ORIGINAL CONTRIBUTION   **Open Access**

Antioxidant and in vivo hepatoprotective effects of *Gardenia gummifera* L.f. fruit methanol extract

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Abstract

Background: *Gardenia gummifera* L.f. (Family: Rubeacea) is used in indigenous system of medicine to cure many diseases. To authenticate the traditional medicinal claim investigation has been under taken to evaluate the antioxidant and hepatoprotective activities of *Gardenia gummifera* L.f. fruit methanol extract (GFME).

Method: GFME was evaluated using various antioxidant assays, including DPPH and Nitric oxide radical scavenging assays. The protective effects of GFME were studied in carbon tetrachloride reduced biochemical markers of hepatic injury such as serum glutamyl oxaloacetate transaminase (SGOT), serum glutamyl pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (TB) and direct bilirubin (DB) and in silico studies were carried out to screen the GFME phytocompounds.

Results: The extract showed significant antioxidant activity in DPPH and Nitric oxide radical scavenging with IC_{50} value of 131.11 and 175.95 respectively. Quantitative phytochemical assay determines the presence of alkaloids 69.1 μg/1 mg and phenolics 76.5 μg/1 mg. GC-MS analysis of aromatic extract resulted in 36 compounds. Among them, compounds 2, 3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one, 2-furancarboxaldehyde 5-(hydroxymethyl) and Quinic acid are the major ones. The fruit methanol extract showed significant in vivo hepatoprotective activity by altering the levels of liver function biochemical parameters such as SGOT, SGPT, ALP, TP, TB and DB. Histology of the liver section also confirms the hepatoprotective activity of GFME. Molecular docking of GC-MS profiled phytocompounds with the target protein TGF-β1and PPARα also confirmed the therapeutic effect with good hydrogen bonding and hydrophobic interactions.

Conclusion: Thus the present study clearly strengthened the traditional medicinal claim of the plant *Gardenia gummifera* L.f. possessing the hepatoprotective drug.

Keywords: *Gardenia gummifera* L.f., Antioxidants, Hepatoprotective activity, Biochemical markers

Background

The liver is the largest organ in the body playing a vital role in the regulation of various physiological processes. Its activity is related to diverse fundamental functions, such as detoxification, secretion, storage and metabolism [1]. It has ability to detoxify exogenous (toxic compounds) and endogenous (waste metabolites) substances of organisms. The liver is also involved in the biochemical processes of providing nutrients and supplying energy. In addition, it aids in the metabolism of fats and carbohydrates, storage of vitamins and secretion of bile [2, 3].

Currently, hepatic diseases become principal threats to public health among worldwide population. Nearly 20, 000 deaths occur each year due to liver illnesses [4]. Hepatic disease is a term that indicates damage to the liver cells, tissues and liver function. The imbalance in the level of antioxidants and pro-oxidants leads to oxidative stress causing potential damage to the organ [5].

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This damage can be induced by various biological factors (bacteria, virus, and parasites) and autoimmune diseases (immune hepatitis, primary biliary cirrhosis), as well as by the action of different chemical, drugs [high doses of paracetamol and anti tuberculosis drugs], toxic compounds [carbon tetrachloride (CCl_4)] and excessive consumption of alcohol [6]. Despite enormous advances in modern medicine, there is no effective drug that stimulate hepatic function. Additionally, some drugs can make adverse effects on body. Thus, it is necessary to identify alternative pharmaceuticals for the treatment of hepatic diseases that are more effective and less toxic [7].

Herbal therapies are in extreme demand in developed and developing countries for primary healthcare [8]. Since ancient times, natural products have been the strength of traditional systems in the supervision of human ailments throughout the globe and also been an integral part of history and culture [9]. It is well established that natural products played precious roles in modern drug development [10]. Even though acceptance of the synthetic merchandises increased due to its making cost, time efficacy, easy quality control, stringent regulation and quick effects, but their safety and efficacy always remained questionable, resulting in the inclination towards the natural products by more than 80% of the total population in the developing world, because of their time tested safety and efficacy [11]. Presently, great attention is being focused on the practice of natural antioxidants to safeguard the human body mainly brain tissues from the oxidative injury affected by free radicals. Synthetic drugs in use have shown harmful side effects compared to natural compounds, therefore, there is a need for additional active, cost-effective and less toxic antioxidants drawn from natural sources [12].

Gardenia gummifera L.f. is a deciduous tree belongs to the Rubiaceae family. It is commonly known as gummy gardenia. It is distributed in the forests of Tamil Nadu, Andhra Pradesh, Kerala and Karnataka, This plant is well known for its application in folk medicine. In the indigenous system of medicine Ayurveda, it is called Nadihingu (Sanskrit), Dikamali (Hindi), Kadbikke (Kannada), Sirukkambil (Tamil), Kambimaram (Malayalam), Chitta mali (Telugu) [13, 14].

This plant is claimed to have numerous medicinal properties possessing astringent and carminative properties that are used in the management of dyspepsia and hemorrhoid. It is also useful in the treatment of flatulence for cleaning foul ulcers and wounds, anticonvulsants [15, 16], Sitosterol, D-mannitol, Oleanoic aldehyde, 19 hydroxyerythrodiol and erythrodiol have been reported from the plant [17]. The preliminary phytochemical investigation of Gardenia gummifera L.f. has shown that it is very rich in phytoconstituents. In view of this study the present study was designed to systematically screen the phytocompounds and carryout the GC-MS analysis. The prophylactic effect of the aromatic compounds of the fruit methanol extract was evaluated along with antioxidant and in vivo hepatoprotective effects. The molecular docking of GC-MS analyzed compounds from fruit methanol extract against the therapeutic target proteins was also performed to authenticate the therapeutic effects of Gardenia gummifera L.f.

Materials and methods
Plant material
The fruits of Gardenia gummifera L.f. were collected in August 2018 (during the rainy season) from Sakarayapatna, Chikmagalur District, Karnataka, India. The plant has been identified and authenticated by an inhouse taxonomist Dr. V. Krishna, Professor, Post Graduate Studies and Research in Biotechnology, Kuvempu University, and the voucher specimen is maintained at Kuvempu University herbaria (KUBPHS80).

Preparation of extract
Plant material was washed with distilled water and dried at room temperature to constant weights. The dried fruit materials were blended separately to powder and subjected to successive solvent extraction from nonpolar to polar solvents like petroleum ether, chloroform and methanol respectively. The extracts were prepared using the soxhlet apparatus. The extract were filtered, pooled and concentrated to dryness under reduced pressure in a rotary evaporator (Buchi, Flawil, Switzerland). In sequential extraction, methanol extract found to give more yield.

Preliminary phytochemical analysis
Qualitative analysis
The qualitative estimation of the Gardenia gummifera L.f. extract was carried out as stated by Lotfy et al. [18].

