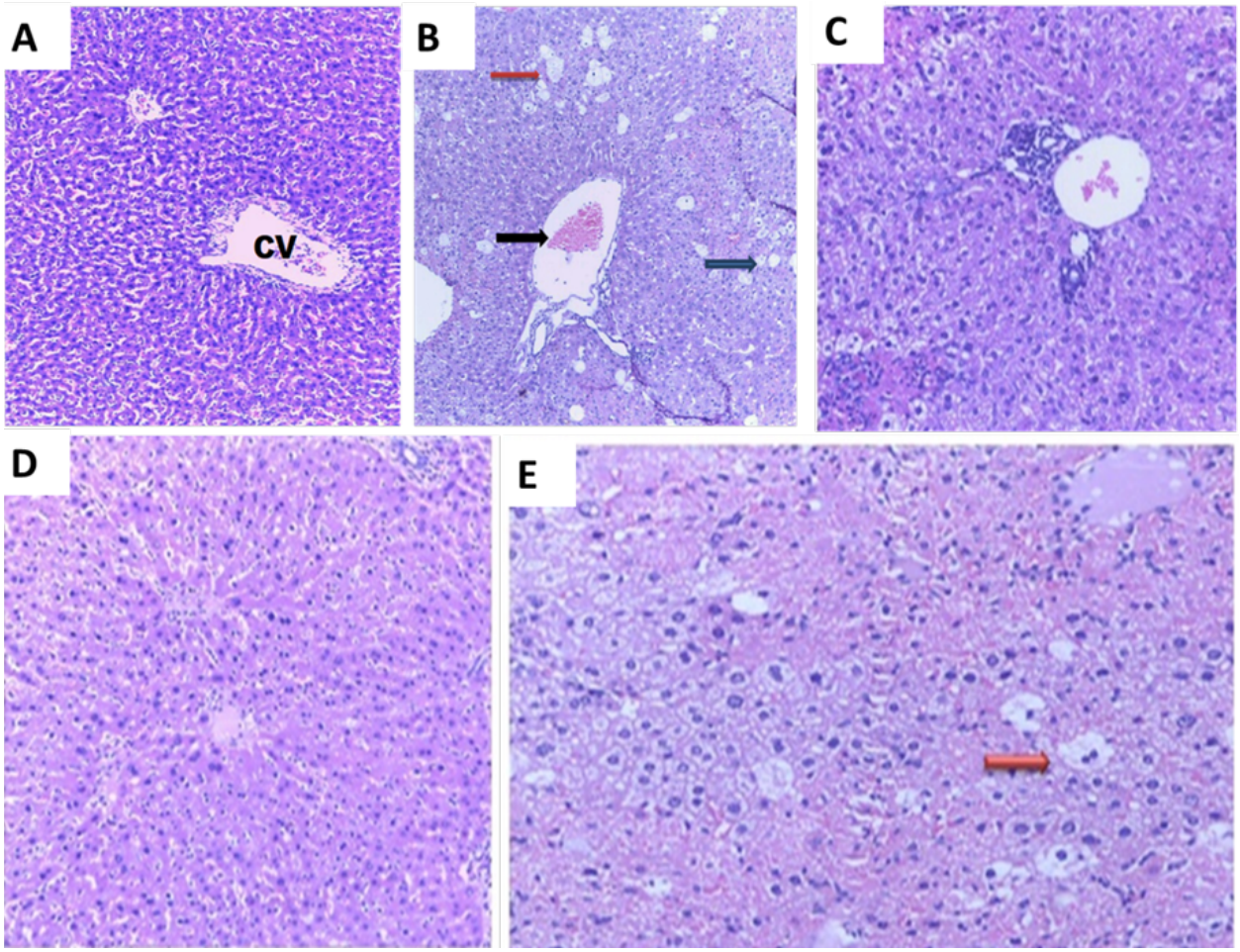

Fig. 6. Photomicrographs of liver from (A) Normal control, (B) Toxic control, (C) Silymarin (100 mg/kg), (D) *DM* stem bark extract (200 mg/kg), (E) *DM* stem bark extract (400 mg/kg). CV: Central vein, Red arrow: Steotohepatitis, Blue arrow: Necrosis, Black arrow: Congestion of the central vein

3.4 Molecular Modelling

In Table 4, the numbers highlighted with yellow labels are the calculated binding energy scores of each compound against catalase, glutathione transferase, and superoxide dismutase. These

compounds showed different binding propensity against the selected antioxidant enzymes. Furthermore, the MM-GBSA binding energy calculations following MD simulations revealed interesting findings as prd in Table 5. The modest binding affinity of compound 11 as indicated in Table 4, a tripeptide; (L-Aesentespartyl-L-Histidyl-L-lysine) against catalase (-13.4 ± 4.0 kcal/mol) compared with the co-crystal ligand (-43.7 ± 7.1 kcal/mol) (Table 5). Similarly, Table 4 shows that compound 10, a nucleotide analogue; (Aminophosphonic acid guanylate ester) displayed weaker binding affinity against glutathione transferase (-30.4 ± 2.0 kcal/mol) compared with the cocrystal ligand (-49.6 ± 3.9 kcal/mol). However, for the superoxide dismutase crystal structure used (PDB ID: 1U3I), a co-crystal ligand was not available, yet compound 12 in the same Table 4, a peptide derivative**; (**Ndelta-(N'-sulphodiamino-phosphinyl)-L-ornithine was found to demonstrate potential binding affinity (-20.0 ± 4.4 kcal/mol) against the enzyme. It is interesting to note that the co-crystal inhibitor of catalase, 3-amino-1,2,4-triazole and the substrate of glutathione transferase, glutathione, bound deeper into and interacted more tightly with the active site of these respective enzymes, thereby demonstrating lower binding energy. The 2D Interaction diagram: (A) docking complex of catalase with compound **11,** (B) docking complex of glutathione transferase with compound 10, (E) docking complex of superoxide dismutase with compound 12 is shown in Figure 7.

Table 4
Docking score of the dominantly active compounds in extracts from *DM* Stem-Bark

Compound number	Compound name	Catalase	SOD	GT
2	**2,6-diazol-1,7-dihydro-8H-imidazole [4,5-] quinazolin-8-one**	-11.24	NA	NA
7	**(4R)-2-(hydroxymethyl)-3-methyl-4-methylsulfanylcyclopent-2-en-1-one**	-4.14	-4.0	-4.08
10	**Aminophosphonic acid guanylate ester**	-10.04	-4.6	-8.23
11	**L-Aspartyl-L-Histidyl-L-lysine**	-10.13	-5.8	-7.54
12	**Ndelta-(N'-sulphodiamino-phosphinyl)-L-ornithine**	-7.06	-7.8	-6.83
13	**(1S,3R,4S)-6,8-dihydroxy-3,4,5-trimethyl-1-[1,3,8-trihydroxy-6-(hydroxymethyl)-9,10-dioxoanthracen-2-yl]-3,4-dihydro-1H-isochromene-7-carboxylic acid**	-4.42	-5.4	-4.66
14	**3-[(1R,2S,3S,4Z,7S,11S,17R,18R)-8,13,17-tris(2-carboxyethyl)-2,7,12,18-tetrakis(carboxymethyl)-1,2,7,11,17-pentamethyl-3,10,15,18,19,21-hexahydrocorrin-3-yl]propanoic acid**	-6.06	-6.7	-7.93

Table 5
MMGSA binding energy scores calculated over 200 ns MD simulation trajectory. The crystal structures of the target enzymes complexed with inhibitors (except for SOD) are highlighted in green

