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Original article

The antioxidant rich active principles of *Clerodendrum* sp. controls haloalkane xenobiotic induced hepatic damage in murine model

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ABSTRACT

Clerodendrum is a plant with potent antioxidant activity and has been frequently employed as a traditional remedy against bronchitis, asthma, liver and stomach disorders. Three species of genus *Clerodendrum* namely *Clerodendrum indicum, C. colebrookianum* and *C. inerme* (Syn. *Volkameria inermis*) were investigated for their possible activity against oxidative stress induced liver injury. Apart from generation of Reactive Oxygen Species (ROS) in the WRL-68 cell line (human hepatic cell line), *in-vitro* and *in-vivo* antioxidant assays were also assessed. Features of immune cell proliferation (MTT) were analyzed thoroughly. Gas Chromatography-Mass Spectrometry (GC–MS) and Fourier Transform Infrared Spectroscopy (FTIR) analyses have been performed to identify the active biological compounds. These active biological compounds were further subjected to molecular docking. The antioxidant activity of three *Clerodendrum* sp. was significantly high in DPPH, nitric oxide, hydroxyl radical and hydrogen peroxide etc. Biochemical parameters like catalase, superoxide dismutase (SOD) and reduced glutathione (GSH) were generated in excess due to CCl_4 administration, which was ameliorated by treating with *Clerodendrum* extract. The phytochemical 24,25-Dihydroxyvitamin D shows excellent binding affinity in Autodock Vina. The present study provided convincing evidences that *C. indicum* and *C. inerme* showed good result but *C. colebrookianum* performed better by almost all means.

1. Introduction

Oxidative stress is a condition where there is an imbalance between the generation of free radicals (reactive oxygen species and reactive nitrogen species) and the inability to detoxify them through protective mechanisms (natural antioxidant). The aerobic cell produces ROS by different endogenous and exogenous factors. ROS is produced by myeloperoxidase (MPO) - Halide - H_2O_2 system where in the presence of chloride ion, H_2O_2 is converted to hypochlorous acid, a potent oxidizing agent (Nimse and Pal, 2015). ROS in low concentration governs imperative physiological functions like cellular growth and expression and subsequently, bolsters defense mechanism against infection (Nimse and Pal, 2015). Unfortunately, due to redox imbalance an increasing risk of diseases like diabetes, cancer, obesity, rheumatoid arthritis, cognitive disorders becomes inevitable (Durackova, 2010; Poyton et al., 2009). In hypoxic environment the mitochondrial respiratory chain can also generate reactive nitrogen species (RNS) (Durackova, 2010). RNS induces excessive lipid peroxidation which may lead to the production of other reactive species like reactive aldehydes and malondialdehyde (Mittler, 2002).

Liver fibrosis is the precursor for different hepatic disorders, of all the liver dysfunctions, contact with toxic reagents and drug is prime (Cichoż-Lach and Michalak, 2014). Carbon tetrachloride (CCl_4) is considered as a haloalkene which is extensively used to

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generate oxidative stress and cause the liver injury (Weber et al., 2003).

Clerodendrum is a member of Lamiaceae family; mostly they are perennial shrub genus and in Indian Ayurvedic system it is used in many of the herbal preparations. The species of *Clerodendrum* are distributed in tropical regions of Asia including India, Myanmar, Bangladesh, Malayasia, Indonesia, Thailand, Bhutan, Nepal and also in temperate Tibet (Hooker, 1885). This genus contains several species; however three species are widely used for medicinal purposes in India. These are *C. indicum* (L.) Kuntze, *C. inerme* (L.) Gaertn. (Syn. *Volkameria inermis* L.) and *C. colebrookianum* Walp. Ethnomedicinally, different parts including leaf, stem and root extracts have been proposed to be instrumental in healing different ailments in North-East India as well as in Chinese medicine (Kar et al., 2014). Although, ethnobotanically they have diverse medicinal properties but their experimental validation is largely incomprehensible. Therefore, the present investigation aims for an in-depth analysis about antioxidant and hepatoprotective potentialities of *C. indicum*, *V. inermis* and *C. colebrookianum* in CCl_4 induced murine model.

2. Materials and methods

2.1. Collection of plant material, extract preparation and determination of free radical scavenging activity

Leaves of all three species namely *C. indicum* (CIL), *V. inermis* (VIL) and *C. colebrookianum* (CCL) were collected from the medicinal plant Garden, NBU and roadside from Azra, Guwahati, Assam and were submitted to the Herbarium. Plant extract was prepared as per the standardized method of Dutta et al. (2018). The plant extracts were used to evaluate a total of twelve *in-vitro* free radical scavenging activity as well as Erythrocyte membrane stabilizing activity (EMSA), MTT, haemolytic assay, phenol and flavonoid content (Dutta et al., 2018).

2.2. Measurement of intracellular ROS generation

The cell line WRL-68 which is a human hepatic was used for general quantification of H_2O_2 production using a fluorescent dye 2′-7′ dichlorofluorescin (H_2DCF). Oxidation of 2′-7′ dichlorofluorescin (H_2DCF) to 2′-7′ dichlorofluorescein (DCF) and detection of intracellular ROS generation was measured as per the modified method of Dutta et al. (2018).

2.3. Hepatoprotective activity

2.3.1. Animal maintenance, acute toxicity study and experimental design

In breed, Swiss albino mice (6–8 weeks of age and 33 ± 2gms body weight; 6 male mice/group, n = 6) were taken from the animal house of Department of Zoology, NBU and were kept in polypropylene cages with proper bedding material with sufficient food and water *ad-libitum*. The animals in the animal house were exposed to constant 12 h dark/light cycle at a temperature of 25 °C. All the experiments were approved by the ethical