Quantitative analysis

Determination of Total Phenolics The total phenolic contents of the extract were quantified by the spectrophotometric method of Singleton. 20 μl of extract (5 mg/ml) was mixed with 0.25 ml of Folin-Ciocalteau reagent and 0.75 ml of 20% sodium carbonate solution. The reaction mixture was allowed to stand in the light for 3 min and incubated for 2 h in dark. The absorbance was measured at 765 nm using UV-Visible Spectrophotometer. Total phenolics were measured by calibration curve achieved from measuring the absorbance of known concentration of Gallic acid standard (0–100 μg/ ml). The concentrations were expressed as μg of Gallic acid equivalents per ml and all the estimations were executed in triplicates [19].

Determination of total alkaloids The quantitative determination of alkaloids was according to the methodology by Harborne. A total of 40 ml of acetic acid (10%) added to 1 g of extract and allowed to stand for 4 h. The filtrate was then concentrated on a water bath to get 1/4th of its original volume. Concentrated ammonium hydroxide was added drop wise to the extracted sample until the precipitation was complete. The complete solution was permitted to settle and the collected precipitate was washed with dilute ammonium hydroxide and then filtered. The residue was dried in an oven and the percentage of the alkaloid is expressed mathematically [20].

Percentage of total alkaloids (%) = Weight of residue× 100/Weight of a sample taken.

GCMS analysis
The fruit methanol extract of *Gardenia gummifera* L.f. was subjected to GC-MS analysis with Shimadzu instrument by GC-MS solutions software and compounds were separated using Rtx-5, capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The Split ratio 1:25, the injector temperature maintained at 300 °C. The column temperature was programmed at 60 °C, followed by 300 °C at 10 min. About 1 μl sample was injected into the column by the split mode. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min and the Mass scanning range was 40–500 m/z. The identification of compounds was based on Willey and NIST libraries as well as the comparison of their retention indices. The compounds were acknowledged after comparison with those available in the computer library (NIST and Willey) attached to the GC-MS instrument and the results obtained have been tabulated [21].

In-vitro antioxidant activity

Determination of DPPH radical scavenging activity 2, 2-diphenylpicrylhydrazyl (DPPH) radical scavenging activity of GFME was conducted by adding 1 ml of an extract with different concentrations in different test tubes followed by addition of 3 ml of 0.004% DPPH in 95% ethanol into all tubes and the mixture was left in the dark for 30 min before its absorbance was read at 517 nm. The absorbance of the reaction mixture was measured at 517 nm. Ascorbic acid was taken as a reference standard. DPPH radical scavenging activity was calculated using the following formula; Percentage of inhibition = [(A control − A test)/A control] × 100, where A control is the absorbance of the control reaction and A test is the absorbance of the extract reaction. The IC_{50} value was calculated using the formula $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extracts concentrations used to test and ΣI is the sum of the percentage of inhibition at different concentrations [22].

Determination of nitric oxide radical scavenging activity Nitric oxide radical scavenging activity of GFME was conducted by adding 250 μl extracts of different concentrations (100, 200, 300 μg) by adding into different test tubes along with sodium nitroprusside (5 mM, in phosphate buffer saline, pH 7.4). The combination was incubated at room temperature under light source (24 W compact fluorescent light bulb). Later 150 min, 0.6 mL of the mixture was transferred into a new tube containing 0.6 mL of Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid). After incubating for 10 min in darkness, absorbance was recorded at 546 nm. Ascorbic acid was used as a standard. Percentage of inhibition was calculated using formula; % Inhibition = [(A control − A test)/A control] × 100, where A control is the absorbance of the control reaction and A test is the absorbance of the extract reaction. The IC_{50} value was calculated using the formula $IC_{50} = [(\Sigma C/\Sigma I) \times 50]$, where ΣC is the sum of extracts concentrations used to test and ΣI is the sum of the percentage of inhibition at different concentrations [23].

Animals and management
Adult male albino rats of Wistar strain, weighing about 160–220 g were used for the acute toxicity studies. Before the experiment, the animals were acclimatized for 14 days to laboratory conditions. The rats were maintained at room temperature, with a 12 h light/dark cycle. During the period of acclimatization, animals were housed in polycarbonate cages with a pellet diet and water. All processes in this study were executed according to the CPCSEA guidelines. The experimental protocol was permitted by the Institutes Animal Ethics Committee (NCP/IAEC/CL/213/01/2012–13) [24].

Acute toxicity study
The acute toxicity test of GFME was performed according to the Organization of Economic Cooperation and Development (OECD) guidelines 423 [25]. In this study, overnight fasted rats were administered orally with extracts prepared in mili Q-water, while the mili-Q water administered to control groups as a vehicle orally. The fixed-dose method OECD guidelines 423 specified by CPCSEA was implemented for toxicity studies. After the administration of *Gardenia gummifera* L.f. extracts animals were observed individually to investigate for toxic effect changes if any for the first 4 h and once daily for 14 days [26].

In vivo hepatoprotective activity
The albino rats weighing between 160 and 220 g of either sex were used in this evaluation. Animals were divided into five groups of each containing six animals.

Olive oil was used as vehicle control for suspending the standard drugs and the extracts. Group I (vehicle control) animals received only olive oil orally (1 ml/kg body weight). Group II (negative control) animals were administered with 0.5 ml/kg of CCl_4 (1:1 ratio, CCl_4: Olive oil). Group III (standard control) received the standard ascorbic acid at a dose of 100 mg/kg b.w for 7 days. Group IV and V (test groups) received the test sample GFME at a dose of 200 and 400 mg/kg b.w respectively. The test extract and vehicle control were administered orally for 7 days at different dosages. On the 7th day after 6 h of the administration, animals of all groups were sacrificed by cervical dislocation and the sample of blood was collected, allowed to clot at room temperature for 30 min. The blood samples was centrifuged and the clear serum was separated at 4000 rpm for 30 min and serum samples was stored at − 40 °C until use for the assessment of different biochemical parameters like serum glutamyl oxaloacetate transaminase (SGOT), serum glutamyl pyruvate transaminase (SGPT), alkaline phosphatase (ALP), total protein (TP), total bilirubin (TB) and direct bilirubin (DB) [27, 28]. Hepatic liver tissue was sensibly removed, washed and homogenized in KCL (1.15%) and phosphate buffer (10 mM) with EDTA (Ph7.4) and centrifuged at 10,000 rpm for 10 min. The supernatant of the homogenate was used for enzyme assays. Leftover hepatic samples were kept for different histological studies [29].

Histological studies

The liver tissue was excised instantly after sacrifice. The tissue was washed with normal saline and dried out by blotting paper fixed by formalin (10%). The fixed liver was dehydrated in gradual ethanol grades (30–100%) and embedded in paraffin. Five micrometers microtome section were prepared and stained using alum hematoxylin and eosin dye then examined under the microscope for the evaluation of histological changes [30].

