GC/MS profile, DNA protectant and hepatoprotective effects of *Praecitrullus fistulosus* fruit methanol extract.

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ABSTRACT

The aim of the present investigation is to evaluate the antioxidant, DNA protectant and hepatoprotective effects of methanol extract of *Praecitrullus fistulosus* fruits. The antioxidant and DNA protectant activity using *in vitro* model and hepatoprotective effect using *in vivo* model was assessed. Antioxidant potential of MeOH was assessed by ABTS, hydroxyl radical, FRAP and H$_2$O$_2$ scavenging activity. Further cell-based antioxidant activity was determined using RBC's cells. Hepatoprotective effects of MeOH were assessed using CCl$_4$ induced liver damage mice model, *in vivo*. Post experimental results have proven the protective role of MeOH extract on the liver was assessed by serum antioxidant and biochemical analysis followed by histopathological studies. Histopathological studies reveal the retained tissue architecture, decreases hepatocellular necrosis, a poor dilated central vein in MeOH treated group. Further *in vivo* antioxidant enzyme activity showed that MeOH significantly protect the liver injury by retaining the antioxidant enzyme level in the CCl$_4$ treated groups. This study provides preliminary evidence that the edible fruits *Praecitrullus fistulosus* as nutraceuticals in food formulation and product development in the pharmaceutical industries.

1. Introduction

Carbon tetrachloride (CCl$_4$) is a well known chlorinated hydrocarbon used as a solvent in various industries including medicine in order to treat hookworm (Das et al., 2007). Individuals working in the industries with continuous exposure to CCl$_4$ would experience with indigestion and inhalation problems. CCl$_4$ induces hepatotoxicity, nephrotoxicity, and hematotoxicity (Nagano et al., 2007). The liver is the primary target for CCl$_4$ followed by the kidney and other organs (Kavita et al., 2018). Longer period exposure to CCl$_4$ can cause fibrosis, cirrhosis and hepatic carcinoma via induction of oxidative stress. An imbalance between oxygen species (O$_2$, H$_2$O$_2$, NO, etc) and endogenous antioxidant system that causes oxidative stress which can lead to liver disorders (Weber et al., 2003). To date, extensive study has been conducted on oriental medicine to develop new therapeutic drugs in order to treat toxic chemicals induced liver damage. In this regard, plant-based medicinal system provides strong evidence that use of phytochemicals to treat various diseases associated with liver (Muriel and Rivera-Espinoza, 2008; Chen et al., 2010; Youssef et al., 2017). With the inspiration of these reports, in the present study, we made an attempt to enlighten the medicinal benefits of edible fruit *Praecitrullus fistulosus* on CCl$_4$ induced liver damage in mice model.

Praecitrullus fistulosus also known as Indian baby pumpkin (common name- Tinda) belongs to Cucurbitaceae family native to India. *Praecitrullus fistulosus fruits* rich in secondary metabolites which exhibit medicinal properties such as anti-inflammatory, antioxidant, anti-helmenthic antidiabetic activity (Dixit and kar, 2010). Previous reports from our group successfully showed the anticancer potential of a lectin from fruit phloem exudates showed anticancer activity against *in vitro* and *in vivo* models (Madhu et al., 2017). In the present study, the antioxidant and hepatoprotective effects of *Praecitrullus fistulosus* methanol extract against CCl$_4$ induced mice model was assessed.

2. Materials and methods

2.1. Collection of plant material

Fruits were collected from the local agricultural fields and authenticated. Collected fruits were washed and sliced into small pieces and dry under shade for 15 days. Dried slices were finely powdered and passed through sieve to obtain fine powder.

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2.2. Preparation of sample

100 g of powder material was mixed with 1 L of methanol and kept for 48 h under constant stirring at ambient temperature. The mixture was filtered and concentrated using vacuum evaporator. Concentrated dark sticky brownish red sample was stored at 4 °C.

2.3. Antioxidant activity

Antioxidant potential of methanol extract was determined using *in vitro* antioxidant assay such as ABTS (Re et al., 1999), FRAP & H_2O_2 (Madhu et al., 2014) and OH^- radical scavenging activity (Ningappa and Srinivas, 2008).

2.4. DNA protection assay

Calf thymus DNA (1 µg/µl) was used for the experiment. Experiment was conducted as described earlier (Upendarrao and Solomon Sunder, 2014). Control: calf thymus DNA, Lysis: Calf thymus DNA + H2O2, Treated: Calf thymus DNA + H_2O_2+ 10 µg sample. After incubation the DNA protectant activity was analyzed using gel electrophoresis analysis and under observed under UV trans illuminator.