System	VDW	Electrostatics	Hydrophobic	Total binding energy
	MMGBSA binding energy (kcal/mol)			
GT (PDB ID: 1U3I)	-32.9 ± 2.7	-7.3 ± 6.1	-6.3 ± 0.5	-43.7 ± 7.1
GT (PDB ID: 1U3I)_10	-12.5 ± 4.7	1.1 ± 3.5	-1.8 ± 1.0	-13.4 ± 4.0
Catalase (PDB ID: 1DGH)	-45.5 ± 13.0	-4.8 ± 5.7	-9.3 ± 3.1	-49.6 ± 3.9
Catalase (PDB ID: 1DGH)-11	-33.4 ± 2.0	4.3 ± 3.0	-5.2 ± 0.5	-30.4 ± 2.0
SOD (PDB ID: 3AK3)-apo	N/A	N/A	N/A	N/A
SOD (PDB ID: 3AK3)_12	-21.8 ± 1.3	5.9 ± 4.3	-7.3 ± 0.6	-20.0 ± 4.4

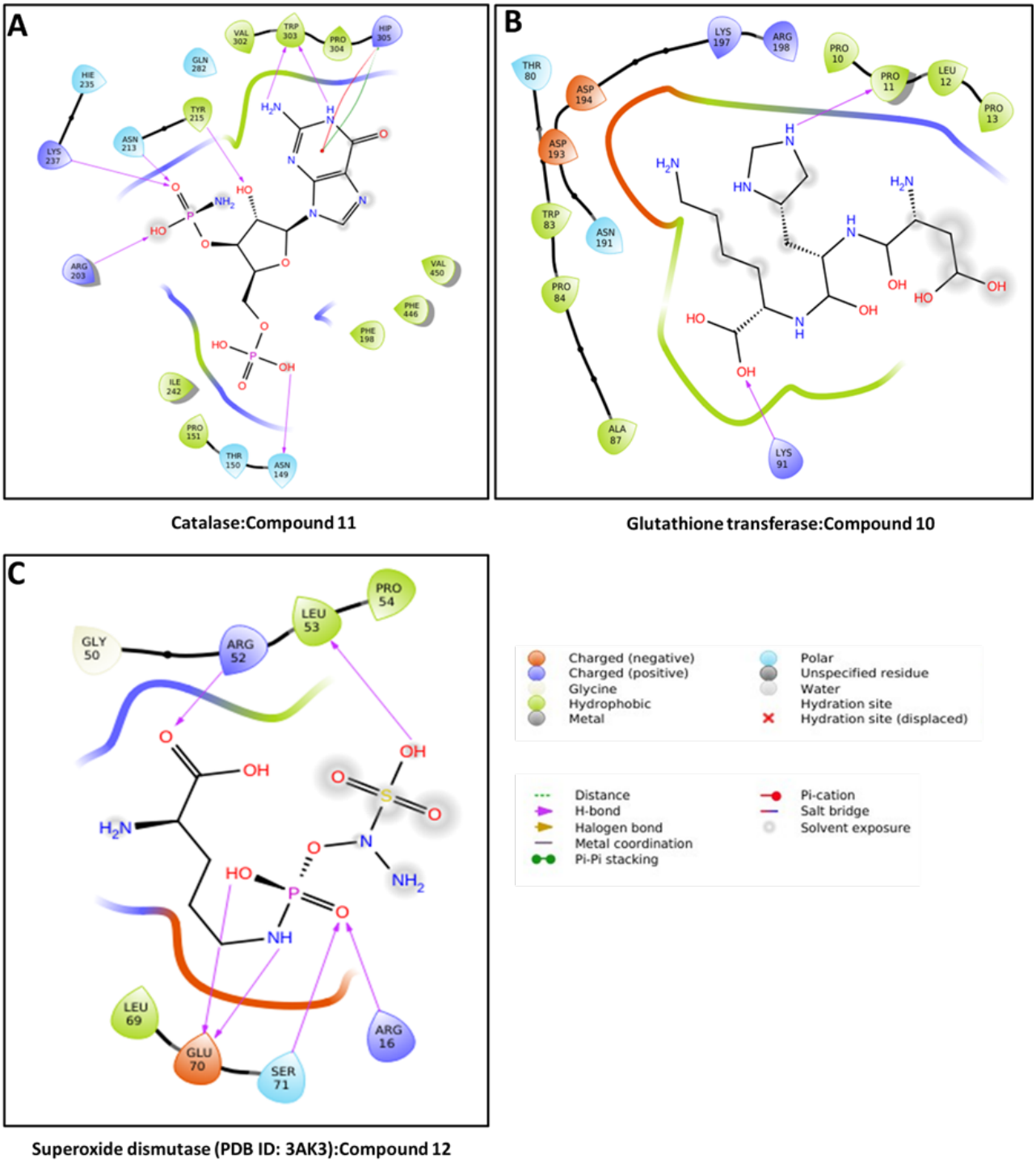

Fig. 7. 2D Interaction diagram: (A) docking complex of catalase with compound 11, (B) docking complex of glutathione transferase with compound 10, (E) docking complex of superoxide dismutase with compound 12

To examine the stability of ligand binding mode, the top docking complexes in terms of binding energy were submitted to MD simulations. RMSD analysis, which measures structural stability of protein and complexes [28] showed that both the catalase crystal structure (Figure 7) and docking complex (Figure 8B) appeared to be stable over time and that the ligand remains bound to the proteins through the simulation. Similar conformational change behaviours were observed with the crystal structure of glutathione transferase (Figure 8C), however, compound 10 appeared to

dissociate from the enzyme and bound again on multiple occasions over time of the simulation. Hence, the stability of the compound against the enzyme may not be ascertained (Figure 8D). While the protein RMSD stabilized through the simulation for the unbound form of the crystal structure of superoxide dismutase (Figure 8E), compound 12 in the docking complex appeared to stabilize beyond 30 nanoseconds until the end of the simulation (Figure 8F).