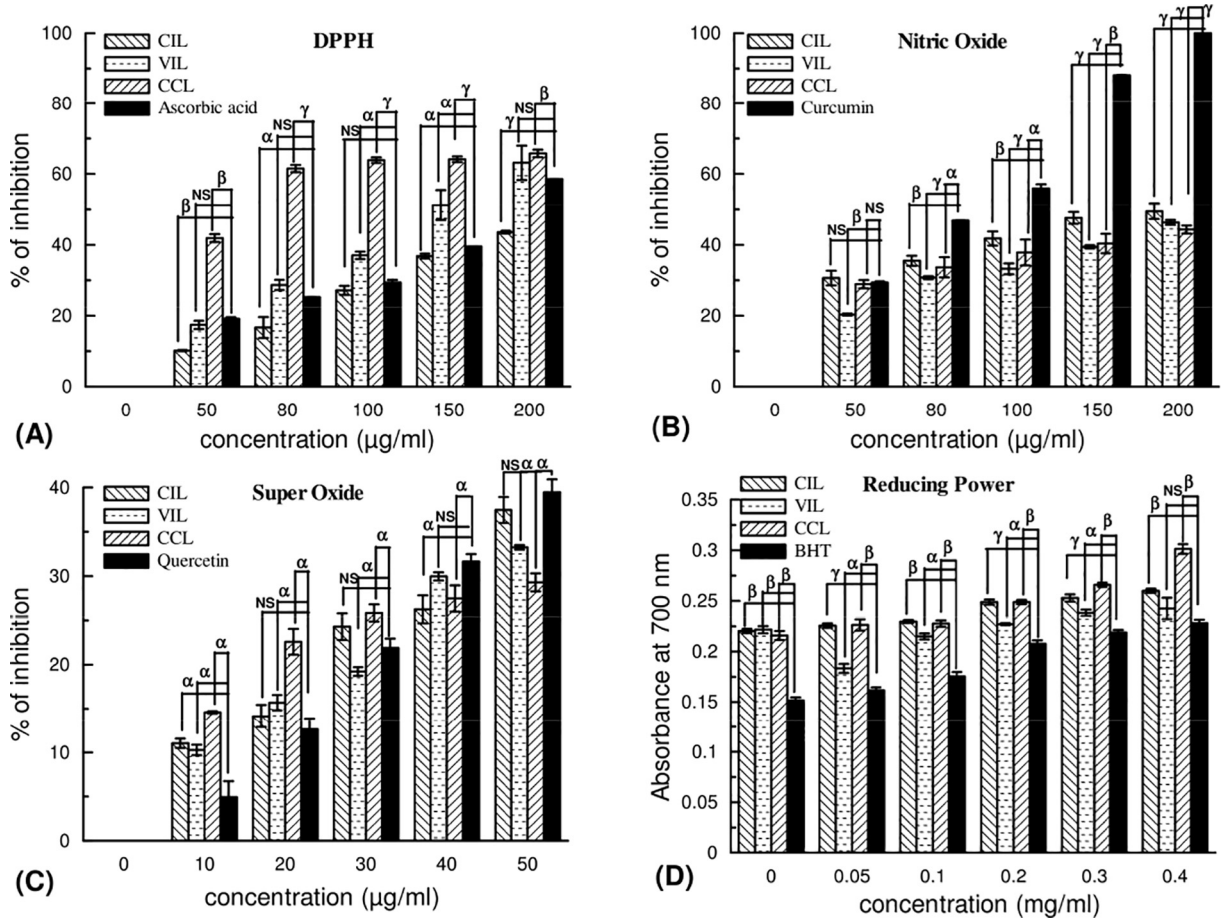

Fig. 1. Antioxidant activity of *C. indicum*, *V. inermis* and *C. colebrookianum*. **(A)** DPPH activity; **(B)** Nitric oxide scavenging activity; **(C)** Super oxide radical scavenging activity; **(D)** Reducing power assay. Data expressed as mean ± S.D (n = 6). $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; NS-Non significant when compared with standard.

committee, Department of Zoology (No. 840/ac/04/CPCSEA and dated: 11/08/2014). Mice were kept on overnight fasting prior to the experiment followed by oral administration of plant extracts (CIL, VIL and CCL) at an increasing dose of 250, 500, 1000 and 2000 mg/kg body weight (BW) to study the acute toxicity following OECD guidelines. The groups were carefully observed for toxicological symptoms at different time intervals, first after 30 min and then at 2, 4, 8, 24 and 48 h. After selection of the doses, Swiss albino mice (54 mice) were randomly divided into nine groups (n = 6) and the plant extracts were administrated daily for 21 days. Control group was given normal saline water; CCl_4 group had 1:1 (v/v) CCl_4 mixed with olive oil; Silymarin group was given 1:1 (v/v) CCl_4 in olive oil as well as 100 mg/kg BW silymarin; low dose group (CILL, VILL and CCLL) received 1:1 (v/v) CCl_4 in olive oil and 50 mg/kg BW plant extract, whereas, high dose extract (CILH, VILH and CCLH) groups received 1:1 (v/v) CCl_4 in olive oil and 200 mg/kg BW plant extract respectively. Hereby 24 hrs after the last dose i.e. on the 22nd day all the animals were sacrificed under proper anesthesia (mild 2% ether) by cervical dislocation. The liver of the diseased mice was collected and the liver supernatant was prepared as per the method of Dutta et al. (2018). For histological studies the remaining liver tissues were chopped into fine pieces and preserved overnight in 10% formaldehyde solution for dehydration. The liver sections were prepared and stained by the method of Dey et al. (2016).

2.3.2. Determination of peroxidase, catalase (CAT), reduced glutathione (GSH), superoxide dismutase (SOD), lipid peroxidation (LPO) and NO release activity

Peroxidase, Catalase (CAT), Superoxide dismutase (SOD), Reduced Glutathione (GSH) activity and NO release activity was measured according to the standard method (Dey et al., 2016; Dutta et al., 2018). The amount of MDA was quantified employing TBARS assay kit (Cayman, USA).

2.4. FTIR, GC–MS analysis and molecular docking study

FTIR and GC–MS analyses were performed according to the standard protocol with slight modifications. The most prominent and active compounds detected through GC–MS were subjected to molecular docking analysis against proteins. The crystallographic structures of the proteins stored in PDB database (http://www.rcsb.org) were downloaded. Molecular docking was performed through AutoDock Vina (Trott and Olson, 2010). Standard method employed by Dutta et al. (2018).

2.5. Statistical analysis

Statistical analysis pertaining to one way analysis of variance (ANOVA) was performed employing KyPlot version 5.0 beta.