2.5. Anti-Hemolytic assay

Hemolytic assay was performed using Rabbit RBC's as prepared earlier (Madhu and Sharada, 2019). Different concentration of methanol extract was mixed with 50 µL of RBC's (2%) and incubates for 1 h at 37 °C. Later the reaction mixture was centrifuged and Hb content in the supernatant was measured spectrophotometrically at 560 nm.

3. Inhibition of lipid peroxidation

The degree lipid peroxidation was assessed by widely used TBARS method (Afsar et al., 2016). RBC's (0.5 ml) was mixed with different concentration of MeOH (0–100 µg/mL) and incubated at room temperature for 20 min H_2O_2 (10 mM) was added to the reaction mixture to provoking oxidative stress of the membrane lipids. Subsequently, the mixture was subjected to centrifugation at 2500 rpm for 10 min and obtained supernatant was spectrophotometrically read at 540 nm. The control tube contains 10 mM PBS, negative control contains 10 mM H_2O_2 and Gallic acid was used as positive control.

4. *In vivo* hepatoprotective experimental design

Hepatoprotective effects of MeOH against CCl_4 induced liver damage experiment was conducted in mice as follows: (1) Group I: Control receives normal saline (2) Group II: CCl_4 received group (1:1 v/v in olive oil, i.p administration of 2 ml/kg on 3rd day) (3) Group III: Treated group (CCl_4 + 100 mg/kg b.w, oral) (4) Group IV: Treated group (CCl_4 + 200 mg/kg b.w, oral). The MeOH treated group received sample daily for three consecutive days. CCl_4 was administered 3 h after the final treatment on the 3rd day. Post experimental studies follows, animals were anesthetized by thiopental and blood was collected through cardiac puncture and processed.

5. Biochemical assays

5.1. Assay for SGOT

Serum AST activity was determined according to the method of Thefeld et al. (1974). 100 µL of serum was mixed with Tris buffer (L-Aspartate 260 mM, LDH 1500 U/L, MDH 900U/L, pH 7.2) and solution containing 12 mM of α-Ketoglutarate and 0.24 mM NADH. Reaction mixture was incubated for 1 min at 37 °C. The change in absorbance was measured per min for 3 min at 340 nm.

SGOT activity (U/L) = (Δ OD / min) x 1745

5.2. Assay for SGPT

Serum SGPT activity was determined according to the method of Thefeld et al. (1974). 100 µL of serum was mixed with 0.5 mL of 110 mM Tris buffer (L-Alanine 660 mM, LDH 1500 U/L) with 0.1 mL of the solution containing (α-Ketoglutarate 16 mM, NADH 0.24 mM; pH 7.5). Reaction mixture was incubated for 1 min at 37 °C. The change in absorbance was measured per minute for 3 min at 340 nm.

SGPT activity (U/L) = (Δ OD / min) x 1745

5.3. Assay for Urea

Serum urea was determined according to the method of Chaney and Marbach, 1962. 100 µL of serum was mixed with the 0.2 mL of reagent (ADP- 0.66 mM, GLDH- 1000 mM/L, Urease > 30000U/L, NADH-0.32 mM & α-Ketoglutarate- 7.5 mM) and 0.8 mL of buffer (pH 7.4) containing 60 mM sodium salicylate, 5 mM sodium nitroprusside. The absorbance was read at 570 nm calorimetrically.

5.4. Assay for Creatinine

Serum creatinine was determined according to the method of Toora and Rajagopal, (2002). 100 µL of serum was mixed with the 0.5 mL of picric acid (8.73 mM/L) and 0.5 mL of reagent containing sodium hydroxide (300 mM/L) and sodium phosphate (25 mM/L). The optical density was read at after 1 min of the incubation with regular intervals.

5.5. Assay for Total protein

Total protein in serum was determined using Bradford assay (Bradford, 1976). Briefly 100 µL of serum was mixed with 975 µL of Bradford reagent and reaction mixture was incubated at room temperature for 5 min. Absorbance was read at 590 nm spectrophotometrically.

6. Evaluation of liver antioxidant enzymes

Sections of liver tissue were removed, weighed and homogenized in phosphate buffer (0.1 M, pH 7.4). Homogenized mixture was subjected to centrifugation at 12000 rpm at 4 °C and supernatant was used for antioxidant enzymes (SOD, Catalase and MDA) tests as described below.