In silico analysis
ADME and toxicity properties analysis
The ADMET can be defined by the following phases: absorption, distribution, metabolism, excretion, and toxicity. Lipinski "Rule of five" is commonly used as a filter for drug-like properties [31]. The inputs for analysis of 2, 3-Dihydro-3, 5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II) and Quinic acid (Compound III) were obtained from the ChemSpider database. They were used as an input for in silico analysis. The physicochemical properties of the compound were calculated using an online Molinspiration cheminformatics server. ADME and Toxic properties of the compounds were calculated using an online PreADMET web-based program [32, 33].

Molecular docking studies
The structure of the target Peroxisome proliferator-activated receptor alpha (PPARα) and Transforming growth factor-beta 1 (TGF-β1), was obtained from Protein Data Bank (PDB ID; 5HYK, and 1VJY). Structures of phytoconstituents 2, 3-Dihydro-3, 5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II) and Quinic acid (Compound III) were drawn and analyzed using ChemDraw Ultra 12.0. 3D coordinates were obtained by using PRODRG online server [34]. Active pockets for proteins were obtained from the CASTp server [35]. Intermediary steps, such as energy minimization, protein and ligands preparation, and grid box creation were completed using the Graphical User Interface program AutoDock Tools (ADT). AutoDock saved the prepared file in PDBQT format. AutoDock/Vina was employed for docking with protein and ligand data along with grid box properties in the configuration file. AutoDock/Vina employs iterated local search global optimizer [36]. During the docking procedure, both the protein and ligands were considered as rigid. After the successful completion of the docking runs, different conformations of the ligands known as Binding modes were obtained with their respective binding affinity and the stable one which happens to be with the lowest binding affinity was picked and aligned with receptor structure for further analysis [37].

Statistical analysis
The results were statistically analyzed using one-way ANOVA using a software ezANOVA followed by one way ANOVA with Dunnett's multiple comparison tests were employed. The difference in values at $P \leq 0.02$ was considered as statistically significant.

Results
Qualitative and quantitative analysis
In sequential extraction methanol extract provided more yield with significant amount of secondary phytocompounds which is proved by qualitative and quantitative phytochemical estimation. Qualitative analysis of GFME revealed the presence of alkaloids, phenolic compounds, terpenoids, and saponins. The quantitative investigation revealed that the presence of phenolics and alkaloid compounds at the concentrations of 76.5 μg/1 mg and 69.1 μg/1 mg respectively.

GC-MS analysis
The chemical compositions of the GFME were investigated using GC-MS. The mass spectra of the compounds found in the GFME were matched by National Institute of Standards and Technology (NIST) library (Fig. 1).
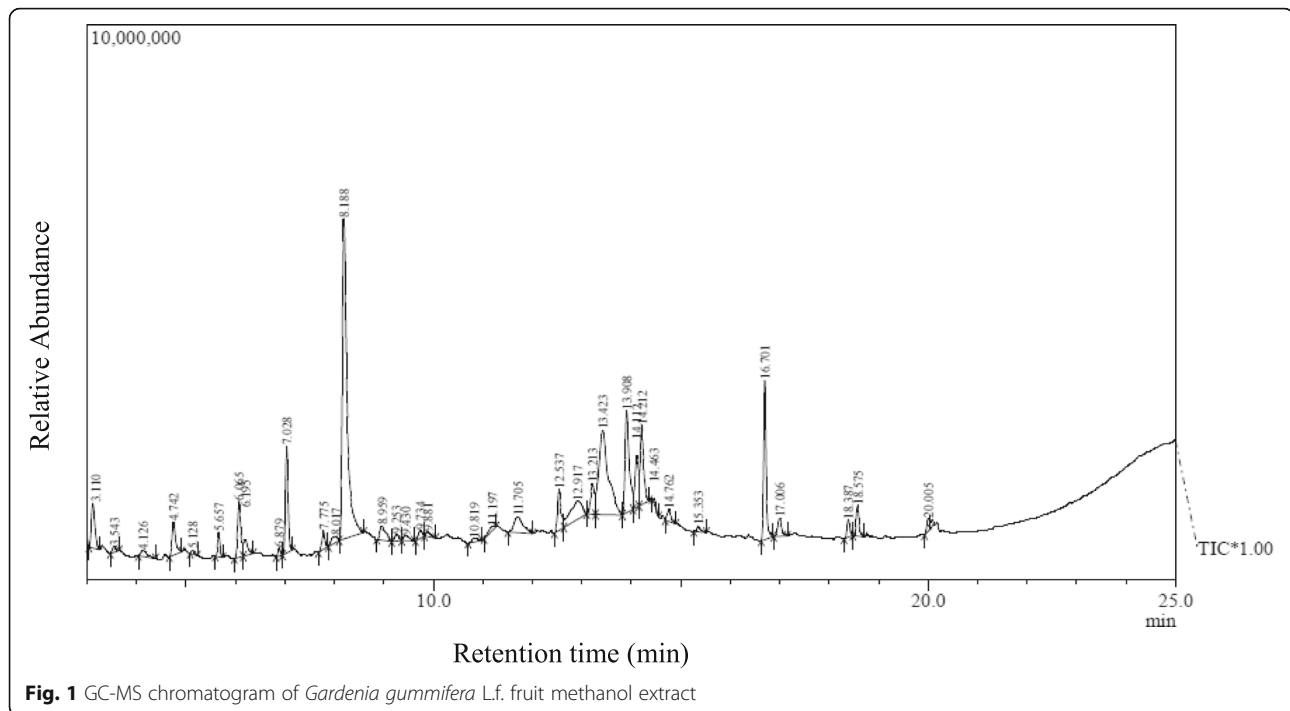
Fig. 1 GC-MS chromatogram of *Gardenia gummifera* L.f. fruit methanol extract

The GC-MS analysis showed the presence of 36 phyto-compounds in the sample (Table 1). Among them, 2-Furancarboxaldehyde, 5-(hydroxymethyl) - ($C_6H_6O_3$) exhibited the highest peak area of 25.94% and the retention time of 8.188 min. Followed by the Quinic acid ($C_7H_{12}O_6$) with an area of 13.60% and retention time of 13.423 min. These are the major phytocompounds present in the GFME.

In vitro antioxidant assay

Plants with antioxidant capacity are useful for medicinal applications. Therefore, in the present study, the antioxidant capacity of *Gardenia gummifera* L.f. extract has been carried out. The free radical scavenging ability of GFME against DPPH and Nitric oxide radicals is shown in Table 2. The GFME showed DPPH radical scavenging activity in a dose-dependent manner in a range of 69.17 ± 0.21 to $82.29 \pm 0.56\%$. The IC_{50} values for extract and standard (ascorbic acid) are 131.11 and 114.93 μg/ml, respectively. In Nitric oxide radical scavenging activity dose-dependent range is 45.10 ± 0.96 to $54.78 \pm 0.21\%$ and the IC_{50} values of extract and standard (ascorbic acid) are 175.95 and 144.45 μg/ml, respectively.