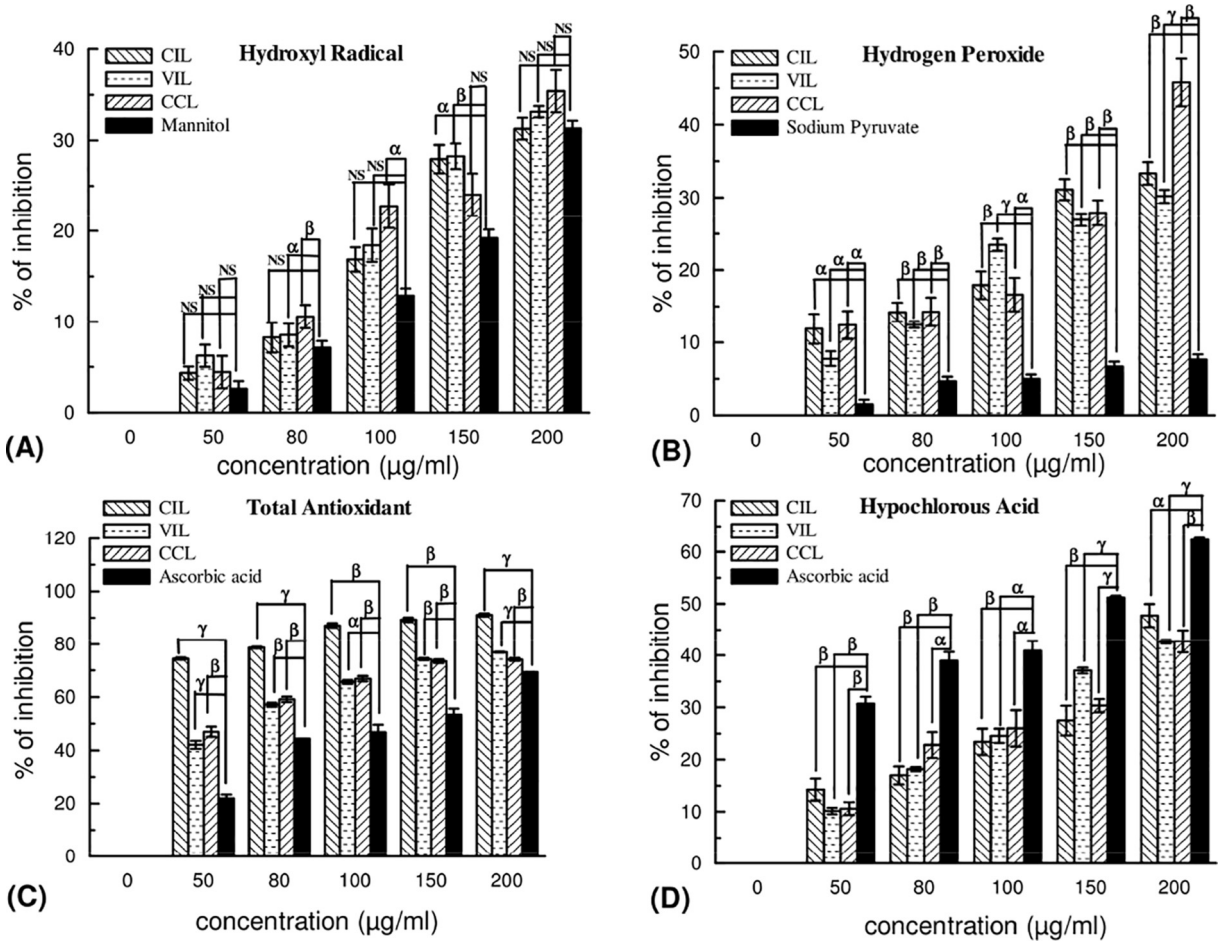

Fig. 2. Antioxidant activity of *C. indicum*, *V. inermis* and *C. colebrookianum*. **(A)** Hydroxyl radical scavenging assay; **(B)** Hydrogen peroxide scavenging activity; **(C)** Total antioxidant scavenging activity; **(D)** Hypochlorous acid scavenging assay. Data expressed as mean ± S.D (n = 6). $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; NS-Non significant when compared with standard.

3. Results and discussion

3.1. In-vitro antioxidant activity

In the present study, all three *Clerodendrum* species, *C. indicum* (CIL), *V. inermis* (VIL) and *C. colebrookianum* (CCL) exhibited higher free radical scavenging activity with respect to standard ascorbic acid (Fig. 1A) (Blois, 1958). Among the three extracts, the CCL (65.78 ± 1.04%) extract showed the highest percent of inhibition. The property of DPPH is to accept an electron or hydrogen radical to attain stability, which changes the colour of the solution due to the presence of natural antioxidant (Huang et al., 2005). Hydroxyl radical (Fig. 2A), peroxinitrate (Fig. 3A) and hydrogen peroxide (Fig. 2B) assays also revealed highly significant scavenging activity. In the peroxisomes, hydrogen peroxide (H_2O_2) is produced from superoxide in the presence of superoxide dismutase (Matés and Sánchez-Jiménez, 2000; Ray and Husain, 2002), it accumulates in cells and converts into Hydroxyl radical ($OH^{\cdot}$) when it comes in contact with other transition metals like Fe^{2+}, Cu^{2+}, etc. (Wanasundara and Shahidi, 1995). In mitochondria, highly toxic superoxide anion ($O_2^{\cdot-}$) are produced which undergoes spontaneous dismutation and generates singlet oxygen. The percent of inhibition of singlet oxygen by *Clerodendrum* sp. showed moderate scavenging activity (Fig. 3B) when compared to standard Lipoic acid. In the present experiment, CIL (77.81 ± 2.45% at 200 μg/ml) and CCL (77.28 ± 0.68% at 200 μg/ml) extracts discolor the ferrozine-complex solution, indicating its iron chelating capacity (Fig. 4Aa and b) and the presence of active components (Blois, 1958). The CCL extract had higher reducing power activity (Fig. 1D) with respect to standard BHT. All concerned extracts exhibited better total antioxidant potential with respect to standard ascorbic acid (Fig. 2C). For nitric oxide scavenging activity (Fig. 1B), superoxide anion activity (Fig. 1C), hypochlorous acid (Fig. 2D) and lipid peroxidation (Fig. 4B) scavenging assay, the extracts showed moderate activity. Nitric oxide, a free radical generated from amino acid L-arginine by nitric oxide synthase (NOS), damages several biological molecules in human body and has been associated with destructive consequences (Gimenez-Garzó et al., 2015; Sehitoglu et al., 2015). *Clerodendrum* extracts show efficient scavenging of nitric oxide. CCL, on the other hand, contained highest phenolic and flavonoid compounds (67.29 ± 3.15 mg/ml keeping gallic acid as standard per 100 mg plant extract and 48.71 ± 1.69 mg/ml keeping quercetin as standard per 100 mg plant extract). Detailed percent of inhibition and IC_{50} values of the respective *in-vitro* antioxidant tests are enlisted in Table 1.

3.2. Erythrocyte membrane stabilizing activity (EMSA)

Erythrocyte Membrane Stabilizing Activity (EMSA) (Fig. 3D) is a vital procedure to indirectly evaluate the superoxide radical induced antioxidant capacity of erythrocyte membrane. CCL extract showed excellent membrane stabilizing activity (30.68 ± 0.77%) which might in turn aid to improve the immune system.

3.3. Haemolytic activity

Haemolytic activity is a parameter to evaluate cytotoxicity, mediated by natural compounds present in the plant extracts. In the mentioned experiment VIL extract showed higher percent of inhibition (24.51 ± 1.12%) than the rest two extracts (Fig. 3C).