6.1. Assay for Superoxide dismutase (SOD)

10 µL of supernatant was mixed with 1.5 mL of tris buffer (0.05 M), 0.5 mL of EDTA (1 mM) and 1 mL of pyrogallol (0.2 mM). Change in absorbance of sample per minute with reference to blank was read at 420 nm. 1.5 mL of Tris buffer and 0.5 mL of EDTA was served as blank.

6.2. Assay for Catalase

10 µL of supernatant was mixed with 1.9 mL of phosphate buffer (0.5 M, pH 7.0). The decreasing in extinction was measured at 240 nm, with 15sec intervals for 1 min immediately after 1 mL of H_2O_2 (30 mM). A sample control was placed in the reference cuvette containing 0.1 mL of tissue homogenate and 2.9 ml of the buffer. Activity of Catalase was calculated using the formula given below and expressed as U/mg protein.

6.3. Assay for malondialdehyde (MDA)

10 μL of supernatant was combined with 0.5 ml PBS, 1 ml of 10% TCA and 1 ml of 0.67% TBA.The samples were boiled for 20 min and centrifuged at 1000rpm for 10 min. The absorbance of the supernatant was measured at 535 nm and the lipid peroxidation was estimated in terms of malondialdehyde content by measurement of thiobarbituric acid reactive substances.

6.4. Histopathology studies (H & E staining)

Histological changes of treated and untreated samples were examined as described earlier (Mahesh et al., 2012). Briefly, collected tissues samples from liver and intraperitoneum tissue were fixed by using 4% proformaldehyde and embedded in paraffin wax and 10 μm sections were made using microtome (SLEE Cryostat). The sections were observed under low-power (910) light microscope to identify the highly vascularized areas. The microvessel density (MVD) was counted in ten fields of these vascularized areas under high-power (940) and the average MVD was noted.

7. Statistical data analysis

The data expressed as mean ± SD. Statistical analysis were carried out using GraphPad Prism 5.1 version. The mean difference groups was evaluated using ONE-WAY analysis of variance (ANOVA) followed by *t*-test. P value less than 0.05 was considered as statistical significant for all analyzers.

8. Results

8.1. Antioxidant activity and GC/MS analysis

Antioxidant potential of MeOH of *Praecitrullus fistulosus* was determined using various *in vitro* hydroxyl radical, ABTS, FRAP, and H_2O_2 model systems. It was observed that MeOH possessed significant hydroxyl radical scavenging, ABTS, FRAP and hydrogen peroxide abilities and their IC_{50} was found to 88.14 μg/mL, 79.43 μg/mL, 58.76 μg mL and 74.98 μg mL respectively (Fig. 1A–D). Although the antioxidant activity of Gallic acid was higher than the MeOH extract.

The major constituents are identified were in the extract are (+)-2-aminoheptane, Phenol, 3,5-bis (1,1-dimethylethyl)-, Hexadecanoic acid-methyl ester, Hexadecanoic acid 15-methyl-, methyl ester, 9-Dodecenoic acid, methyl ester (E), 9, 12-Octadecadienoic acid, methyl ester, 9, 12, 15-octadecatrienoic acid, methyl ester, (Z,Z,Z-), 1, 3, 5-trimethoxy-2-propenylbenzene were found predominantly and their 3D structure was depicted in Fig. 2(A–G). Overall 90 compounds are identified which has been listed in Table 1 (Supplementary Data 1) and many other compounds were identified as low level.

8.2. MeOH inhibits H_2O_2 induced DNA damage and lipid peroxidation

Chelating ability of MeOH extract was confirmed by H_2O_2 induced DNA damage assay. As shown in Fig. 1E. MeOH offered strong protection against H_2O_2 induced DNA sugar damage compared to control. Antioxidant potential of MeOH sample was tested using cell-based antioxidant model using erythrocytes. The protective effects of MeOH against H_2O_2 induced lipid peroxidation using erythrocytes and results showed that the MeOH showed 68% of protection at 100 μg/mL concentration whereas Gallic acid showed 87% protection at 10 μg/mL (Fig. 4D). Meanwhile, we also tested for anti-hemolytic/toxicity property of MeOH against erythrocytes in a dose-dependent manner. Data demonstrated that MeOH extract exhibits negligible effects at 500 μg/mL and complete lysis was observed at 1000 μg/mL concentration.