Acute toxicity studies

Acute toxicity studies were undertaken according to a fixed-dose method as per the OECD 423 test guidelines. An effort was also made to detect LD_{50} of GFME. Physical observations showed no signs of behavioural pattern and there was no mortality and weight loss observed at

the concentration of 2000 mg/kg b.w. It is supposed that 2000 mg/kg is the cut off dose. As per the toxicology guidelines, 1/10th and 1/5th of the dosage i.e. (200 mg/kg) and (400 mg/kg) were taken as effective doses for all further experiments with rat models.

In vivo hepatoprotective activity
Assessment of liver function by the level of biochemical markers
The present study has been attempted to evaluate the role of hepatoprotective activity of fruit methanol extract of plant *Gardenia gummifera* L.f.

The study showed a significant increase ($p < 0.0001$) in serum SGOT (137.38 ± 1.30) and SGPT (217.15 ± 1.45) levels on CCl_4 toxicity (Fig. 2). After the treatment with GFME, the toxicity levels of SGOT (123.36 ± 2.37 in 200 mg/kg and 116.71 ± 2.14 in 400 mg/kg) and SGPT (155.17 ± 2.73 in 200 mg/kg and 142.36 ± 1.57 in 400 mg/kg), decreased when compared to the control groups. In ALP determination there is a significant increase in the level of enzyme observed ($p < 0.0001$) in CCl_4 toxicity (187.91 ± 1.53) as depicted in Fig. 2. However, after the treatment with the GFME samples, the increased ALP level was controlled (159.12 ± 1.45 and 145.16 ± 1.79 in 200 mg/kg and 400 mg/kg respectively) when compared to control groups. In the estimation of total bilirubin and direct bilirubin there is significant increase ($p < 0.02$) in CCl_4 toxicity (2.16 ± 0.5 and 1.74 ± 0.04) (Fig. 3), but later treatment with GFME sample, the total bilirubin (1.77 ± 0.12 in 200 mg/kg and $1.44 \pm$

Table 1 Chemical composition of *Gardenia gummifera* L.f. fruit extract

SL. No	R Time (min)	Area %	Compound
1.	3.110	2.58	Furfural
2.	3.543	0.33	Butanoic acid, 2-ethyl-3-oxo-, methyl ester
3.	4.126	0.61	1,2-Cyclopentanedione
4.	4.742	1.97	Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one
5.	5.128	0.22	Maltol
6.	5.657	1.04	Pentanoic acid, 4-oxo-
7.	6.065	3.16	1,3,5-Triazine-2,4,6-triamine
8.	6.195	1.08	Furyl hydroxymethyl ketone
9.	6.879	0.36	2-Propanamine, N-methyl-N-nitroso-
10.	**7.028**	**4.89**	**2,3-dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one**
11.	7.775	1.02	Thiophene, 2,3-dihydro-
12.	8.017	0.65	3-Methoxy-hexane-1,6-diol
13.	**8.188**	**25.94**	**2-Furancarboxaldehyde, 5-(hydroxymethyl)-**
14.	8.959	1.44	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-
15.	9.253	0.47	Cyclohexane, 1,4-diethoxy-, trans-
16.	9.430	0.49	1-Carbmethoxy-3,3-dimethyldiaziridine
17.	9.734	0.52	Pentanoic acid, propyl ester
18.	9.881	0.37	2-Ethyl-3-methoxycyclopropanecarboxylic acid, methyl ester
19.	10.819	0.38	2-Propenoic acid, 3-phenyl-
20.	11.197	0.44	1,2:5,6-Dianhydrogalactitol
21.	11.705	2.42	D-Allose
22.	12.537	2.57	Benzoic acid, 4-hydroxy-3-methoxy
23.	12.917	3.86	.beta.-D-Lyxofuranoside, 5-O-(.beta.-D-lyxofuranosyl)-decyl-
24.	13.213	2.18	Azelaic Acid
25.	**13.423**	**13.60**	**(1R,3R,4R,5R)-(−)-Quinic acid**
26.	13.908	7.17	Hydrazinecarboxamide, 2-(2-methylcyclohexylidene)-
27.	14.112	3.10	Acetate, [4-hydroxy-4-(1-methylethyl)-5-methyl-2-hexynyl] ester
28.	14.212	4.81	Pyrimidine-2,4(1H,3H)-dione, 6-hydroxy-5-methyliminomethyl
29.	14.463	0.25	2-Propanone, 1-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-
30.	14.762	0.72	Acetate, [4-hydroxy-4-(1-methylethyl)-5-methyl-2-hexynyl] ester
31.	15.353	0.37	3-Hydroxy-4,5-dimethoxybenzoic acid
32.	16.701	6.91	l-(+)-Ascorbic acid 2,6-dihexadecanoate
33.	17.006	1.16	Hexadecanoic acid, ethyl ester
34.	18.387	0.86	Oleic Acid
35.	18.575	1.39	Octadecanoic acid
36.	20.005	0.69	14,15,16-Trinor-8.xi.-labdan-6.beta.-ol, 8,13-epoxy-

0.05400 mg/kg) and direct bilirubin (0.86 ± 0.05 in 200 mg/kg and 0.66 ± 0.03 in 400 mg/kg) levels decreased when compared to the control groups. Similarly, in total protein estimation there is a significant decrease ($p < 0.0001$) in CCl_4 toxicity (2.47 ± 0.12) as shown in Fig. 3, but after the treatment with GFME sample, the total protein (4.58 ± 0.11 and 5.52 ± 0.13 in 200 mg/kg and 400 mg/kg respectively) level increased when compared to control groups.

Histological studies

In the histological studies of the liver tissue, the section of normal control animal showed normal hepatic cells with a well-preserved cytoplasm, prominent nucleus and central vein. The CCl_4 treated hepatic liver tissue cells showed a total loss of normal hepatic architecture with cell necrosis and evident vacuolation of hepatocytes. Pre-treatment with GFME at the dose of 200 and 400 mg/kg b.w exhibited protective capacity and regaining of

Table 2 In vitro antioxidant assay of GFME

SL. No	Activity	Extract concentration (µg/ml)	% of activity	IC$_{50}$ µg/ml	Standard IC$_{50}$ in µg/ml
1	DPPH radical scavenging assay	100	69.17 ± 0.21	131.11	114.93
		200	77.35 ± 0.39		
		300	82.29 ± 0.56		
2	Nitric oxide radical scavenging assay	100	49.08 ± 0.87	175.95	144.45
		200	54.78 ± 0.21		
		300	66.64 ± 0.39		

The results shown are averages of three independent experiments, values are Mean ± SEM

normal histological appearance (Fig. 4). The administration of GFME at a dose of 400 mg/kg b.w offered protection more effectively when compared to the lower dose of 200 mg/kg b.w. Silymarin exhibited a potential hepatoprotective effect with the appearance of normal histological features and reduced the degenerative changes in the liver tissue. Thus, the results clearly indicate the hepatoprotective potential of GFME against CCl$_4$ induced liver damage in experimental rats.