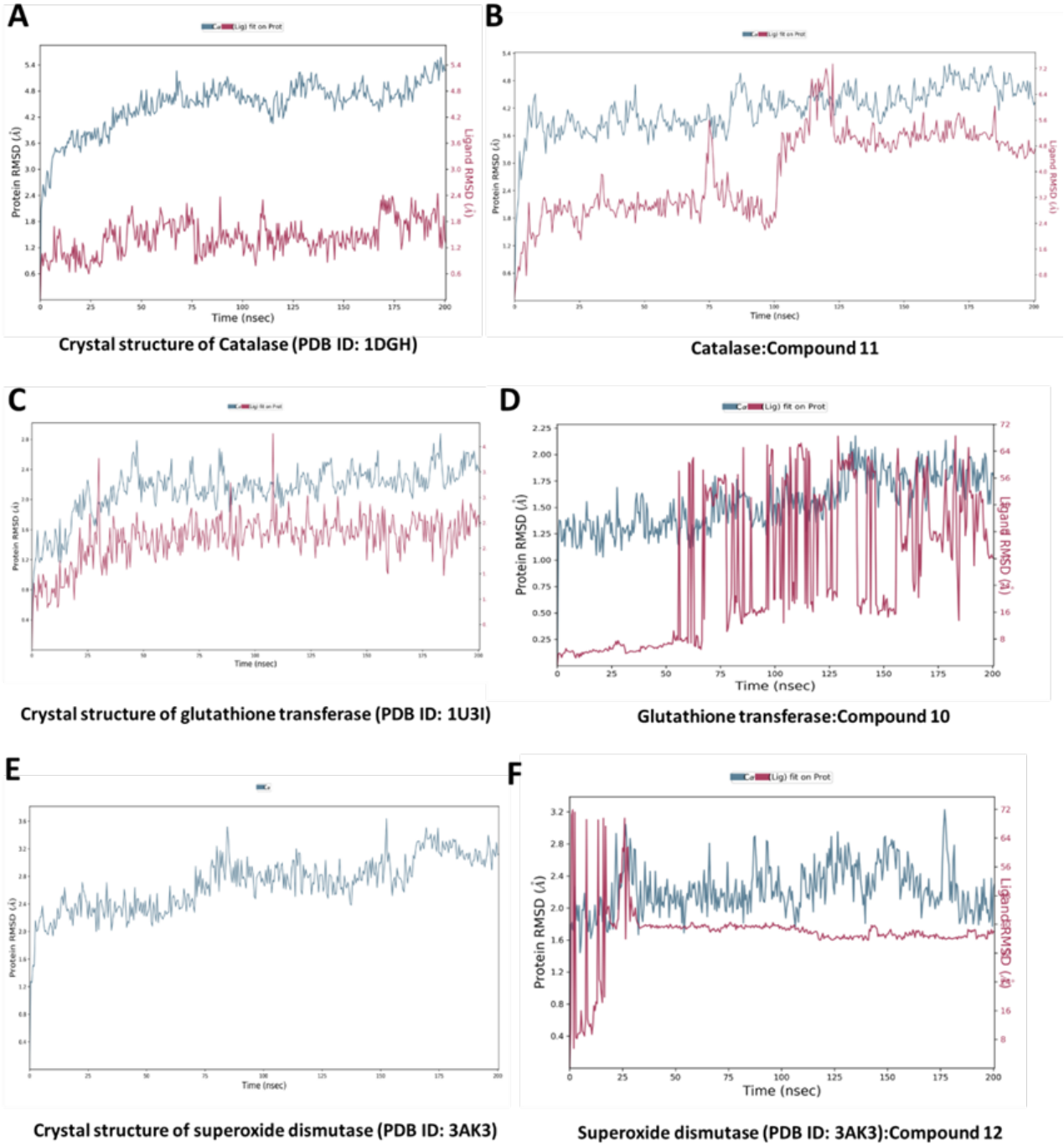

Fig. 8. Root-mean-square displacement (RMSD) profile over 200 ns simulation of complexes: (A) crystal structure of catalase (PDB ID: 1DGH), (B) docking complex of catalase with compound **11,** (C) crystal structure of glutathione transferase (PDB ID: 1U3I), (D) docking complex of glutathione transferase with compound **10,** (E) crystal structure of superoxide dismutase (PDB ID: 3AK3), and (F) docking complex of superoxide dismutase with compound **12.** The timelines of the protein-ligand interactions over 200 ns simulation are provided in Figure 9

These interactions comprised mainly H-bonds and hydrophobic contacts. In particular, the docking complexes showed comparable patterns of persistent interactions to those in the crystal structures of catalase and glutathione transferase (Figure 9A-D), however, the docking complex of superoxide dismutase with compound 12 showed few persistent interactions through the simulation (Figure 9F). The corresponding interaction timelines are consistent with the interaction fraction for each system (Figure 10).

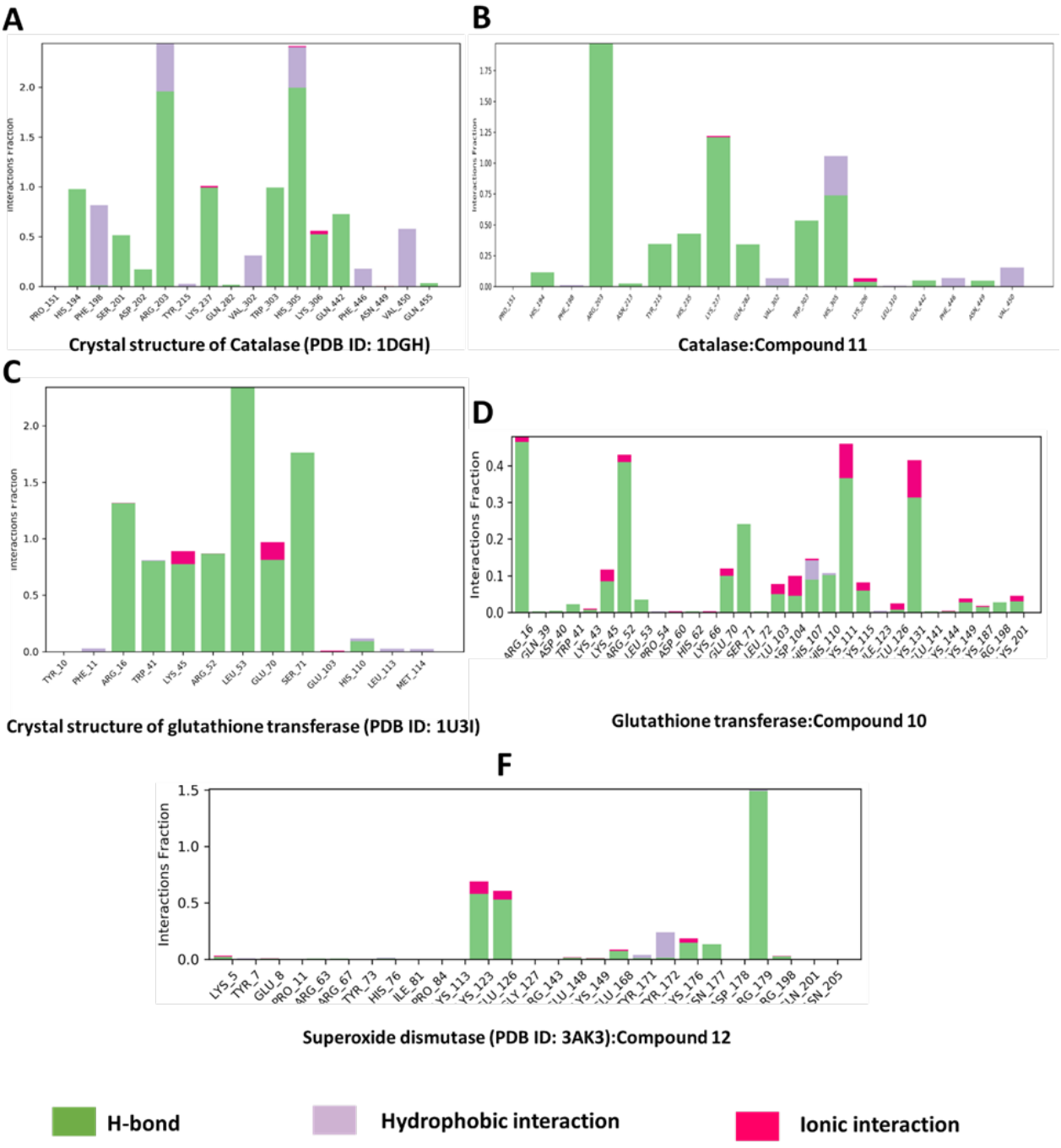

Fig. 9. Protein-ligand interaction fraction over 200 ns simulation: (A) crystal structure of catalase (PDB ID: 1DGH), (B) docking complex of catalase with compound 11, (C) crystal structure of glutathione transferase (PDB ID: 1U3I), (D) docking complex of glutathione transferase with compound 10, (E) crystal structure of superoxide dismutase (PDB ID: 3AK3), and (F) docking complex of superoxide dismutase with compound 12

There were more interactions through the simulation in the crystal structure of catalase (Figure 10A) and its docking complex with compound 11 (Figure 10B), the crystal structure of glutathione transferase (Figure 10C) compared with its docking complex (Figure 10D), and quite a few in the case of the docking complex of superoxide dismutase (Figure 10E).