8.3. Hepatoprotective effects of MeOH against CCl_4 induced liver damage

The mean weight of each group of mice is shown in Fig. 4B. The data demonstrated that a significant increase in liver weight was observed in CCl_4 treated group (1.65 g) compared to normal mice (1.26 g). In contrast, mice received the MeOH showed an effective decrease to 1.45 g (100 mg/kg #$p < 0.05$) and 1.32 g (200 mg/kg ##$p < 0.005$) in the liver weight compared to the CCl_4 treated group mice. Histopathological examination of mice liver was assessed by Hematoxylin & Eosin staining method. As illustrated in Fig. 4A CCl_4 received mice showed congestion of the central vein, blood vessel congestion, alters the lobular tissue architecture, changes in hepatic sinusoids, necrotic foci, lipid deposition, nuclear degeneration, etc. This specific morphology was markedly diminished as dose of the sample increases with mild liver damage.

8.3.1. Serum biochemical parameters

Table 1 illustrates the protective effects of MeOH on CCl_4-induced hepatotoxicity. This study finds the elevated level of AST (###$p < 0.001$), ALT (##$p < 0.001$) and ALP (###$p < 0.001$) levels in CCl_4 administered group compared normal group mice. MeOH administered orally for 3 days before CCl_4 induced hepatoxicity. MeOH (100 mg/kg & 200 mg/kg b.w) significantly reduced AST (###$p < 0.001$), ALP (###$p < 0.001$) and ALT (###$p < 0.001$) levels compared CCl_4 treated group. Other serum biochemical metabolites such as urea, creatinine and albumin showed a negligible difference compare with the control group. Upon pre-treatment with 100 mg/kg and 200 mg/kg of MeOH, significantly reduced the MDA level in liver homogenate showed the protective effects of *Praecitrullus fistulosus* fruits.

8.3.2. Effects of MeOH on liver antioxidant enzyme assessments

Fig. 4C–E represents the *in vivo* antioxidant status of liver enzymes. Significantly decreased in the SOD (###$p < 0.0001$) and CAT (###$p < 0.0001$) was observed after intoxication with CCl_4 when compared to normal mice group. As it is shown in Fig. 4C, mice treated with MeOH effectively restore the SOD activity (###$p < 0.0001$) compared to CCl_4 treated control mice. On the other hand liver CAT content was significantly reduced in CCl_4 administered group (###$p < 0.005$). However, upon treatment with *MeOH* significantly decreases the levels of CAT compared with CCl_4 treated control group (Fig. 3D). The hepatic lipid peroxidation swas determined by measuring MDA level. CCl_4 treated control group significantly elevates the MDA content of liver when compared with normal mice. Whereas mice administered with MeOH showed gradual decrease 100 mg/kg (##$p < 0.05$) and 200 mg/kg (###$p < 0.005$) in the MDA indicates the antioxidant potential of the sample (Fig. 4E).

9. Discussion

Famous Greek scholar Hippocrates quotes as "*Let food be thy medicine and medicine be the food*". With this quote we selected one of the major vegetable, fruit used in the south Asian countries in their dietary lifestyle to evaluate its health benefits. Consumption of dietary antioxidants found in fruits and vegetables has been shown to minimize the oxidative stress in the body, thereby it prevents several oxidative stress associated diseases such as cancer, cardiovascular disorder, inflammation, cataracts, so on (Farzaneh and Carvalho, 2015). Earlier studies showed that the methanol extract of the fruit peel contains high amount of polyphenols and flavanoids is responsible for their antidiabetic property (Dixit and Kar, 2010). In the present study, we investigate the antioxidant, toxicity and hepatoprotective effects of methanol extract of *Praecitrullus fistulosus* (MeOH) against CCl_4 induced liver injury in a mouse model.