In silico analysis
Physicochemical properties of phytocompounds
The prediction of physicochemical properties such as, octanol-water partition coefficient (milogP), Molecular polar surface area (TPSA), molecular weight (MW), number of rotatable bonds (norbt) and number of OH atoms (nOHNH) affect absorption and bioavailability of drug molecule. According to Lipinskis Rule of Five, there were no violations in the compound I (2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one), compound II (2-furancarboxaldehyde 5-(hydroxymethyl)-) and compound III (Quinic acid) identified by GC-MS analysis of GFME

(MW > 500 and NH or OH > 5). The TPSA value and number of rotatable bonds indicated good bioavailability. The TPSA values for the above compounds are 66.76 Å, 50.44 Å, and 118.21 Å and the number of rotatable bonds found to be 0, 2, and 1 respectively. Rotatable polar atomic bonds increased the flexibility of molecules for more adaptable and efficient interaction with the enzyme active site and the values of TPSA of compound I (2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one), compound II (2-furancarboxaldehyde 5-(hydroxymethyl)-) and compound III (Quinic acid) indicated the good oral bioavailability. The data are presented in Table 3.

ADME and toxic properties
The HIA (human intestinal absorption) values for the compound I (2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one), compound II (2-furancarboxaldehyde 5-(hydroxymethyl)-) and compound III (Quinic acid) are 75.91%, 89.88%, and 20.28% respectively. The data of ADME and toxic properties of the compounds are shown in Table 4. These data indicate that among the tested compounds, compound II (2-furancarboxaldehyde
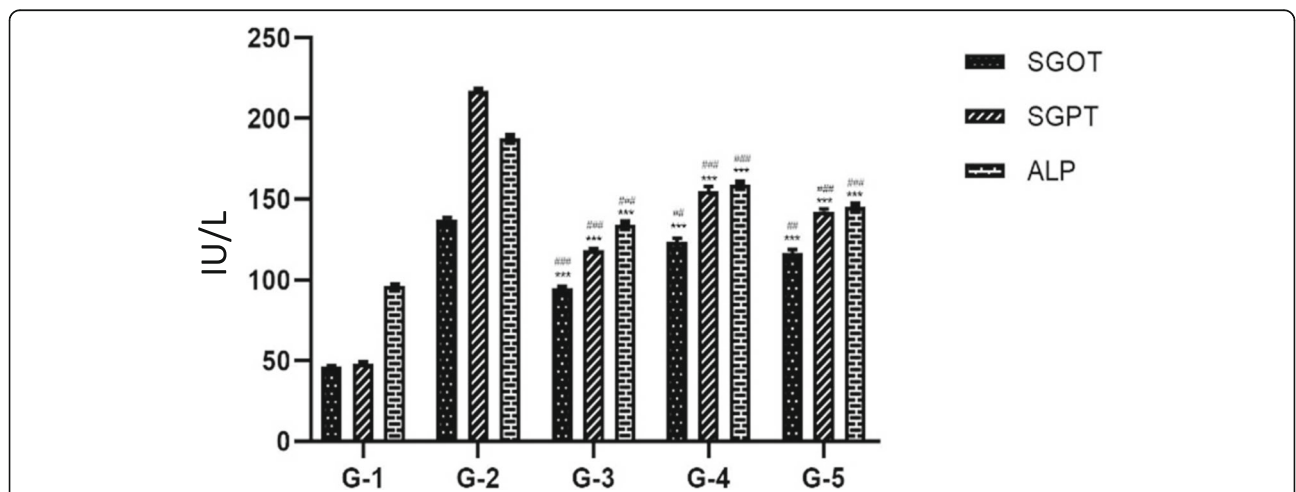

Fig. 2 Effect of GFME on enzyme SGOT, SGPT, ALP levels in blood serum of CCl$_4$ induced hepatotoxicity. Values are mean ±S.E.M. symbol represent statistical significance; *#p<0.02, **##p<0.001 and ***###p<0.0001.* indicates the comparison of G- 1 with G-3, G- 4, G-5; # indicates the comparison of G- 2 with G-3, G- 4, G-5.SGOT- Serum glutamyl oxaloacetate transaminase, SGPT- Serum glutamyl pyruvate transaminase, ALP-Alkaline phosphatase. G1- normal control, G2-negative control, G3- standard, G4- GFME (200mg), G5- GFME (400mg)
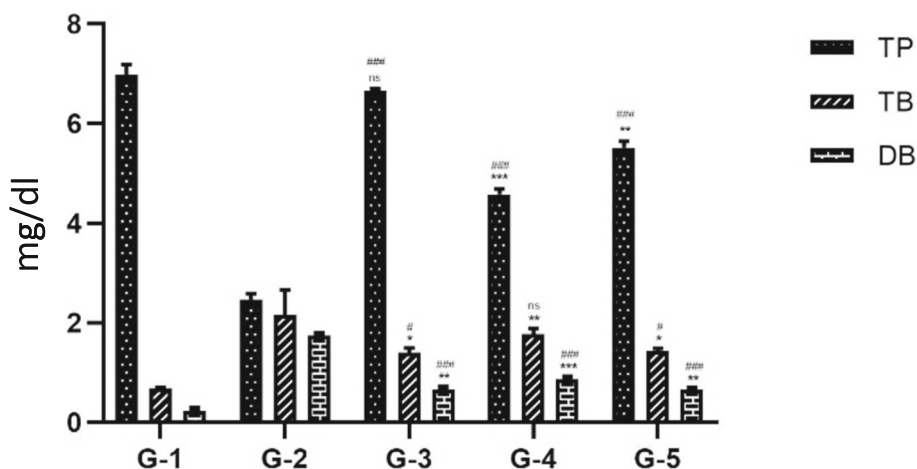
Fig. 3 Effect of GFME on enzyme TP, TB, and DB levels in blood serum of CCl₄ induced hepatotoxicity. Values are mean ±S.E.M. symbol represent statistical significance; *#p<0.02, **##p<0.001 and ***###p<0.0001.* indicates the comparison of G- 1 with G-3, G- 4, G-5; # indicates the comparison of G- 2 with G-3, G- 4, G-5. TP- Total protein, TB- Total bilirubin, DB- Direct bilirubin. G1- normal control, G2-negative control, G3- standard, G4- GFME (200mg), G5- GFME (400mg)

5-(hydroxymethyl)-) can be well absorbed via the intestinal tract. In addition, compound III (Quinic acid) showed good permeability to Cacok2 cell (8.27242) and to MDCK cell model (0.530). In the distribution phase, the PPB binding assessment of compound I exhibited strong binding energy with plasma proteins (predicted value of 38.437%). In addition, BBB penetration revealed that the compound showed low absorption in CNS (predicted value 0.273, 0.511 and 0.428%). The skin permeability is another important risk assessment factor of the compounds during accidental contact with the skin and the compounds had revealed the least skin permeability

(− 4.75733, − 2.61671 and − 4.96717). In the toxicity phase, the carcinogenic effects of the compound were evaluated.