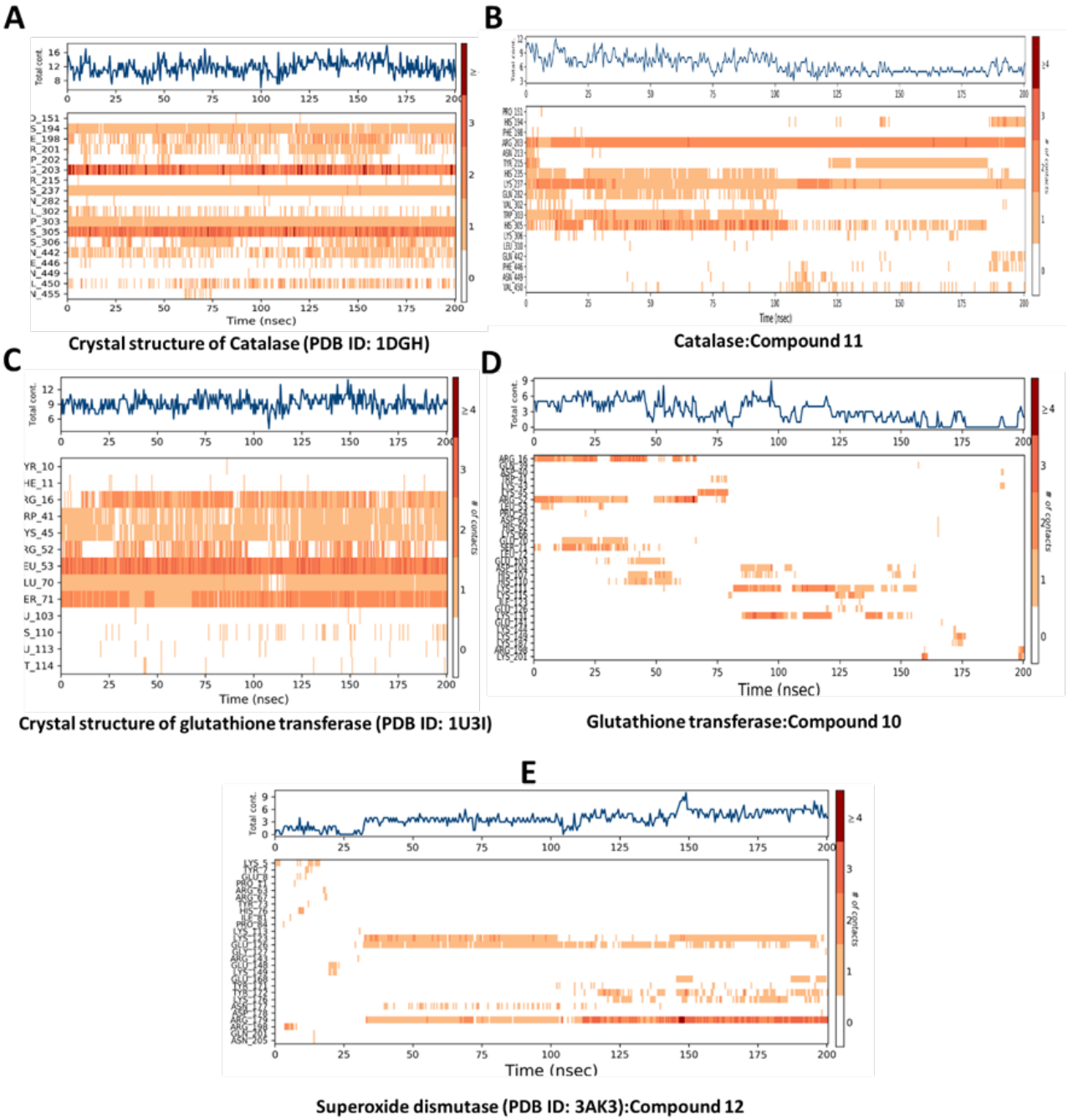

Fig. 10. Protein-ligand interaction timeline over 200 ns simulation: (A) crystal structure of catalase (PDB ID: 1DGH), (B) docking complex of catalase with compound 11, (C) crystal structure of glutathione transferase (PDB ID: 1U3I), (D) docking complex of glutathione transferase with compound 10, (E) crystal structure of superoxide dismutase (PDB ID: 3AK3), and (F) docking complex of superoxide dismutase with compound 12

4. Discussion

The qualitative profile of *DM* stem-bark ethanol extract using modern LC-MS/MS analysis operated in positive ion mode offers intuition in the phytochemical significance of the extract as a groundwork step to reveal quality assessment of it. LC-MS analysis of *DM* stem-bark extract detected fifteen peaks. Nevertheless, all compound detected were identified using global natural product molecular networking data base. Although compounds identification was limited to only LC-MS/MS in this research, only compounds identified with minimal error *m/z* values of ±0.0001 were selected as confirmed metabolites present in DM stem-bark extract. This was to ensure the metabolites identification was valid even though other spectroscopic methods were not exploited which might have been a limitation to this study. Alkaloids were represented by five compounds from peak 1 to 5, and are the major phytometabolites detected in this extract. The detected compound annotated peak 1 was identified as 5-Methoxydimethyl-tryptamine a powerful psychoactive tryptamine. Other alkaloid with relevant biological activity was calcimycin, a polyether antibiotic and divalent cation ionophore, exhibit cytotoxicity against cancer cells. This compound was reported to have induces the expression of the growth arrest in colon cancer cells [29] and modulates DNA damage inducible gene, *gadd153* in response to DNA damaging agents [30].

Flavonoids were also detected in the form of 8-C-Methylvelloquercetin 3,5,3'-trimethyl ether, a quercetin derivative. This finding corroborates with earlier report by Ramadwa *et al.,* [53], that quercetin 3-O-β-glucoside and quercetin 3-O-α-rhamnoside were tentatively identified in DM extract. This compound is a class of flavonol quercetin derivatives with great number of pharmacological activities including anticancer, antioxidant, antimicrobial, anti-inflammatory [31]. Some of the activities were attributable to the hydroxyl group at position 3,5,7,3',4' of the A and B rings, also the double bond between second and third carbons and fourth carbon carbonyl group have main role in antioxidant activity of quercetin. The amino acids derivatives detected were Asp-His-Lys, a tripepetide and Ndelta-(N'-sulphodiamino-phosphinyl)-L-ornithine, a peptide analogue. Tripeptides comprises of three amino acids linked by a peptide bond were considered to be more diverse than monomers of peptide. The molecular weight of antioxidant peptides ranges from 400-650 which correspond to the molecular weight of compound 11 detected. Such peptides exhibit robust antioxidant activities against *in-vitro* antioxidant models [32]. However, others reported, the isolation of Lupeol, a pharmacologically active triterpenoid with nociceptive activity in the *D.mespiliformis* extract.

4.1 In-vivo Antioxidant Activity

Adult albino mice were used to assess the protective effect of *DM* stem-bark extract on CCl_4 induced hepatotoxicity. CCl_4 was given via intraperitoneal injection twice weekly for 28 days. This procedure is a model that represents reproducible fibrosis, which can also be reversible after discontinuation of the treatment [33]. Thus, this archetype is often used in study of fibrosis development and analysis of liver repair mechanisms, emphasizing the systematic importance of this model. In many aspects CCl_4-induced hepatic fibrosis and cirrhosis in murine models mirrors the pattern of toxic damage seen with human disease [34].

Changes in body weight are correlated with the protracted inflammatory disease conditions and it might be due to less intake of food. The CCl_4 toxicity marginally reduced the body weight, while the positive control and extract treated group exhibited no such decrease in the body weight compared to the control. Also the liver weight was assessed in all the experimental groups. The CCl_4-treated mice suffered from hepatomegaly with striking increase in the liver weight compared to the normal

control group. This scenery could be explained in light of the progression of liver fibrosis [35]. In addition, swelling is very common as organs become opaque and boggy during inflammatory conditions due to increased vascular permeability and vasodilatation [36]. However, the groups treated with the silymarin and extract showed marginal changes in the liver weight as compared to the control. Thus, interestingly, the relative liver weight was preserved in those groups as shown in present study.