The MeOH extract of the dried fruits was dark brownish red color at ambient temperature and had a mild odor. Analysis of *Praecitrullus*
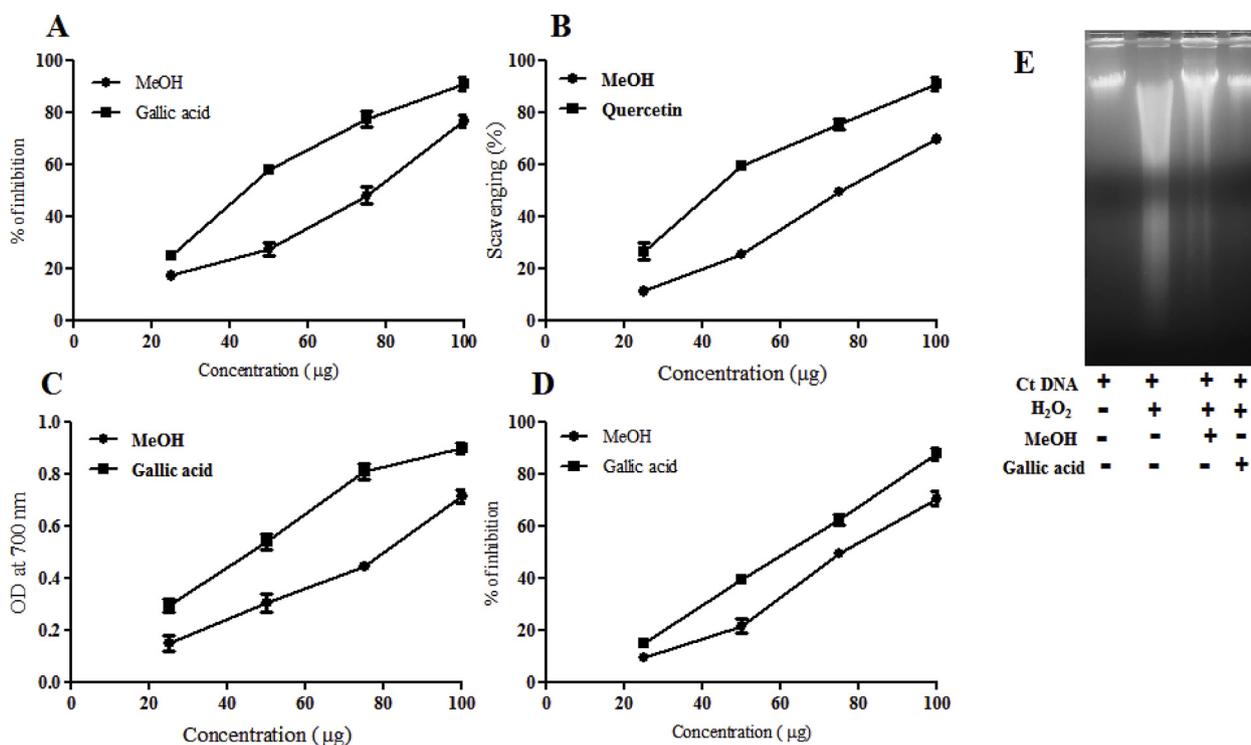
Fig. 1. Antioxidant activities of MeOH extract of *Praecitrullus fistulosus* A) ABTS assay B) Hydroxyl radical scavenging activity C) Ferric ion reducing power assay D) Hydrogen peroxide inhibition assay E) DNA protectant assay. Statistical data represents SD ± mean, n = 3.