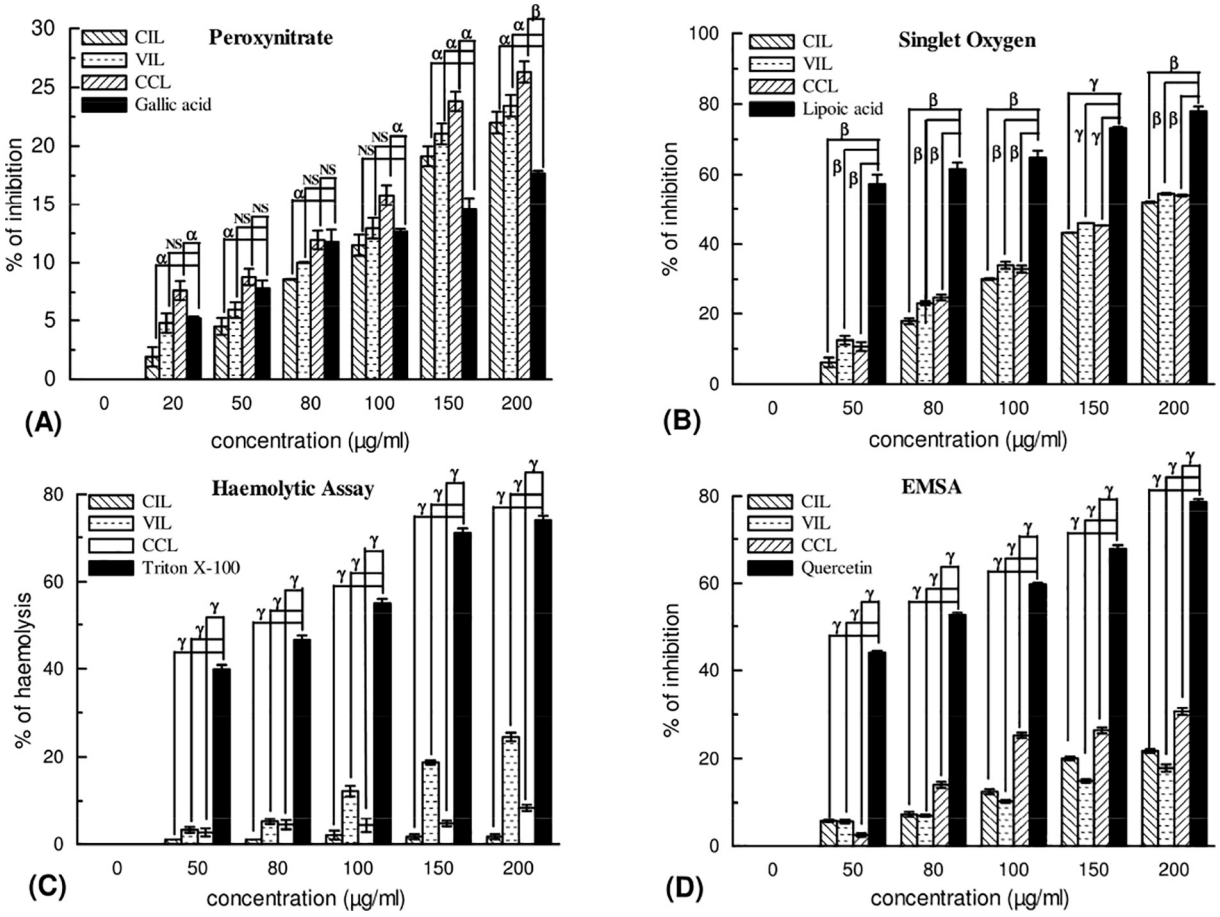

Fig. 3. Antioxidant activity of *C. indicum*, *V. inermis* and *C. colebrookianum*. **(A)** Peroxynitrate scavenging assay; **(B)** Singlet oxygen scavenging activity; **(C)** Haemolytic assay; **(D)** Erythrocyte membrane stabilizing activity. Data expressed as mean ± S.D (n = 6). $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; NS-Non significant when compared with standard.

3.4. Measurement of cell viability (MTT assay)

Cell viability effects of CIL, VIL and CCL extracts were estimated using MTT colorimetric assay on splenocyte cells. After incubating the splenocyte cells at different concentrations of CIL, VIL and CCL for 4 h the results were obtained. Fig. 4C showed that the extracts were non-toxic to splenocyte cells. However, CCL extract of the present study stimulated the proliferation of mice splenocyte with increasing order of their doses.

3.5. Measurement of ROS generation in WRL-68

Cellular response in case of ROS is generally characterized by the up regulation of antioxidants such as superoxide dismutase

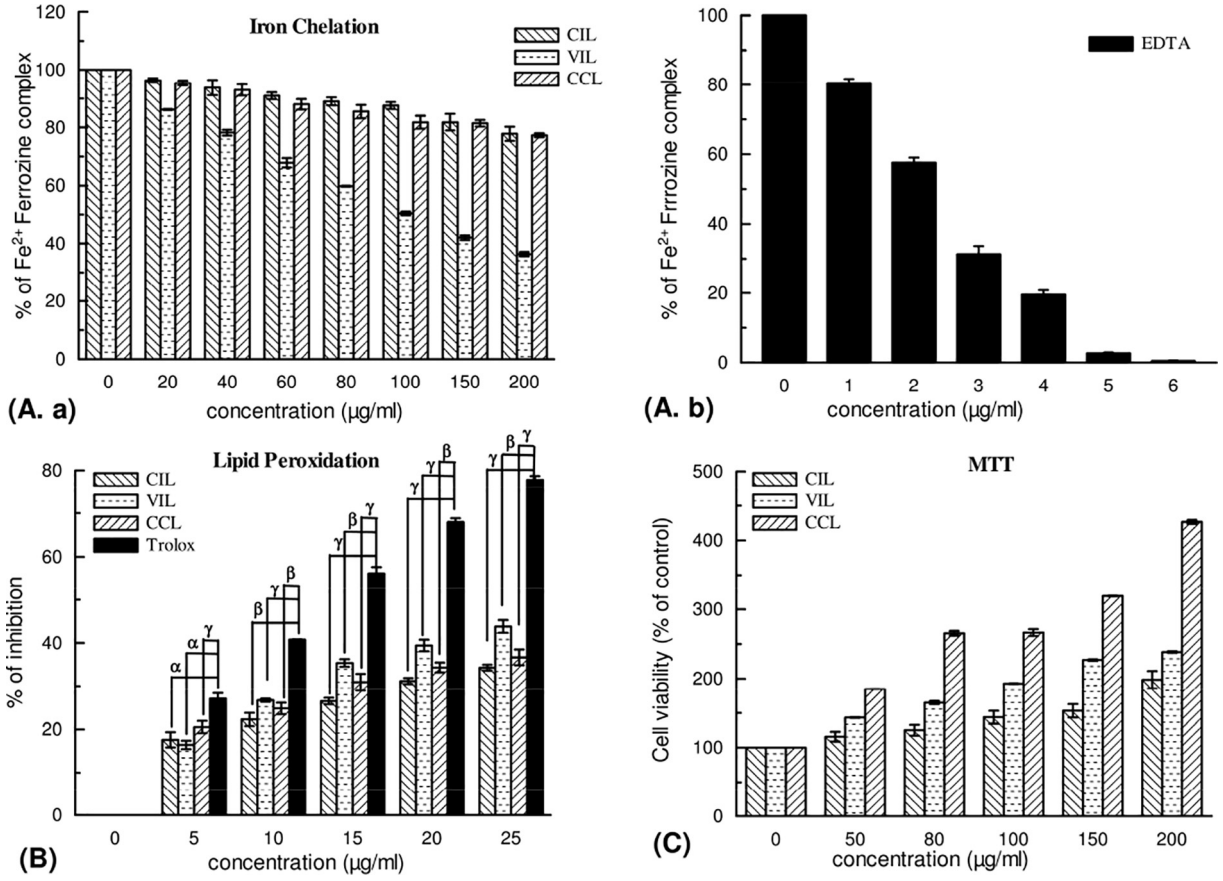

Fig. 4. Antioxidant activity of *C. indicum*, *V. inermis* and *C. colebrookianum*. **(A)** Iron chelation assay; **(B)** Lipid peroxidation activity; **(C)** MTT cell proliferation assay. Data expressed as mean ± S.D (n = 6). Data expressed as mean ± S.D (n = 6). $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; NS-Non significant when compared with standard.