The ALT, AST, ALP and bilirubin were employed clinically to assess the hepatocytes destruction, while the total protein is widely used to assess the metabolic and functional ability of the liver. Challenging experimental animal model with CCl_4 significantly derange serum hepatocellular injury biomarkers such as the amino transferases, bilirubin, albumin and total protein from the normal values. The hepatotoxic effect of CCl_4 is attributed to its immediate cleavage by cytochrome P450 (CYP2E1) in hepatocytes, which generates trichloromethyl radicals ($CCl_3\cdot$) leading to lipid peroxidation and subsequently to membrane damage. This study showed that CCl_4 toxicity was associated with marked increased in serum ALT, AST and bilirubin, also striking reduction in albumin and total protein were noticed. The marked increased in serum enzyme level may be ascribed to disruption of hepatocytes by CCl_4 whereby the enzyme leaked to the plasma. High level of direct bilirubin, which is considered as indicator of cholestasis and pathological alterations of the biliary flow; thus, uppermost concentration of direct bilirubin observed in the serum obtained from CCl_4 induced group is an indication of liver injury. The marked reduction in total protein and albumin observed in response to CCl_4 treatment, may in part attributable to hypomethylation of cellular components. Thus, mRNA could be dislocated from rough endoplasmic reticulum and the outcome is thought to be inhibition of protein synthesis [37]. CCl_4 induced hypoproteinemia and hypoalbuminemia in albino rats have been reported by others [38] which corroborates with our findings in albino mice.

Intriguingly, *DM* stem-bark extract counteracted these alterations by restoring serum levels ALT, AST, bilirubin, as well as albumin and total protein to almost near normal. This ameliorative effect of the plant extract mimics silymarin, a herbal drug with several bioactivities such as antioxidant, anti-inflammatory, immunomodulatory as well as liver regenerating mechanism [39]. In this study, 100mg per kg BW of silymarin was used as standard drug. The antioxidant and hepatoprotective effects of the extract may in part attributable to the phytochemical components present. In present research, several bioactive compounds were detected including alkaloids, amino acids and peptide analogues, and anthraquinones, flavonoids, nucleoside and nucleotide analogues, polyphenols, and vitamin B precursor. A quercetin derivative, was detected 8-C-Methylvelloquercetin 3,5,3'-trimethyl ether. Several studies have indicated the therapeutic potentials of quercetin to treat various diseases such as aging, cancer, diabetes, cardiovascular disease and also enhances antioxidant capacity of endogenous antioxidant defence system [40]. Also a tripeptide, L-Aspartyl-L-histidyl-L-lysine was detected, the antioxidant effects of the extract may be ascribed to this molecules acting as antioxidant peptides. Several studies showed that antioxidant peptides, in addition to radical scavenging effects [41], these molecules also have potentials to resist radiotoxicity and enhance body's resistance to oxidative stress [42].

Oxidative stress is an important cause of liver dysfunction and also the pathophysiological basis of hepatic injury [43]. Previous report demonstrated that increased oxidative stress causes lipid peroxidation in cells, mitochondrial calcium overload, inflammatory reaction, abnormal function of organelles, oxidative DNA damage and even cancerization [44]. The efficacy of any hepatoprotective drug is essentially dependent on its ability in reducing the harmful effects hepatotoxin, or maintaining the normal hepatic physiology that has been disturbed thereof. In this study, the effects of the plant extract on endogenous antioxidant defence system were assessed. Antioxidant enzymes such as CAT, GST, SOD, constitute a helpful team of defence against ROS, and any other environmental toxicity.

The mechanism of antioxidant enzymes such as GST, GSH, catalase and SOD were markedly compromised by the CCl_4 toxicity. Depletion of the antioxidant system in CCl_4 treated group could be attributed to CCl_4 generated cellular ROS production and the subsequent depletion of the antioxidant cellular system [45], whilst *DM*stem-bark extract treatment strikingly reversed the antioxidant mechanism as compared to the CCl_4 treated group. Likewise, glutathione is a first line of defence and scavenges ROS. Additionally, GSH-dependent enzymes offer an important line of protection as they detoxify noxious by-products generated by ROS [46]. In this experiment the depletion of Glutathine-S transferase activity in the liver in response to CCl_4 toxicity may be due to enhanced GSH utilization in the elimination of peroxides or NADPH reduction activity. Several studies showed that GSH plays a key role in detoxifying the toxic metabolites of CCl_4 and that liver injury begins when GSH stores are markedly depleted [47]. Furthermore, the CCl_4 administration significantly enhanced the oxidative stress, thereby increasing the concentration of the MDA, which is the end product of the lipid peroxidation [48]. In the liver, CCl_4 is bio-transformed into active free radicals (CCl_3• and CCl_3OO•) which are responsible for the increase in MDA level as a result of peroxidation of polyunsaturated fatty acids of the cell membrane [49]. However, the extract treatment significantly attenuated the production of the MDA and hence inhibited the oxidative stress in contrast to the CCl_4 induced group. The *in-vitro* antioxidant model data assured the radical scavenging potential of the extract. Several previous reports demonstrated that the presences of the antioxidants in an extract were determined by assessing the ability of extract to reduce the ferric cyanide complex to the ferrous form. The reducing power of a compound may serve as a significant indicator of its potential antioxidant activity [50]. Samples with higher reducing power have better abilities to donate electron to the free radical to form stable substances by accepting the donated electrons, resulting in the termination of radical chain reaction [51]. In the present study, the reducing ability of the extracts of *DM*was quite comparable with previous studies [9,52].

The histological results corroborate the biochemical findings. The control untreated group showing normal histological structure of hepatic parenchyma. Microscopic examination of CCl_4 treated group showed various histopathological alterations indicating acute liver injury associated with fibrosis. Hepatic parenchyma showed disruption of hepatic lobular structure with bridging fibrosis. The hepatocytes showed diffuse macrovesicular steatosis associated with inflammatory reaction apoptosis and appearance of mitotic was detected. Oval cell proliferation along with bile ductular hyperplsia and kupffer cells activation with marked polyploidy of hepatocytes was noticed and was represented by hepatocytomegally, karyomegally, anisokaryosis and increased number of binucleated hepatocytes.

The microscopic examination of silymarin group revealed reduction in histopathological hepatic alterations that was induced by CCl_4 treatment. The hepatic lobular architecture was maintained macrovesicular steatosis of hepatocytes was scarce involving individual hepatocytes with incidental mitosis of hepatocytes associated with moderate kupffer cells activation. Oval cell proliferation was observed in portal area. Thus, silymarin showed a high protective activity against the CCl_4-induced hepatic toxicity because of its antioxidant, cytoprotective, radical scavenging, and anti- inflammatory properties nearly restored normal cytological architecture of the hepatocytes.

Group treated with extract was more similar to silymarin treated group but the extent of fibrosis was pronounced with more disruption of hepatic architecture. Treating CCl_4-induced toxicity with *DM*extract partially improved the liver tissues. It showed mild inflammation of hepatocytes and mild fatty changes. This improvement is ascribed to the antioxidant activity of some bioactive metabolites detected such as quercetin derivatives, tripeptides, and alkaloids. Similarly, silymarin also protected the liver by reducing oxidative stress within the liver tissues and nearly restored them to their normal architecture.

In the molecular docking data, the patterns of interaction may suggest that the bioactive compounds may be binding transiently to enhance the activity of the antioxidant enzymes. The MM-GBSA binding affinity scores were reported to be more reliable than the docking scores given that the protein flexibility and solvation effects are taken into account during the calculations [27]. Protein-ligand interaction analysis on the top compound (see Table 4,5, and Figures 7-10) for each target was performed. The key interactions formed between the selected compounds and the enzymes were H-bonds with polar residues and hydrophobic contacts with non-polar residues all over the active sites. The specific residues involved in the formation of these interactions, which are mostly polar are shown in the two-dimensional interaction. The fact that these compounds bound to the target enzymes superficially and less strongly compared with the co-crystal inhibitors may suggest their roles as enhancers. Nonetheless, the compounds may possess the potential to enhance the antioxidant activities of these enzymes.