Molecular docking

The docking results have proven that, the ligand molecules of compound I (2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one), compound II (2-furancarboxaldehyde 5-(hydroxymethyl)-) and compound III (Quinic acid) showed the binding affinity of − 4.0, − 4.6 and − 4.3 respectively. There is 1 to 2 hydrogen bonding and good hydrophobic interactions against the amino acid molecules of TGF-β1 protein (Fig. 5).
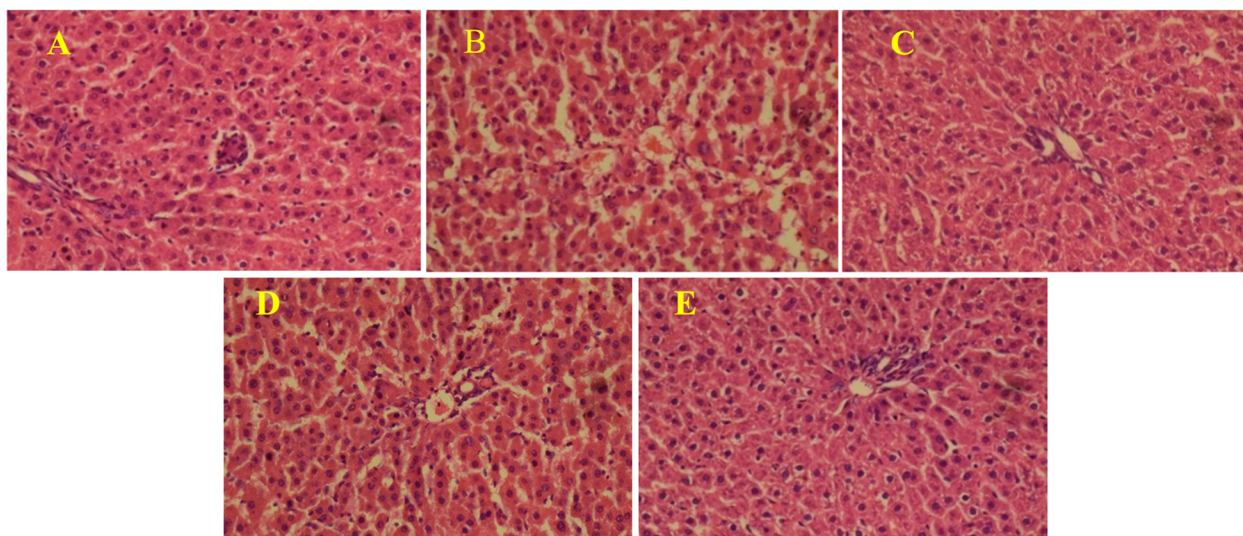

Fig. 4 Histological picture of liver architecture in CCl₄ induced hepatotoxicity in rat. **a** (Liver architecture of Normal control), **b** (Liver architecture of CCl₄ treated), **c** (Liver architecture CCl₄ treatment + 50 mg/kg Silymarin treatment)), **d** (Liver architecture CCl₄ treatment+ 200 mg/kg of GFME), **e** (Liver architecture CCl₄ threatment+ 400 mg/kg of GFME)

Table 3 Physicochemical properties

Compound name	Compound (Chemspider ID)	Physicochemical properties						
		MW	miLogP	TPSA	nON	nOHNH	nrotb	Volume
Sylimarine		482.44	1.47	155.15	10	5	4	400.86
I	106,997	144.12	−0.46	66.76	4	2	0	123.40
II	26,000,801	126.11	0.56	50.44	3	1	2	109.42
III	10,246,715	192.17	−2.33	118.21	6	5	1	161.46

2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II), and Quinic acid (Compound III)

Docking of compound I (2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one), compound II (2-furancarboxaldehyde 5-(hydroxymethyl)-) and compound III (Quinic acid) ligand with PPARα protein also showed a good level of inhibition with the binding affinity of – 5.5, – 5.1 and – 6.2 respectively. There are comparatively good hydrogen bonding and good hydrophobic interactions against the amino acid molecules in relation to the PPARα protein (Fig. 6) (Tables 5 and 6).

Discussion

The intensity of liver damage, ranging from subclinical icteric hepatitis to necro inflammatory hepatitis, cirrhosis and carcinoma have been proved to be associated with redox imbalance and oxidative stress [38]. A balance between oxidant and antioxidant intracellular systems is hence vital for cell function, regulation, and adaptation to diverse growth conditions. Excessive production of ROS may, however lead to oxidative stress, loss of cell function and ultimately apoptosis or necrosis. Recent research in the field of free radical biology suggested an important pathophysiological role of free radicals and oxidative stress in the development and progression of liver diseases [39]. Hence, antioxidants are frequently used to treat oxidative liver injury and the consumption of antioxidants is known to be an important means of preventing or delaying the appearance of liver diseases. The phytochemicals have effective antioxidant potential to treat various human diseases [40]. Antioxidant properties of bioactive compounds are responsible for the hepatoprotection by plant extracts. These compounds effectively reduce the oxidative stress and other inflammatory responses [41].

Plants are the potential source of bioactive compounds for the improvement of new therapeutical agents. The traditional medicine all over the world is at present revalued by an extensive activity of research on different plant species and their therapeutic principles. The utilization of plants as remedies existed thousands of years ago. The knowledge of utilizing specific plants for specific disorders was passed down via oral practice. Ultimately, herbal pharmacopeia records indication regarding medicinal plants [42]. Systematic surveys for medicinal plants have been commenced in many countries because of their contributions to health care. It was clear that the primary benefits of using plant-derived medicines are relatively safer than synthetic alternatives [43].

Natural products include herbals, extracts, formulations, etc., which have been used for the cure of many liver sicknesses in traditional systems of medicine. In the absence of reliable hepatoprotective drugs in modern medicine, a large number of medicinal plants with well-established traditional use have been recommended for the treatment of liver disorders [44]. In this study, the hepatoprotective effectiveness of *Gardenia gummifera* L.f. fruit extracts has been evaluated. This plant is known for its medicinal properties in indigenous medicine in India. It is very rich in phytocompounds, which gives a very strong purpose to select this plant for pharmacological assessment [45]. Earlier reports on medicinal plants suggest that alkaloids [46] and phenolic compounds [47] have wide potential biological properties. Numerous studies propose that the plant is having phenolic and alkaloids which has attracted an extreme deal of scientific and public interest due to the health-

Table 4 ADME and toxic properties

ADME								Toxicity	
Compound name	Chemspider ID	HIA (%)	PPB (%)	BBB (%)	Cacok2 (nm/sec)	Skin Permeability	MDCK (nm/sec)	Rodent Carcinogenicity	
								Carcino Mouse	Carcino Rat
Silymarine		78.55	87.70	0.0596	7.852	−4.236	0.0691	negative	negative
I	106,997	75.91	38.437	0.273	1.85622	−4.75733	24.0956	negative	negative
II	26,000,801	89.88	2.783	0.511	7.647	−2.61671	158.521	negative	positive
II	10,246,715	20.28	6.595	0.428	8.27242	−4.96717	0.530	negative	positive