Table 1
Percent (%) of inhibition and IC_{50} (in parenthesis) values of three *Clerodendrum* species (CIL, VIL and CCL) and standard for different antioxidant and free radical scavenging assays.

Parameters	CIL	VIL	CCL	Standard
DPPH	43.6 ± 0.4 (287.8 ± 11.9**)	63.1 ± 4.8 (160.5 ± 16.6*)	65.7 ± 1.0 (67.8 ± 2.5***)	58.5 ± 0.02 (203.2 ± 1.9)
Hydroxyl Radical	31.3 ± 1.2 (484.6 ± 28.2*)	33.13 ± 0.6 (452.1 ± 29.0*)	35.39 ± 2.3 (438.3 ± 39.9*)	31.3 ± 0.8 (597.2 ± 11.9)
Hydrogen Peroxide	33.3 ± 1.5 (396.4 ± 24.4**)	30.2 ± 0.8 (433.5 ± 16.8**)	45.8 ± 3.3 (348.74 ± 26.7**)	7.64 ± 0.68 (2185.2 ± 187.4)
Nitric Oxide	49.4 ± 2.2 (155.06 ± 8.05**)	46.4 ± 0.7 (211.3 ± 6.3***)	44.31 ± 1.1 (188.7 ± 21.02**)	100 ± 0.00 (61.17 ± 0.41)
Superoxide Anion	37.4 ± 1.4 (373.4 ± 25.2**)	33.3 ± 0.3 (395.1 ± 9.3***)	29.3 ± 1.02 (366.7 ± 20.3**)	39.5 ± 1.5 (94.5 ± 3.7)
Hypochlorous Acid	47.7 ± 2.2 (308.3 ± 33.3*)	42.6 ± 0.3 (293.6 ± 7.6**)	42.7 ± 2.1 (299.9 ± 17.9**)	62.4 ± 0.4 (130.07 ± 5.12)
Total Antioxidant Activity	91.1 ± 0.6 (18.03 ± 0.3**)	76.8 ± 0.2 (58.9 ± 1.8**)	74.1 ± 0.5 (55.7 ± 2.7**)	69.3 ± 0.07 (116.5 ± 5.9)
Peroxynitrite	22.0 ± 0.9 (724.0 ± 40.2***)	23.4 ± 0.9 (637.8 ± 25.9*)	26.3 ± 0.9 (525.9 ± 22.06**)	17.6 ± 0.2 (799.3 ± 36.9)
Singlet Oxygen	52.02 ± 0.3 (240.4 ± 3.8^{NS})	54.5 ± 0.2 (202.2 ± 4.5***)	54.05 ± 0.3 (205.9 ± 4.8***)	77.9 ± 1.3 (48.4 ± 3.6)
Lipid Peroxidation	34.3 ± 0.6 (316.6 ± 13.8**)	43.8 ± 1.4 (226.3 ± 7.5*)	36.6 ± 1.7 (270.3 ± 20.3**)	77.8 ± 0.9 (11.1 ± 0.2)
Iron chelation	77.8 ± 2.4 (676.2 ± 45.2)	36.3 ± 0.7 (116.4 ± 3.5)	77.3 ± 0.6 (576.2 ± 57.4)	0.4 ± 0.2 (1.4 ± 0.0)

Units in µg/ml. Data expressed as mean ± S.D (n = 6). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; NS-Non significant when compared with standard.

(SOD) and plays a vital part in apoptosis. In the control group of WRL-68 (Fig. 5A), the scattered green fluorescence light indicates that ROS formation was of minimal quantity. Whereas, H_2O_2 and CCl_4 groups of WRL-68 (Fig. 5B and C) showed a large amount of ROS generation. Among the three plants, CCL group (Fig. 5F) induced light fluorescence indicating a low amount of ROS generation compared to CIL (Fig. 5D) and VIL (Fig. 5E). The change in fluorescence intensity indicates that CIL, VIL and CCL extracts has an upper hand in the resultant production of intracellular ROS. Thus, it has been evident that *Clerodendrum* extracts possesses both *in-vivo* and *in-vitro* antioxidant capacity and might have potential to prevent different types of oxidative stress related disorders.

3.6. Hepatoprotective activity

3.6.1. Acute toxicity study

CIL, VIL and CCL extracts were administered orally and none of the mice died even at 2000 mg/kg dose. Therefore, 1/40th (50 mg/kg) and 1/10th (200 mg/kg) of the maximum dose were considered safe for *in-vivo* studies.

3.6.2. Body weight changes

Changes in mouse body weight after the treatment of CCl_4, silymarin, CIL, VIL and CCL have been displayed in Table 2. Treated mice displayed no significant gain in weight.

3.6.3. In-vivo antioxidant assays

In the present study, we tried to further investigate how free radicals are linked to hepatic damage and the potential therapeutic role of *Clerodendrum* extracts in this regard. Haloalkane CCl_4 is extensively used in industrial sectors; however, it is always associated with environmental toxicity and occupational hazards (Ruprah et al., 1985). Partial pressure of reactive oxygen in tissues is responsible for carbon tetrachloride (CCl_4) induced hepatotoxicity. CCl_3^* and $CHCl_2^*$ radicals are produced by the low partial pressure of oxygen (De Groot et al., 1988). Lipid peroxidation, resulting from oxidative stress, is a vital reason for hepatic injury (De Groot et al., 1988). In the present study there is a significant inhibition of catalase, reduced glutathione (GSH) and superoxide dismutase (SOD) activity by *Clerodendrum* (CIL, VIL and CCL) extract, which occurred in CCl_4 intoxicated mice compared to the control group (Fig. 6A, C and D). *C. colebrookianum* treatment showed a significant increase in the percent of inhibition of catalase and reduced glutathione when compared to other *Clerodendrum* species (CIL and VIL) and CCl_4 intoxicated groups. CCl_4 treatment significantly lowers the peroxidase enzyme activity in hepatic tissue (Fig. 6B). Peroxidase activity enhanced significantly (15.37 ± 0.45 unit/mg tissues) after CCLH administration and was eventually better than standard silymarin treated case (12.73 ± 0.93 unit/mg tissue). Interestingly, MDA level was significantly decrease when treated with CCLH which was even lower than the MDA level in control group (Fig. 6E). NO level was increased due to CCl_4 toxicity (Fig. 6F) and significant ($p < 0.001$) reduction of NO level was distinctly evident in the treated group

Table 2
Effects of three *Clerodendrum* species (CIL, VIL and CCL) on the body weight of the treated mice.