fistulosus methanol extracts obtained by GC/MS analysis resulted in the identification of several compounds, which is accounted for total extract (Supplementary Data 1). The *in vitro* antioxidant studies showed that the MeOH extract exhibits promising antioxidant property by quenching the free radicals generated in the *in vitro* system. Antioxidant activity of MeOH is due to presence of high amount of phytochemicals such as polyphenols, flavanoids, sugars and other bioactive components responsible for the antioxidant potentials of the extracts (Kaur and
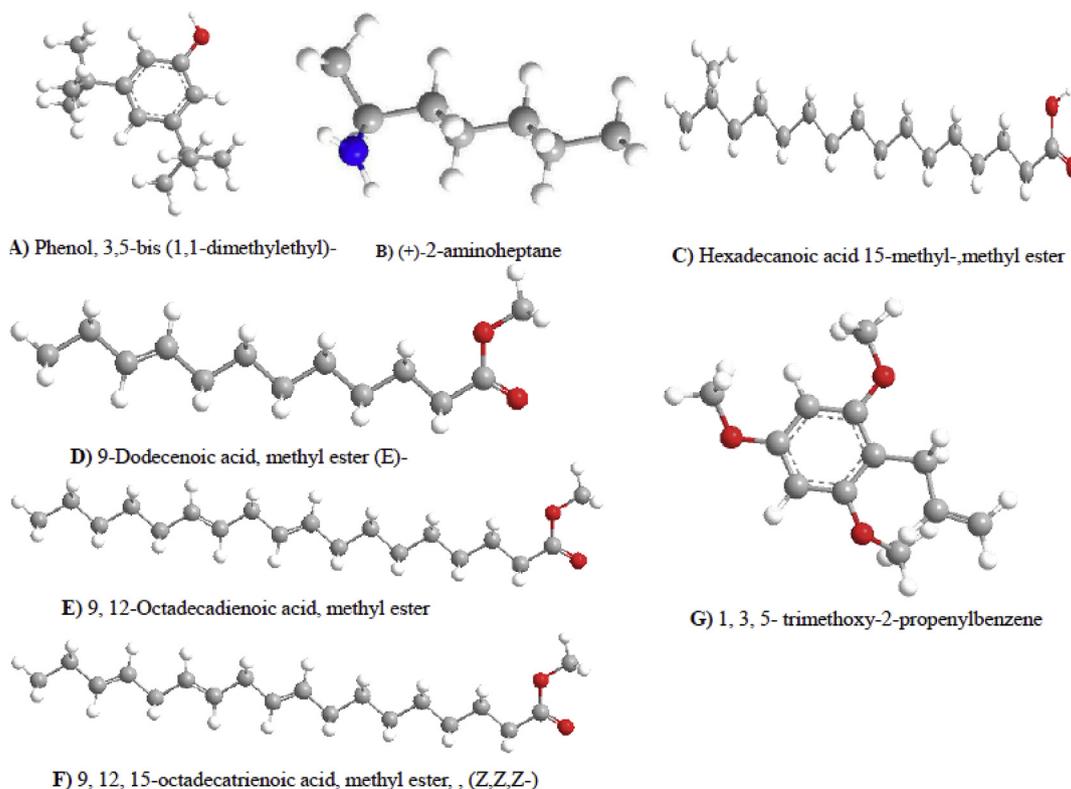

Fig. 2. Three dimensional models of compounds detected in GC/MS analysis: Seven major compounds were detected in GC/MS analysis and their 3D structure was drawn using *ChemBio* Draw software (version).

Table 1

Examinations	Albumin	Creatinine	Urea	AST	ALT	ALP
Normal	4.01 ± 0.123	0.71 ± 0.023	48.76 ± 2.34	298.56 ± 4.78	167.56 ± 12.65	432 ± 7.03
CCl4 control	3.91 ± 0.54	0.79 ± 0.091	49.87 ± 3.2	709 ± 18.65	643.23 ± 31.13	891.96 ± 51.01
CCl4 + 100 mg/kg b.w	4.14 ± 1.06	0.77 ± 0.34	50.43 ± 4.77	417.67 ± 28.2 [###]	456.89 ± 41.2 [##]	659.45 ± 32.5 [###]
CCl4 + 200 mg/kg b.w	4.10 ± 2.01	0.73.23 ± 0.76	50.21 ± 6.87	312.32 ± 14.78[###]	381.87 ± 29.9[###]	511.98 ± 41.4 [###]

Abbreviations: ALP, Alkaline phosphatase; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; CCl4, Carbon tetrachloride, MeOH, Methanol extract.
Data are expressed as the mean ± SD.
[##]$P < 0.01$ and [###]$P < 0.001$ compared with CCl4 control group.

Kapoor, 2008). Earlier reports showed that the presence of high amount of polyphenols and flavanoids in the MeOH extract exhibit promising antioxidant potential by neutralizing free radicals, quenching singlet and triplet species or decomposing peroxides (Farzaneh et al., 2018). Overall, antioxidant results showed that MeOH showed an effective antioxidant capacity at 100 µg/mL concentration which is due to the presence of various phytochemicals (Fig. 1A–D). The overall antioxidant potential is due to the presence of various compounds in the sample contributed significant capacity of the medicinal plants.

Based on the above results, we further demonstrated the protective effects of the MeOH extract using RBC's cell based model, to evaluate the degree of lipid peroxidation which is relevant to the biological system. Results showed that the MeOH extract prevents the lysis of RBC's cells from H_2O_2 induced hemolysis of the cell membrane induced by lipid peroxidation. This results resemblance to the evidenced by the Gallic acid showed 86% at 100 µg concentration. Based on these results we further evaluate the protective effects of MeOH against H_2O_2 induced DNA damage using calf thymus DNA. Free radicals generated from H_2O_2 which introduces strand break in either strand of the DNA. Previous reports clearly showed that different medicinal plants from various species protect H_2O_2 induced DNA damage, *in vitro* (Sabahi et al., 2018). Electrophoresis results showed that the MeOH extract effectively protects DNA from H_2O_2 induced DNA strand break. These results enlighten and proven the beneficiary role of antioxidants in biological system (Farzaneh and Carvalho, 2017).