2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II), and Quinic acid (Compound III). HIA: human intestinal absorption, PPB: plasma protein binding, BBB: the blood-brain barrier

Fig. 5 Ligplot analysis and docking in relation to TGF-β1. **a** Crystal structure of TGF-β1, ligand Silymarin (Standard drug), **b**, **c**, **d** Crystal structures of TGF-β1 with the ligands 2, 3-Dihydro-3, 5-dihydroxy-6-methyl-4 h-pyran-4-one, 2-furancarboxaldehyde 5-(hydroxymethyl)- and Quinic acid

promoting effects exhibited through a large array of bio-logical activities as well as antioxidant properties [48]. CCl_4 metabolism initiates with the trichloromethyl free radical by the action of the cytochrome P-450 oxygenase system. These free radicals, which are primarily formed as relatively unreactive, reacts very rapidly with oxygen to produce a highly reactive peroxytrichloromethyl (CCl_3OO^-) [49]. Both these derivatives are capable of covalently binding to proteins or lipids or abstracting a

hydrogen atom from an unsaturated lipid, thus initiating lipid peroxidation of the cell membrane [50]. Thus, causing damage to cell membranes through disturbing Ca2+ homeostasis, varying enzyme activities, and lastly inducing hepatic damage. This lipid peroxidative deg-radation of the liver cell plasma membrane results in the release of a variety of enzymes such as SGPT, SGOT, and ALP. These are generally positioned in the cytosol and enters into the bloodstream. Estimating the

Fig. 6 Ligplot analysis and docking in relation to PPARα. **a** crystal structure of PPARα, ligand Silymarin (Standard drug), **b**, **c**, **d** Crystal structures of PPARα with the ligands 2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one, 2-furancarboxaldehyde 5-(hydroxymethyl)- and Quinic acid

Table 5 Binding energy, No. of H- Bonds, H- Bond length, H-bond with and hydrophobic interactions of TGF-β1 ligands with receptors

Ligands	Target Protein	Affinity (kcal/mol)	No. of H-bonds	H-bond length (Å)	H-bond with	Hydrophobic interactions
Silymarin	TGF-β1	−6.8	3	3.14, 3.00 3.04	Ser241 Arg240	Phe243, Ile367, Arg244, Glu247, Leu354, Gly353, Val373, Phe216, Glu238, Arg237
Compound I		−4.0	1	2.87	Ser241	Arg244, Leu354, Gly353, Phe216, Arg240
Compound II		−4.6	2	2.82 3.28	Asp351 Lys232	Leu260, Tyr249, Glu245, Phe262, Leu278, Ser280
Compound III		−4.3	2	3.20 3.25	Ser241 Arg244	Leu354, Gly353, Arg240, Ile367

2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II), and Quinic acid (Compound III)

actions of these serum marker enzymes facilitates the assessment of liver function [51]. In this study, the fruit methanol extract of *Gardenia gummifera* L.f. has been used.

The GFME showed the presence of a richness of phytochemicals. The phytochemical screening exposed that the GFME is rich in phytoconstituents such as alkaloids, phenolic compounds, terpenoids, and saponins. Similarly, the quantitative analysis showed the presence of phenolic and alkaloid compounds at the concentrations of 76.5 μg/1 mg and 69.1 μg/1 mg respectively. GC-MS results of GFME revealed the presence of 36 compounds. Among them, 2-Furancarboxaldehyde 5-(hydroxymethyl) - ($C_6H_6O_3$) showed the highest area of percentage (25.94), possessing anti-oxidant and proliferative activities. These compounds are potentially used for the cancer chemoprevention, inhibits the formation of sickled cells in the blood and also possesses antibacterial activity [52]. Another compound Quinic acid ($C_7H_{12}O_6$) showed an area of percentage 13.60, which has been found to possesses potential antibacterial activity [53], used against colon cancer [54]. It has potent antioxidant [55] and anti-inflammatory activities [56].

GFME was evaluated for the DPPH and Nitric oxide radical scavenging assay with ascorbic acid as a standard compound (Table 2). The results revealed that as the concentration of the extract increases the activity also increases, Thus, indicates that the GFME possesses DPPH and Nitric oxide radical scavenging activities with the IC_{50} value of 131.11 and 175.95 μg/ml respectively. It is opined that the fruit extract possesses potent proton donating capacity and could serve as a free radical scavenger or inhibitor.
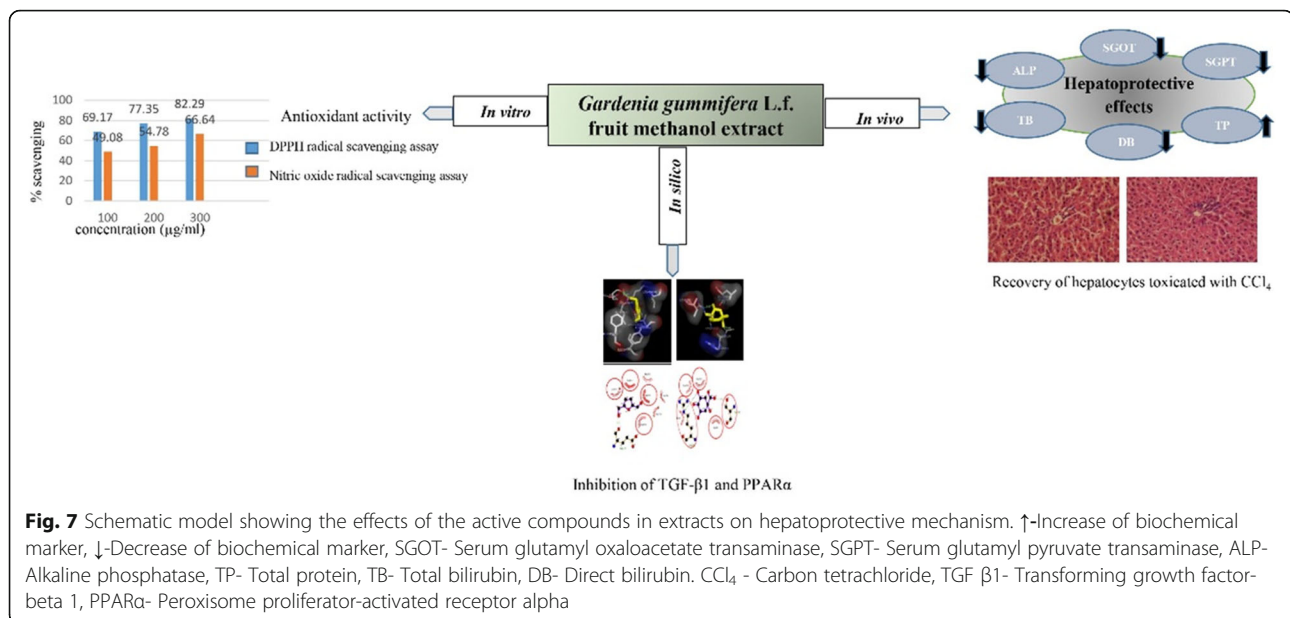
Antioxidants from natural products detoxify the toxins, removes excessive ROS, actively involves in anti-lipid peroxidation and scavenging of free radicals. In the present study, pre-treatment with GFME (200 mg/kg and 400 mg/kg b.w) showed significantly reduced levels of SGPT, SGOT, ALP, TB and DB whereas the total protein levels increased significantly in the extract-treated group. The decrease in the levels of SGOT and SGPT indicates that the extract protected the structural integrity of the hepatocyte cell membrane by stimulating the restoration process of damaged cells. The significant increase in the total protein levels is an indication of the stabilization of endoplasmic reticulum leading to protein synthesis. Reduction in ALP, TB and DB levels reveals that the extract restored the stability of the biliary function during injury with CCl_4. Thus, the results indicate that the GFME possesses protective activity against CCl_4 induced toxicity.