Group	Initial weight	Final weight	% Body weight change
Control	33.98 ± 1.65	35.99 ± 0.71^{NS}	5.61 ± 3.06▲
CCl_4	36.95 ± 1.34	$32.71 \pm 1.86^{**}$	−13.05 ± 2.28▼
Silymarin	35.57 ± 1.26	36.93 ± 1.63^{NS}	3.64 ± 1.99▲
CIL 50 mg/kg	33.01 ± 1.97	35.32 ± 0.82^{NS}	6.59 ± 3.44▲
CIL 200 mg/kg	33.08 ± 1.93	35.36 ± 0.88^{NS}	6.49 ± 3.15▲
VIL 50 mg/kg	33.05 ± 1.95	35.34 ± 0.8^{NS}	6.53 ± 3.41▲
VIL 200 mg/kg	33.01 ± 1.92	$35.42 \pm 0.98^{*}$	6.86 ± 2.80▲
CCL 50 mg/kg	32.05 ± 1.04	$35.51 \pm 1.1^{**}$	9.73 ± 1.29▲
CCL 200 mg/kg	31.34 ± 0.86	$35.09 \pm 0.47^{*}$	10.67 ± 2.50▲

[a]Weight (mean ± SD) in gram, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, NS = Non significant. Final body weight was compared with initial body weight of corresponding group. ▲ Increase weight; ▼ Decrease weight.

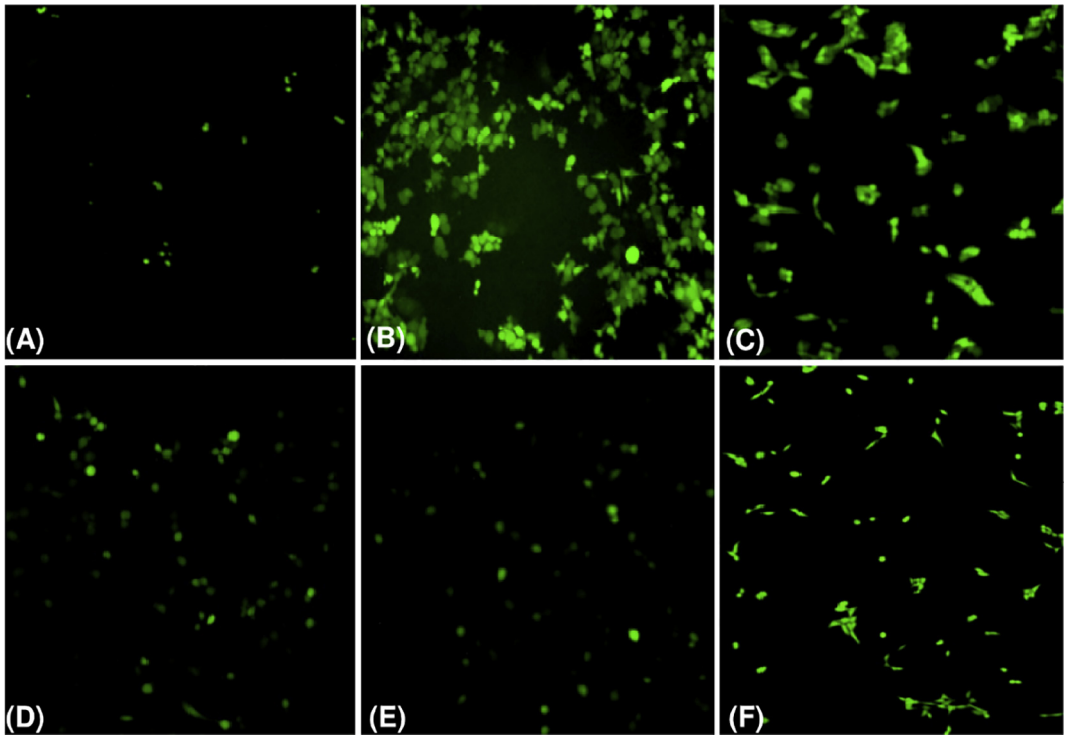

Fig. 5. Effects of *Clerodendrum* species (CIL, VIL and CCL) on oxidative stress in the Human Hepatic Cell Line (WRL-68). (**A**) Control cell culture; (**B**) Cells exposed to 200 µg/ml concentration of H_2O_2; (**C**) Cells exposed to 200 µg/ml concentration of CCl_4; (**D–F**) Cells exposed to 200 µg/ml concentration of CIL, VIL and CCL for 24 h.