5. Conclusions

This study ascribes a full representation of the ethanol extracts of *DM.* Applying modern LC-MS/MS analysis operated in positive ion mode a total of 15 compounds were identified. Where, five alkaloids, two amino acids, one Anthraquinone, one nucleoside derivative, three phenolic acid derivatives, one fatty amide, one flavonoid, one vitamin B12.

The *DM* stem-bark extract administration showed marked hepatoprotection against chronic CCl_4-induced liver injury model. The extract significantly improved the biochemical and histological parameters, while enhanced the activity of the endogenous antioxidant biomarkers. Furthermore, the treatment exhibited no observable toxicity against the animals. These identified compounds rationalize the treatment effect of the extracts against CCl_4 induced toxicity, since *in silico* modelling data demonstrated an enhanced interaction between the bioactive compounds and the endogenous antioxidant enzymes.

List of abbreviations

CCL_4-carbon tetrachloride; DM-*Diopyros mespliformis*; DPPH 2,2 , diphenyl-1-picryl-hydrazl;H_2O_2-hydrogen peroxide; GST-glutathione S transferase; MDA-malondialdehdye; GNPS-global natural product social molecular network; LC/MS-liquid chromatogramhy/mass spectrometry; qRTPCR-quantitative reverse transcriptase polymerase chain reaction; FRAP-ferric reducing antioxidant power; CH_3CCL_3- trichloromethyl; $CH_3CCL_3O_2$- trichloromethylperoxy; UHPLC-ultra high performance liquid chromatography; NITR-national institute of trypanosomiasis research; IP-intraperitoneal; AST-aspartate amino transferase; ALT-alanine amino transferase; RMSD-root mean square displacement; RMSF – root mean square fluctuation; MM-GBSA-molecular mechanism- general born surface.

Declarations

- Consent for publication: All authors have read and provided their consent for the publication of the data in the manuscript.
- Availability of data: All raw data for the manuscript can be accessed via the following link: https://drive.google.com/drive/folders/1hjznhn4LTEdWOwD5s6MNQm624YAOGMI0?usp=drive_link
- Competing interest: The authors declare that there is no competing of interests.
- Funding: not applicable

Author Contributions

Conceptualization, H.H.; Data curation, M.B.S., A.I.U., and M.U.A.; Formal analysis, M.B.S., N.A., .A.I.L, ; Funding acquisition, H.H.; Investigation, M.B.S. and M.U.A.; Project supervision: H.H and H.A.P.; Validation, H.H.; Visualization, H.H. and H.A.P.; Writing—original draft, H.H. and H.A.P.; Writing—review & editing, M.B.S. H.H. and H.A.P. All authors have read and agreed to the published version of the manuscript.

Acknowledgement

The authors would like to acknowledge the contribution of the following, towards the realisation and success, for the conduct of the research and its output: Abdullahi Ibrahim Uba, Nicholas Joseph Paradis, Chun Wu, Zurina Binti Zainal of Natural Medicines and Products Research Laboratory, Institute of Bioscience Universiti Putra Malaysia for LCMS analysis; AbdulGaniyu AbdulHakeem Raji of Chemical Pathology Laboratory Federal Teaching Hospital Gombe for serum chemistry analysis; Abubakar Ibrahim of Department of Department of Human Physiology for animal care and treatment.

References

[1] Al-Seeni, Madeha N., Haddad A. El Rabey, Mazin A. Zamzami, and Abeer M. Alnefayee. "The hepatoprotective activity of olive oil and Nigella sativa oil against CCl 4 induced hepatotoxicity in male rats." *BMC complementary and alternative medicine* 16 (2016): 1-14. https://doi.org/10.1186/s12906-016-1422-4

[2] Lawal, B., O. K. Shittu, P. C. Ossai, A. N. Abubakar, and A. M. Ibrahim. "Antioxidant activities of Giant African snail (Achachatina maginata) Haemolymph against CCl4-induced hepatotoxicity in albino rats." *Br J Pharm Res* 6, no. 3 (2015): 141-54. https://doi.org/10.9734/BJPR/2015/15887

[3] Jyothi, K. S. N., P. Hemalatha, A. Avanthi, and Suresh Challa Suresh Challa. "A comparative analysis on the alpha amylase inhibitory potential of six ornamental medicinal plants." (2013): 1-6.

[4] Delli Bovi, Anna Pia, Francesca Marciano, Claudia Mandato, Maria Anna Siano, Marcella Savoia, and Pietro Vajro. "Oxidative stress in non-alcoholic fatty liver disease. An updated mini review." *Frontiers in medicine* 8 (2021): 595371. https://doi.org/10.3389/fmed.2021.595371

[5] Arhoghro, E. M., K. E. Ekpo, E. O. Anosike, and G. O. Ibeh. "Effect of aqueous extract of bitter leaf (Vernonia Amygdalina Del) on carbon tetrachloride (CCl4) induced liver damage in albino Wistar rats." *Eur J Sci Res* 26, no. 1 (2009): 12-30.

[6] Tibiri, André, Sébastien Boria, Tata Kadiatou Traoré, Noufou Ouédraogo, Aude Nikièma, Souleymane Ganaba, Jean-Marie Compaoré, Issiaka Ouédraogo, Innocent Pierre Guissou, and Maëlle Carraz. "Countrywide survey of plants used for liver disease management by traditional healers in Burkina Faso." *Frontiers in Pharmacology* 11 (2020): 563751. https://doi.org/10.3389/fphar.2020.563751

[7] Ying, X. U., Ping-Ping Zhong, and T. A. O. Yan-Yan. "Metabolic profiling of endogenous bile acids: a novel method to assess hepatoprotective effect of Tanreqing capsule on carbon-tetrachloride-induced liver injury in rats." *Chinese journal of natural medicines* 16, no. 4 (2018): 271-283. https://doi.org/10.1016/S1875-5364(18)30057-8

[8] Dong, Shu, Qi-Long Chen, Ya-Nan Song, Yang Sun, Bin Wei, Xiao-Yan Li, Yi-Yang Hu, Ping Liu, and Shi-Bing Su. "Mechanisms of CCl4-induced liver fibrosis with combined transcriptomic and proteomic analysis." *The Journal of toxicological sciences* 41, no. 4 (2016): 561-572. https://doi.org/10.2131/jts.41.561

[9] Adamu, H. M. "Phyto chemical screening and antioxidant activity of the stem bark extracts of Diospyros mespiliformis: a medicinal plant in Bauchi." *International Journal of Pharmacy Research & Technology (IJPRT)* 10, no. 1 (2020): 37-43.

[10] Abubakar, M. S., A. M. Musa, A. Ahmed, and I. M. Hussaini. "The perception and practice of traditional medicine in the treatment of cancers and inflammations by the Hausa and Fulani tribes of Northern Nigeria." *Journal of Ethnopharmacology* 111, no. 3 (2007): 625-629. https://doi.org/10.1016/j.jep.2007.01.011

[11] David, Oluwole Moses, John Oludele Olanlokun, Bisola Evelyn Owoniyi, MoyinOluwa Ayeni, Oluwakemi Ebenezer, and Neil Anthony Koorbanally. "Studies on the mitochondrial, immunological and inflammatory effects of solvent fractions of Diospyros mespiliformis Hochst in Plasmodium berghei-infected mice." *Scientific Reports* 11, no. 1 (2021): 6941. https://doi.org/10.1038/s41598-021-85790-6

[12] Nitiéma, Mathieu, Pegdwendé Esther Ouédraogo, Tata Kadiatou Traoré, Oumarou Maman Noura, Boukaré Kaboré, Wendkouni Leila Marie Esther Bélem-Kabré, Windingoudi Rimwagna Christian Ouédraogo et al. "Phytochemical Profile, Antioxidant, and Anti-Inflammatory Activities, Safety of Use and Spasmolytic Effects of