CCl4 induced mice have been extensively used to study the liver

toxicant and its metabolic biproducts are involved in the pathogenesis of liver and kidney. CCl4 is converted to trichloromethyl free (CCl_3) and trichloromethylperoxyl (Cl_3COO^-) by cytochrome P450 enzyme. The free radicals generated by CCl4 covalently bind to biological macromolecules include proteins, nucleic acids and lipids. This lead to membrane damage followed by lysis and mitochondrial membrane damage results to increase in the serum, liver transaminases (Lin et al., 1996). More importantly CCl4 causes formation of TBARS; one of the major reactive aldehyde which is responsible for peroxidation of polyunsaturated fatty acids (Khan et al., 2012). Post experimental studies showed that mice treated with CCl4 gain weight in the liver, whereas mice treated with MeOH reduced the weight nearer to the normal range (Fig. 4B). Histopathological studies showed that CCl4 provokes liver damage by alter the tissue architecture, dilated central vein and accumulation of lipid, necrotic cells in the control group. More importantly gradual increase in the intracellular lipid peroxidation leads to altering the phospholipids structure and resulting in the damage the cell structure. In contrast to the control group, mice treated with MeOH retain the hepatic architecture with few necrotic cells (Fig. 4A). This is due to the mice administered with MeOH sample restores the antioxidant enzyme and reduced the MDA level in serum of treated group animals. These data, supports with the results of the serum enzymes viz., AST, ALT and ALP activities and hepatic lipid peroxidation level as well. Recent studies showed that *Rosmarinus officinalis* leaves extract protects the CCl4 induced liver damage in mouse model by restoring SOD, GSH and Catalase content in the serum of the
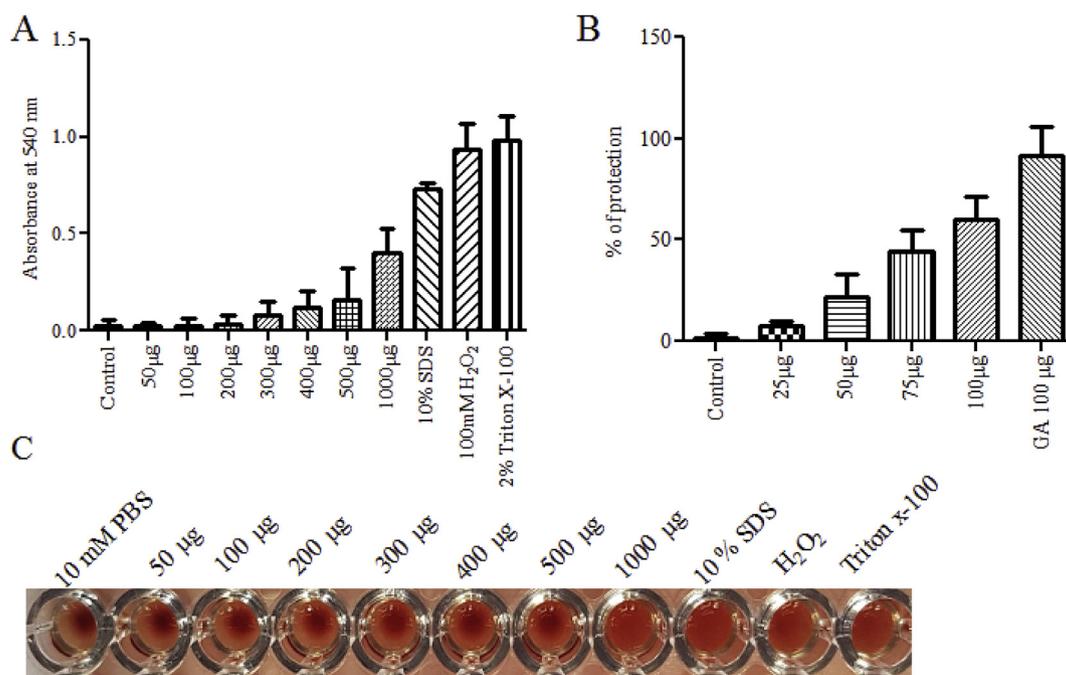