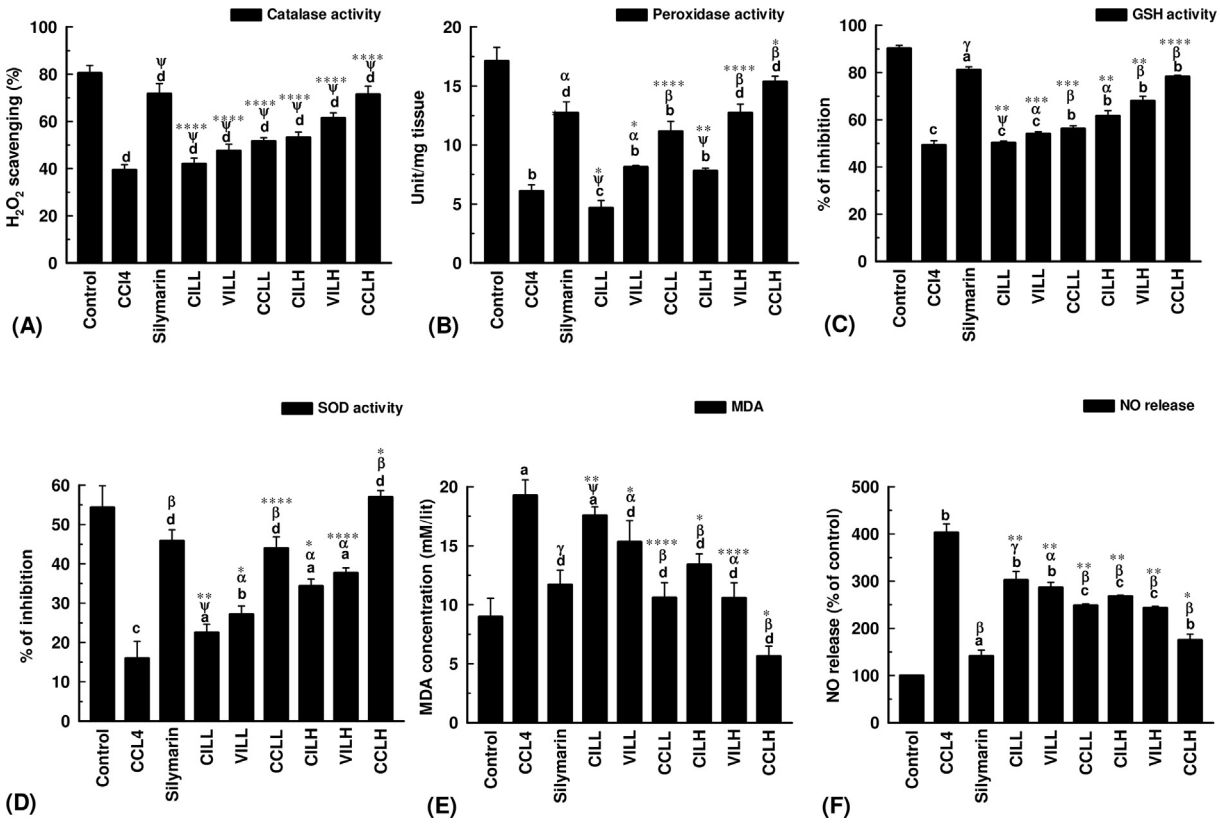

Fig. 6. The effect of *Clerodendrum* extracts (CIL, VIL and CCL) on (**A**) Catalase activity; (**B**) Peroxidase activity; (**C**) Reduced Glutathione (GSH) activity; (**D**) Superoxide Dismutase (SOD) activity; (**E**) Lipid Peroxidation (LPO) activity; (**F**) NO release. Data expressed as mean ± S.D (n = 6). $^{a}p < 0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$; ^{d}p = non significant vs control group; $^{\alpha}p < 0.05$; $^{\beta}p < 0.01$; $^{\gamma}p < 0.001$; $^{\psi}p$ = non significant vs CCl_4 group; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p$ = non significant vs silymarin group.

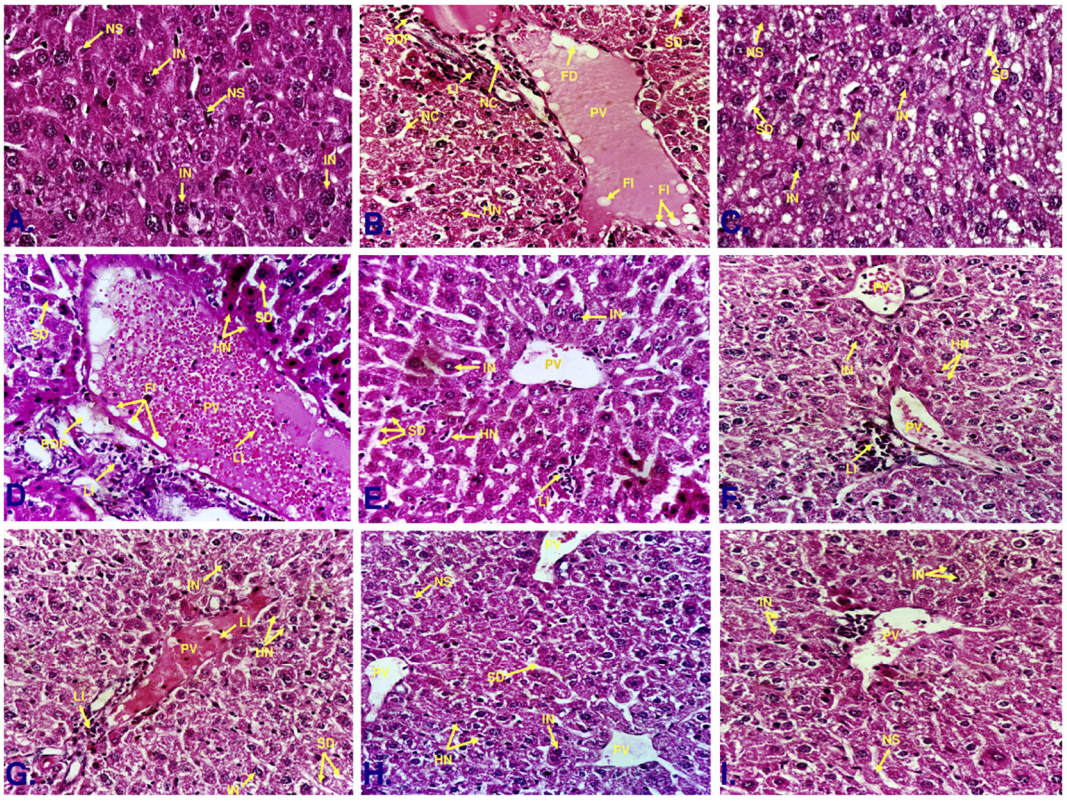

Fig. 7. Photomicrographs (400×) of the histopathological examinations of the liver samples of different groups. (**A**) Control group (**B**) CCl_4 group (**C**) Silymarin group (**D**) CILL group (**E**) CILH group (**F**) VILL group (**G**) VILH group (**H**) CCLL group (**I**) CCLH [intact nucleus (IN), normal sinusoids (NS), fatty infiltrations (FI), Necrotic hepatocytes (N), leukocyte infiltrations (LI), prominent calcification (C), congested vesicles (VC), bile duct proliferations (BdP), sinusoidal dilations (SD), haemorrhagic necrosis (HN), portal veins (PV)].

as well as in control. Catalase is an antioxidative enzyme; it limits the generation of $OH^{\cdot}$ by H_2O_2 scavenging. SOD (super oxide dismutase) and GSH (Glutathione reductase) are other enzymes that catalyze the formation of ordinary molecular oxygen or hydrogen peroxide from superoxide radicals. The administration of *Clerodendrum* increases the activity of catalase, peroxidase, reduced glutathione, superoxide dismutase levels and stabilizes the elevated level of MDA and NO.

3.6.4. Histopathological examination

Histopathological results demonstrate structural changes in hepatic tissue of different treatment groups (Fig. 7). The haematoxilin-eosin staining of CCl_4 groups showed different types of liver damage (Fig. 7B), whereas, administration of silymarin significantly regulated the injury level (Fig. 7C). Interestingly, in the present study it came out that CCLH (Fig. 7I) brought down the injury more effectively when compared to the standard silymarin. The detailed histopathological study also supports the *in-vitro* antioxidant and *in-vivo* hepatoprotective activity and helps in attenuation of deformed hepatic cellular architectures. Cellular metabolism is also responsible for the production of oxidative species which takes part in proliferation, cell activation and migration (Droge, 2002).