The histological investigation of the liver sections revealed that the normal liver architecture was disturbed by hepatotoxin intoxication in the CCl_4 treated group

Table 6 Binding energy, No. of H- Bonds, H- Bond length, H-bond with and hydrophobic interactions of PPARα ligands with receptors

Ligands	Target Protein	Affinity (kcal/mol)	No. of H-bonds	H-bond length (Å)	H-bond with	Hydrophobic interactions
Silymarin	PPARα	−7.6	4	2.69, 2.84	Cys276 Thr283 Tyr334	Glu282, Glu286, Met220, Leu321, Leu331, Val324, Ile317, Phe318, Ser280, Met355, Met330, Thr279, Val332, Cys278, Cys275, Ala333
Compound I		−5.5	1	2.82	Ala455	Ala454, Lys448, val444, Ile447, Leu456, Glu277
Compound II		−5.1	1	2.75	Glu451	Lys448, Val444, Phe351, Ile447, Ile354, Phe273, Leu456
Compound III		−6.2	1	3.09	Gln277	His274, Glu451, Phe273, Ile354, Phe351, Ala455, Leu456, Ala454

2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one (Compound I), 2-furancarboxaldehyde 5-(hydroxymethyl)-(Compound II), and Quinic acid (Compound III)

Fig. 7 Schematic model showing the effects of the active compounds in extracts on hepatoprotective mechanism. ↑-Increase of biochemical marker, ↓-Decrease of biochemical marker, SGOT- Serum glutamyl oxaloacetate transaminase, SGPT- Serum glutamyl pyruvate transaminase, ALP- Alkaline phosphatase, TP- Total protein, TB- Total bilirubin, DB- Direct bilirubin. CCl_4 - Carbon tetrachloride, TGF β1- Transforming growth factor-beta 1, PPARα- Peroxisome proliferator-activated receptor alpha

compared to the normal group (groups A and B) (Fig. 4). The combination of GFME + CCl_4 (200 mg/kg and 400 mg/kg), (group D and E) exposure showed the recovery of damaged cells by retaining the usual arrangement of the central vein, radiating pattern of cell plates and non-appearance of fat droplets when compared to the hepatocytes of CCl_4 damaged groups and further regeneration of hepatic cells was seen in the group C animals (Silymarin + CCl_4). The histological observations provide supplementary support to the efficiency of the Gardenia fruit methanol extract in reducing liver damage caused by CCl_4 intoxication.

The phytocompounds identified from GC-MS profiling obtained in higher concentrations were subjected to in silico analysis. A total of 36 phytocompounds were identified in the GC-MS analysis. Out of the total 03 were subjected for in silico analysis based on the area of percentage and their efficacy in terms of their antioxidant and pharmacological properties as established from the earlier reports [57–59].

In the process of drug development preliminary in silico analysis play a key role. The GC-MS analysis and screening of in vitro and in vivo efficacy of *Gardenia gummifera* L.f. have strengthened the traditional medicinal claim. Further studies were carried out to screen the ADMET properties and molecular docking. The targets were chosen based on the specificity of the literature data on the hepatoprotective mechanism [60]. The proteins, transforming growth factor-beta 1 (TGF-β1) play a major role in redox imbalance by up-regulation of ROS production and the down regulation of the anti-oxidant defense system. Similarly, peroxisome proliferator-activated receptor α (PPARα), is a ligand-activated transcription factor and

member of the superfamily of the nuclear hormone receptor, which plays a prominent role in maintaining glucose and lipid homeostasis by modulating gene expression of hepatocytes [61]. The in silico study predicted that the compounds I, II and III have not strictly violated any rule. Comparatively compound II has good bioactivity, good flexibility, and permeability and thus indicating its potentiality to be a good drug candidate. The above data may help for further analysis of pure compounds. Inhibition of proteins TGF-β1 and PPARα has been proposed as the most likely mechanism of the hepatoprotective effect. The effects of the active compounds in extracts on hepatoprotective mechanism depicted in Fig. 7. In general, it is predicted that among the phytocompounds tested the inhibition efficiency of compound II and compound III is nearly equal to a standard drug concerning target protein TGF-β1 and PPARα respectively. Hence, the in silico studies support the hepatoprotective activity *Gardenia gummifera* L.f. fruit methanol extract and the present prediction may help for the future screening of drug development on hepatic diseases.

Conclusion
The findings of this study showed that fruit methanolic extract of *Gardenia gummifera* L.f. exhibited potential protective action against the hepatotoxicity induced by CCl_4. The hepatoprotective role of GFME might be due to the presence of chemical constituents like phenolic compounds and alkaloids. These phytocompounds may offer antioxidant activity and thus preventing the oxidative stress-induced in the liver. Hence, GFME may act as a prophylactic as well as a curative drug in treating hepatotoxic conditions.

Abbreviations
GFME: *Gardenia gummifera* L.f. fruit methanol extract; ADME/T: Absorption distribution metabolism excretion toxicity; CCl$_4$: Carbon tetrachloride; PPARα: Peroxisome proliferator-activated receptor alpha; TGF-β1: Transforming growth factor-beta 1; DPPH: 2,2-diphenylpicrylhydrazyl

Authors' contributions
Concept – VNM; Design – VNM; RM; KV; Supervision – RM; KV; Resources – VNM; RM; KV; Materials – VNM; RM; RB;KV;SLS; Data Collection and/or Processing – VNM; RB; SLS; Analysis and/or Interpretation – VNM; RM; KV; SLS; Literature Search – VNM;RM; RB; SLS; Writing – VNM; RM; KV; Critical Reviews – VNM; RM; KV; RB; SLS; All authors read and approved the final manuscript.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article.

Ethics approval
The study protocol was approved by the National College of Pharmacy, Shimoga.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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