Fig. 3. Effects of MeOH on RBC's cells A) Anti-hemolytic assay B) Lipid peroxidation assay C) Hemolytic assay using microplate reader-where red button indicates anti-hemolysis, mat button indicates lysis of the RBC's cells. Statistical data represents SD ± mean, n = 3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
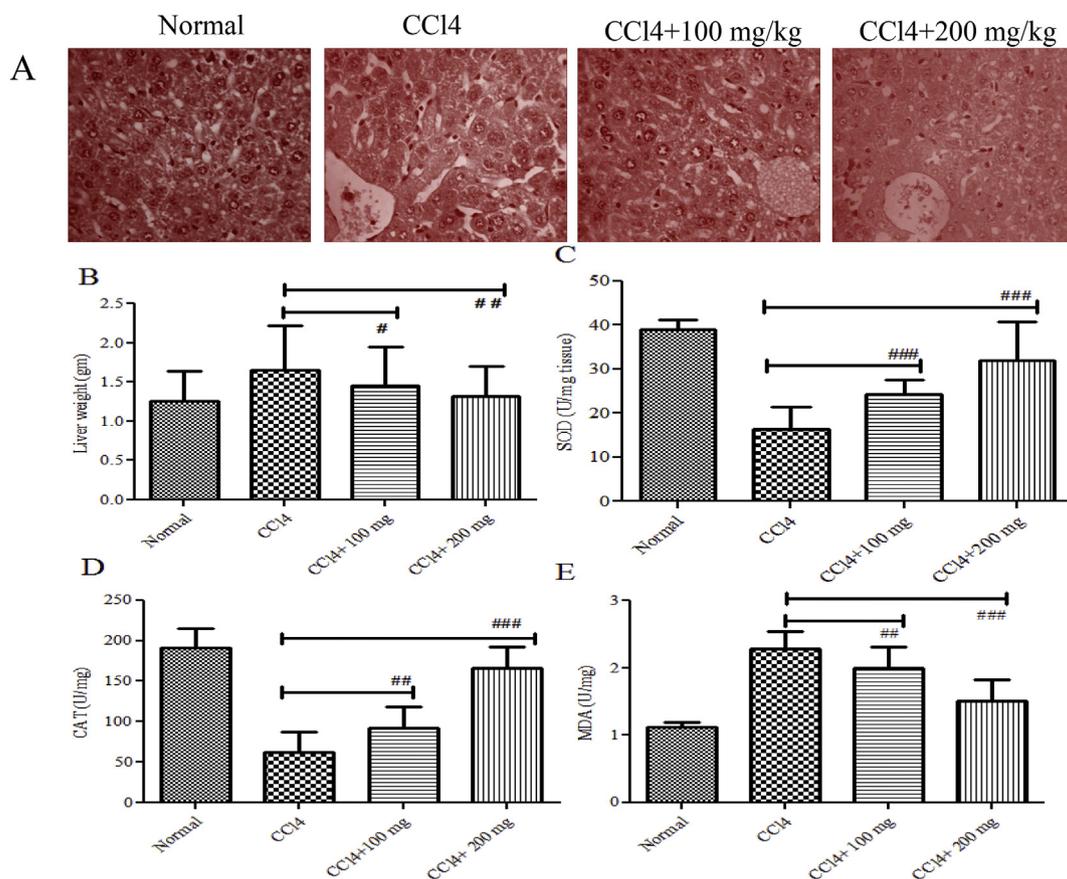
Fig. 4. *In vivo* hepatoprotective effects of MeOH on CCl4 induced liver damage: A) Histopathology analysis of liver B) Liver weight C) SOD activity D) Catalase activity E) MDA content (#*p* < 0.5, ##*p* < 0.05, ###*p* < 0.005). Statistical data represents SD ± mean, n = 3.

treated group animals (Anderson et al., 2001; Hamed et al., 2019).

10. Conclusion

In the present study antioxidant, DNA protectant and hepatoprotective effects of methanol extract of *Praecitrullus fistulosus* (MeOH) were evaluated using *in vitro* and *in vivo* models respectively. GC-MS analysis indicated the presence of various phytoconstituents that responsible for their ethanomedicinal benefits of the plant fruits. Furthermore, MeOH shown to provide hepatoprotective effect against CCl$_4$ intoxication in mice models by retaining the serum and antioxidant enzyme level in the liver. At last this study provides a comprehensive report for its possible use of fruits as nutraceuticals in food formulation and product development in pharmaceutical industries.

Conflicts of interest

The Authors declare that there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101272.

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