Aqueous Decoction Extract of Diospyros mespiliformis Leaves Hochst. ex A. DC.(Ebenaceae) on the Isolated Duodenum of Rat." *Pharmacology & Pharmacy* 14, no. 12 (2023): 513-529. https://doi.org/10.4236/pp.2023.1412034

[13] DAHIRU, Mubarak Muhammad, and Neksumi MUSA. "Phytochemical Profiling, Antioxidant, Antidiabetic, And Admet Study Of Diospyros Mespiliformis Hochst. Ex A. Dc.(Ebenaceae) Leaf." *Journal of Faculty of Pharmacy of Ankara University/Ankara Üniversitesi Eczacilik Fakültesi Dergisi* 48, no. 2 (2024). https://doi.org/10.33483/jfpau.1354293

[14] Yakubu, M. Y., M. N. Ibrahim, A. M. Umar, U. F. Abubakar, and A. Isiyaku. "Effect of Diospyrus mespiliformis Hochst (Ebenaceae) Stem Bark Extract on Some Biochemical and Hematological Parameters in CCl4 Induced Oxidative Stress in Albino Mice." *BIMA Journal Of Science And Technology (2536-6041)* 8, no. 3B (2024): 39-45.

[15] Pantami, Hamza Ahmed, Muhammad Safwan Ahamad Bustamam, Soo Yee Lee, Intan Safinar Ismail, Siti Munirah Mohd Faudzi, Masatoshi Nakakuni, and Khozirah Shaari. "Comprehensive GCMS and LC-MS/MS metabolite profiling of Chlorella vulgaris." *Marine drugs* 18, no. 7 (2020): 367. https://doi.org/10.3390/md18070367

[16] Chinedu, Enegide, David Arome, and Fidelis Solomon Ameh. "A new method for determining acute toxicity in animal models." *Toxicology international* 20, no. 3 (2013): 224. https://doi.org/10.4103/0971-6580.121674

[17] McGill, Mitchell R., and Hartmut Jaeschke. "Metabolism and disposition of acetaminophen: recent advances in relation to hepatotoxicity and diagnosis." *Pharmaceutical research* 30 (2013): 2174-2187. https://doi.org/10.1007/s11095-013-1007-6

[18] Chen, Yue, Bo Huang, Jingsheng He, Li Han, Yichao Zhan, and Youwei Wang. "In vitro and in vivo antioxidant effects of the ethanolic extract of Swertia chirayita." *Journal of ethnopharmacology* 136, no. 2 (2011): 309-315. https://doi.org/10.1016/j.jep.2011.04.058

[19] Benzie, Iris FF, and J. J. Strain. "[2] Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration." In *Methods in enzymology*, vol. 299, pp. 15-27. Academic press, 1999. https://doi.org/10.1016/S0076-6879(99)99005-5

[20] Senhaji, B., B. Chebli, E. Mayad, A. Hamdouch, N. Heimeur, A. Chahid, and Z. Ferji. "Phytochemical screening, quantitative analysis and antioxidant activity of Asteriscus imbricatus and Pulicaria mauritanica organic extracts." *International Food Research Journal* 24, no. 6 (2017): 2482-2489.

[21] Sarma, Rahul, Sima Kumari, Ramakrishnan Elancheran, Meetali Deori, and Rajlakshmi Devi. "Polyphenol rich extract of Garcinia pedunculata fruit attenuates the hyperlipidemia induced by high fat diet." *Frontiers in Pharmacology* 7 (2016): 294. https://doi.org/10.3389/fphar.2016.00294

[22] Kasangana, Pierre Betu, Pierre Selim Haddad, and Tatjana Stevanovic. "Study of polyphenol content and antioxidant capacity of Myrianthus arboreus (Cecropiaceae) root bark extracts." *Antioxidants* 4, no. 2 (2015): 410-426. https://doi.org/10.3390/antiox4020410

[23] Nakamura, Tsutomu, Kasumi Torikai, Koichi Uegaki, Junji Morita, Kodai Machida, Atsushi Suzuki, and Yasushi Kawata. "Crystal structure of the cambialistic superoxide dismutase from Aeropyrum pernix K1–insights into the enzyme mechanism and stability." *The FEBS journal* 278, no. 4 (2011): 598-609. https://doi.org/10.1111/j.1742-4658.2010.07977.x

[24] Putnam, Christopher D., Andrew S. Arvai, Yves Bourne, and John A. Tainer. "Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism." *Journal of molecular biology* 296, no. 1 (2000): 295-309. https://doi.org/10.1006/jmbi.1999.3458

[25] Friesner, Richard A., Jay L. Banks, Robert B. Murphy, Thomas A. Halgren, Jasna J. Klicic, Daniel T. Mainz, Matthew P. Repasky et al. "Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy." *Journal of medicinal chemistry* 47, no. 7 (2004): 1739-1749. https://doi.org/10.1021/jm0306430

[26] Uba, Abdullahi Ibrahim, Nicholas Joseph Paradis, Chun Wu, and Gokhan Zengin. "Phenolic compounds as potential adenosine deaminase inhibitors: molecular docking and dynamics simulation coupled with MM-GBSA calculations." *Amino Acids* 55, no. 12 (2023): 1729-1743. https://doi.org/10.1007/s00726-023-03310-4

[27] Genheden, Samuel, and Ulf Ryde. "The MM/PBSA and MM/GBSA methods to estimate ligand-binding affinities." *Expert opinion on drug discovery* 10, no. 5 (2015): 449-461. https://doi.org/10.1517/17460441.2015.1032936

[28] Aier, Imlimaong, Pritish Kumar Varadwaj, and Utkarsh Raj. "Structural insights into conformational stability of both wild-type and mutant EZH2 receptor." *Scientific reports* 6, no. 1 (2016): 34984. https://doi.org/10.1038/srep34984

[29] Sack, Ulrike, Wolfgang Walther, Dominic Scudiero, Mike Selby, Jutta Aumann, Clara Lemos, Iduna Fichtner, Peter M. Schlag, Robert H. Shoemaker, and Ulrike Stein. "S100A4-induced cell motility and metastasis is restricted by

the Wnt/β-catenin pathway inhibitor calcimycin in colon cancer cells." *Molecular biology of the cell* 22, no. 18 (2011): 3344-3354. https://doi.org/10.1091/mbc.e10-09-0739

[30] Wu, Hao, Jingdan Liang, Jialiang Wang, Wei-Jun Liang, Lixia Gou, Qiulin Wu, Xiufen Zhou, Ian J. Bruce, Zixin Deng, and Zhijun Wang. "Cezomycin is activated by CalC to its ester form for further biosynthesis steps in the production of calcimycin in Streptomyces chartreusis NRRL 3882." *Applied and Environmental Microbiology* 84, no. 12 (2018): e00586-18. https://doi.org/10.1128/AEM.00586-18

[31] Mirza, Mohd Aamir, Syed Mahmood, Ayah Rebhi Hilles, Abuzer Ali, Mohammed Zaafar Khan, Syed Amir Azam Zaidi, Zeenat Iqbal, and Yi Ge. "Quercetin as a therapeutic product: evaluation of its pharmacological action and clinical applications—a review." *Pharmaceuticals* 16, no. 11 (2023): 1631. https://doi.org/10.3390/ph16111631

[32] Ozawa, Hitoshi, Taiki Miyazawa, Gregor Carpentero Burdeos, and Teruo Miyazawa. "Biological functions of antioxidant dipeptides." *Journal of Nutritional Science and Vitaminology* 68, no. 3 (2022): 162-171. https://doi.org/10.3177/jnsv.68.162