3.7. FTIR analysis

FTIR is a suitable method to identify and validate the components present in herbal formulations (Liu et al., 2006). Functional groups present in three *Clerodendrum* extracts were identified by using Fourier transform infrared spectroscopy (FTIR). The IR spectrum of CIL, VIL and CCL extracts indicated the presence of major peaks like amines, ether, alcohol and carboxylic acid at 1081 cm^{-1}, 1245 cm^{-1}, 3294 cm^{-1}, 1734 cm^{-1} respectively. The peak ratio of FTIR analysis revealed that CIL, VIL and CCL extracts are natural sources of several vital phyto-compounds.

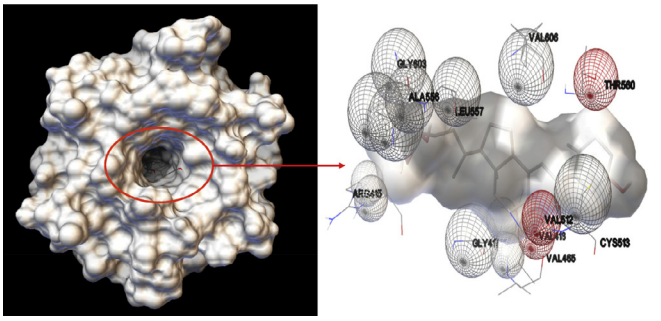

Fig. 9. Molecular docking (molecular surface view) between Nrf2 protein and 24,25-Dihydroxyvitamin D: This molecular docking figure show compounds at their binding site on the left and on the right the amino acids that interact with the ligand to give resultant binding energy.

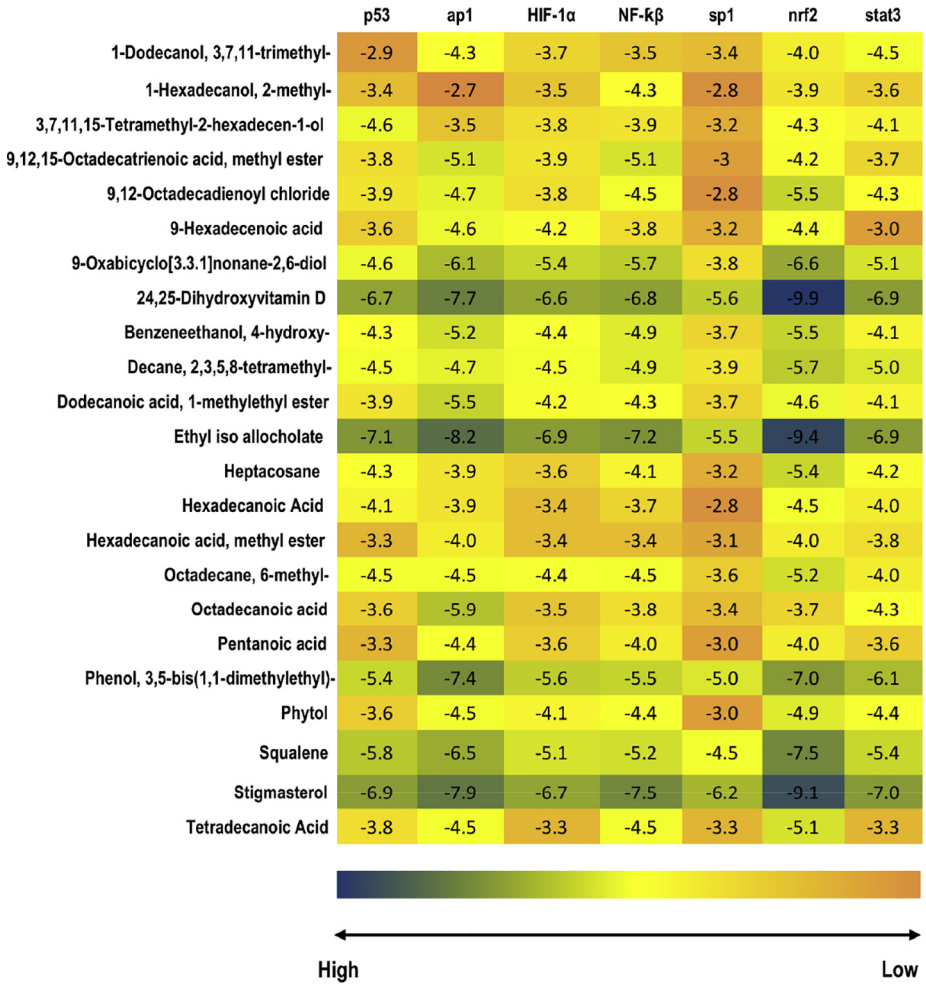

Fig. 8. Heatmap based on binding energy among proteins and phytochemicals. The phytochemicals which served as ligands for molecular docking experiment are along the Y axis and the proteins are placed on the X-axis.

3.8. GC–MS analysis

Functional groups, revealed from FTIR analysis, were extensively correlated with GC–MS data. Subsequently, our analysis involved identification of active compounds in CIL, VIL and CCL using GC–MS method. A total number of thirty three (33) phytocompounds have been identified in CIL (9 compounds), VIL (14 compounds) and CCL (10 compounds).

3.9. Molecular docking

The molecular docking results came out with new insights. While phytochemicals were analyzed with each of the proteins, at least one compounds from each plant showed exhilarating results. 24,25-Dihydroxyvitamin D of *C. indicum* (CIL) (−9.9 kcal/-mol), Ethyl iso allocholate of *V. inermis* (VIL) (-8.2 kcal/mol) and Stigmasterol of *C. colebrookianum* (CCL) (−9.1 kcal/mol) showed higher binding affinity compared to other compounds (Fig. 8). Among them on an average stigmasterol was found to have a slight upper hand compared to the remaining compounds. However, when individual interactions were compared, 24, 25-Dihydroxyvitamin D displayed the highest binding affinity with Nrf2 protein which is a transcription factor in humans encoded by NFE2L2 gene (Nuclear factor erythroid-derived 2) (Fig. 9) (Kovac et al., 2015). Interestingly, Nrf2 has the best interaction pattern with all the ligands. However, ap1 protein (activator protein 1) also had decent binding energies with most of the ligands; likewise, NF-κβ also had good interactions on an average. A heatmap of docking scores showing the binding affinity between the selected proteins and the chemicals present in the plants is shown in Fig. 8. Thus, these herbal alternatives can provide essential antioxidants as well as have no evident toxic effect even at the face of oxidative stress.

4. Conclusion

The present study shows an in depth antioxidant and hepatoprotective assessment of *Clerodendrum* species. CCl_4 induced liver damage involves overproduction of ROS and RNS. Present findings reveal that *Clerodendrum* species might be instrumental in the recovery of oxidative stress and protection from hepatic damage. The herbal formulation of *Clerodendrum* extracts especially CCL extract seems to be effective in combating against the oxidative stress induced hepatic damage.

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