[33] Liedtke, Christian, Tom Luedde, Tilman Sauerbruch, David Scholten, Konrad Streetz, Frank Tacke, René Tolba, Christian Trautwein, Jonel Trebicka, and Ralf Weiskirchen. "Experimental liver fibrosis research: update on animal models, legal issues and translational aspects." *Fibrogenesis & tissue repair* 6 (2013): 1-25. https://doi.org/10.1186/1755-1536-6-19

[34] Brol, Maximilian Joseph, Felicitas Rösch, Robert Schierwagen, Fernando Magdaleno, Frank Erhard Uschner, Steffen Manekeller, Alexander Queck et al. "Combination of CCl4 with alcoholic and metabolic injuries mimics human liver fibrosis." *American Journal of Physiology-Gastrointestinal and Liver Physiology* (2019). https://doi.org/10.1152/ajpgi.00361.2018

[35] ElBaset, Marwan A., Rana S. Salem, Fairouz Ayman, Nadeen Ayman, Nooran Shaban, Sherif M. Afifi, Tuba Esatbeyoglu, Mahmoud Abdelaziz, and Zahraa S. Elalfy. "Effect of empagliflozin on thioacetamide-induced liver injury in rats: role of AMPK/SIRT-1/HIF-1α pathway in halting liver fibrosis." *Antioxidants* 11, no. 11 (2022): 2152. https://doi.org/10.3390/antiox11112152

[36] Ullah, Hadayat, Ashrafullah Khan, Muhammad Waleed Baig, Naseem Ullah, Naveed Ahmed, Muhammad Khalid Tipu, Hussain Ali, and Salman Khan. "Poncirin attenuates CCL4-induced liver injury through inhibition of oxidative stress and inflammatory cytokines in mice." *BMC complementary medicine and therapies* 20 (2020): 1-14. https://doi.org/10.1186/s12906-020-02906-7

[37] Schwarz, Dianne S., and Michael D. Blower. "The endoplasmic reticulum: structure, function and response to cellular signaling." *Cellular and molecular life sciences* 73 (2016): 79-94. https://doi.org/10.1007/s00018-015-2052-6

[38] Alamri, Eman S., Haddad A. El Rabey, Othman R. Alzahrani, Fahad M. Almutairi, Eman S. Attia, Hala M. Bayomy, Renad A. Albalwi, and Samar M. Rezk. "Enhancement of the protective activity of vanillic acid against tetrachloro-carbon (CCl4) hepatotoxicity in male rats by the synthesis of silver nanoparticles (AgNPs)." *Molecules* 27, no. 23 (2022): 8308. https://doi.org/10.3390/molecules27238308

[39] Karimi, Gholamreza, Maryam Vahabzadeh, Parisa Lari, Marziyeh Rashedinia, and Mohammad Moshiri. ""“Silymarin”, a promising pharmacological agent for treatment of diseases." *Iranian journal of basic medical sciences* 14, no. 4 (2011): 308.

[40] Jia, Hai, Yunchang Zhang, Xuemeng Si, Yuhang Jin, Da Jiang, Zhaolai Dai, and Zhenlong Wu. "Quercetin alleviates oxidative damage by activating nuclear factor erythroid 2-related factor 2 signaling in porcine enterocytes." *Nutrients* 13, no. 2 (2021): 375. https://doi.org/10.3390/nu13020375

[41] Uno, Shinya, Daisuke Kodama, Hiroko Yukawa, Hiroyuki Shidara, and Miki Akamatsu. "Quantitative analysis of the relationship between structure and antioxidant activity of tripeptides." *Journal of Peptide Science* 26, no. 3 (2020): e3238. https://doi.org/10.1002/psc.3238

[42] Yang, Nanxiang, Yongyan Pei, Yan Wang, Limin Zhao, Ping Zhao, and Zhanchao Li. "Identifying the antioxidant activity of tripeptides based on sequence information and machine learning." *Chemometrics and Intelligent Laboratory Systems* 238 (2023): 104845. https://doi.org/10.1016/j.chemolab.2023.104845

[43] Allameh, Abdolamir, Reyhaneh Niayesh-Mehr, Azadeh Aliarab, Giada Sebastiani, and Kostas Pantopoulos. "Oxidative stress in liver pathophysiology and disease." *Antioxidants* 12, no. 9 (2023): 1653. https://doi.org/10.3390/antiox12091653

[44] Hajam, Younis Ahmad, Raksha Rani, Shahid Yousuf Ganie, Tariq Ahmad Sheikh, Darakhshan Javaid, Syed Sanober Qadri, Sreepoorna Pramodh et al. "Oxidative stress in human pathology and aging: molecular mechanisms and perspectives." *Cells* 11, no. 3 (2022): 552. https://doi.org/10.3390/cells11030552

[45] El-Boshy, Mohamed E., Fatma Abdelhamidb, Engy Richab, Ahmad Ashshia, Mazen Gaitha, and Naeem Qustya. "Attenuation of CCl4 induced oxidative stress, immunosuppressive, hepatorenal damage by fucoidan in rats." *J Clin Toxicol* 7, no. 3 (2017): 1-7. https://doi.org/10.4172/2167-7972.1000348

[46] Li, Ying, Zhi Li, Changwen Li, Xin Ma, Yicong Chang, Chenxi Shi, Jingshan He, Rui Li, Ishfaq Muhammad, and Fangping Liu. "Evaluation of hepatoprotective activity of Syringa oblata leaves ethanol extract with the indicator of glutathione S-transferase A1." *Revista Brasileira de Farmacognosia* 28 (2018): 489-494. https://doi.org/10.1016/j.bjp.2018.05.011

[47] Conde de la Rosa, Laura, Leire Goicoechea, Sandra Torres, Carmen Garcia-Ruiz, and José C. Fernandez-Checa. "Role of oxidative stress in liver disorders." *Livers* 2, no. 4 (2022): 283-314. https://doi.org/10.3390/livers2040023

[48] Ito, Fumiaki, Yoko Sono, and Tomoyuki Ito. "Measurement and clinical significance of lipid peroxidation as a biomarker of oxidative stress: oxidative stress in diabetes, atherosclerosis, and chronic inflammation." *Antioxidants* 8, no. 3 (2019): 72. https://doi.org/10.3390/antiox8030072

[49] Mas-Bargues, Cristina, Consuelo Escriva, Mar Dromant, Consuelo Borras, and Jose Vina. "Lipid peroxidation as measured by chromatographic determination of malondialdehyde. Human plasma reference values in health and disease." *Archives of biochemistry and biophysics* 709 (2021): 108941. https://doi.org/10.1016/j.abb.2021.108941

[50] Pal, Ranju, Kundlik Girhepunje, Nidhi Shrivastava, Mohammed Misbah Hussain, and N. Thirumoorthy. "Antioxidant and free radical scavenging activity of ethanolic extract of Morinda citrifolia." *Research Journal of Pharmacy and Technology* 4, no. 8 (2011): 1224-1226.

[51] Hg, Hamza, My Musa, A. B. Bababe, and S. Modu. "Phytochemical analysis and in-vitro antioxidant activity of Xeromphis nilotica stem-bark extract." *BIMA Journal of Science and Technology (2536-6041)* 5, no. 02 (2021): 138-147.

[52] Ebbo, A. A., D. Sani, M. M. Suleiman, A. Ahmad, and A. Z. Hassan. "Acute and sub-chronic toxicity evaluation of the crude methanolic extract of Diospyros mespiliformis hochst ex a. Dc (ebenaceae) and its fractions." *Toxicology reports* 7 (2020): 1138-1144. *https://doi.org/10.1016/j.toxrep.2020.08.028*

[53] Ramadwa, Thanyani Emelton, and Stephen Meddows-Taylor. "Traditional uses, pharmacological activities, and phytochemical analysis of Diospyros mespiliformis Hochst. ex. A. DC (Ebenaceae): A review." *Molecules* 28, no. 23 (2023): 7759. https://doi.org/10.3390/molecules28237759