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HEPATOPROTECTIVE EFFECT OF *EUGENIA UNIFLORA* ACTIVE FRACTION AGAINST CCL_4 INDUCED HEPATOTOXICITY IN MALE WISTAR RATS

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Keywords:

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ABSTRACT: *Eugenia uniflora* known as pitanga or Surinam cherry, belongs to the Myrtaceae family and has essential nutrients that are prerequisites for human health. In the present study, *Eugenia uniflora* active fraction (EF18) that was obtained from the partial purification of crude ethanolic extract of *Eugenia uniflora* leaves were evaluated for its carbon tetrachloride (CCl_4) induced liver toxicity in rats. Acute toxicity studies were carried out as per OECD-423 guidelines for identifying the correct and safe dose of EF18. Results of the present acute toxicity study showed that oral administration of dried up to the dosage of 2000 mg/kg was nontoxic. On the basis of the acute toxicity studies, two doses of EF18 were selected, a higher dose (500 mg/kg) and a lower dose (250 mg/kg). For the hepatotoxicity studies, Silymarin was used as a standard drug. In the study, EF18 significantly reduced the impact of CCl_4 toxicity on the liver in a dose-dependent manner. Results of the histopathological analysis also support that EF18 can effectively reduce liver toxicity. The results of preliminary phytochemical screening of EF18 revealed the presence of various pharmacologically active phytochemicals in it. The hepatoprotective effect of EF18 was may be due to the presence of these phytochemicals in it. The overall study results suggested that *Eugenia uniflora* active fraction (EF18) can be used as a potent hepatoprotective agent against the CCl_4 induced liver toxicity.

INTRODUCTION: Most of the populations rely on medicinal plants for their health care needs [1]. In recent days the usage of medicinal plants is more prevalent. Herbal medicines are safe to use since it does not have any side effects. The liver is the largest and most important organ in the human body. Hepatocytes constitute about 75% of the hepatic parenchyma. Major roles of the liver in our body include hormonal balance, maintenance of glucose level during fasting, lipid digestion, and detoxification of xenobiotics. Bile, the end product of hemoglobin metabolism, is secreted by the liver.

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This bile has a role in lipid digestion. Any forms of liver injuries or abnormalities lead to liver disease or necrosis. Hepatotoxicity may also result in severe metabolic disorders to mortality [2]. Hepato-toxic chemicals act by producing free radicals that can damage the liver cells. Excessive exposure to toxic chemicals may overcome the natural defense mechanism, which in turn, results in the peroxidation of membrane lipids and in elevated serum enzyme levels [3].

Many herbal formulations were used as liver protective agents. Liver diseases are of two types acute, which exist for a short time and chronic that persists for a prolonged time. Patients having liver damage usually express clinical symptoms like fibrosis, necrosis, liver cirrhosis, elevated serum marker levels, hepatic veno-occlusive disease, vomiting, and bleeding [4]. Synthetic drugs used for the treatment of liver disease showed side effects

and are very expensive. So, there exists a need for plant-derived drugs, which are very cheap and have no side effects. Ancient time onwards, various herbal medicines were used to prevent liver disease [5]. Phytochemicals present in plants possessed a significant interest as they are the natural alternatives to synthetic medicines. Before consumption, the plant must be examined for its toxicity [6]. Different toxicity investigations of medicinal plants were carried out. All the newly developed drugs were tested for its toxicity and clinical trials in order to avoid toxicity before usage [7].

Acute toxicity studies are very much important in determining the correct and safe dose that could be used successively. *Eugenia uniflora*, the plant taken for the study, is used in popular medicine because of its anti-rheumatic, anti-febrile, and also as a therapeutic agent for stomach diseases [8]. Leaves of *Eugenia uniflora* contain substances with biological properties that are advantageous for human health, and this favors its usage as a medicinal plant [9]. Pitanga is also known as *Eugenia uniflora* is mainly distributed in tropical and subtropical America [10]. In the present study, *Eugenia uniflora* active fraction (EF18) was evaluated for its toxicity and its hepatoprotective effect. Free radicals are responsible for most of the diseases. Liver disease is one of the major threats to humans, and its prognosis in the later stages was very difficult. Reactive oxygen species also have a role in liver diseases. Antioxidants exhibited a curative potential and play a vital role in preventing liver diseases and thereby promoting human health [11, 12, 13, 14, 15].

MATERIALS AND METHODS:

Preparation of *Eugenia uniflora* Active Fraction (EF18): The most active crude ethanolic extract of *Eugenia uniflora* was obtained by the Soxhlet extraction using powdered leaves of *Eugenia uniflora* leaves. Solvents used were selected on the basis of increasing polarity. For the preparation of EF18, the crude ethanolic extract of *Eugenia uniflora* was subjected to partial purification by using column chromatography technique. Different solvents of increasing polarity were used for the preparation of gradient in column chromatography. The stationary phase used for the study was the silica gel of 60-120 mesh size. The mobile phase includes solvents of increasing polarity. The solvents used include petroleum ether, chloroform, ethyl acetate, and ethanol. The solvent ratio used was 9:1. The mobile phase consists of various solvent gradients that were passed through the column, and the eluent was collected in tubes. In order to detect the most active fraction, all the fractions were subjected to phytochemical analysis and antioxidant evaluation. All these were carried out using standard procedures. The results of these assays revealed that EF18 fraction showed the maximum activity. Hence, this fraction was considered as the most active and selected for further studies.

Animals: Male Wistar rats, about 150 ± 5 g were taken for the study. Animals were obtained from the small animal breeding station, Mannuthi, Trissur. The studies were performed according to the regulations of the Institute Animal ethics committee that was approved by the committee for the control purpose and supervision of experimental animals (CPCSEA) Reg: number: B11042016-7/MGU/SBS/IAEC/11-04-2016.

Acute Toxicity Studies: Acute oral toxicity study was performed as per OECD-423 guidelines (acute toxic class method). Acute toxicity of *Eugenia uniflora* active fraction (EF18) was carried out (using standard protocol [16]) mainly to determine whether it is toxic and also to detect its correct and safe dose. The animals were randomly divided into four groups, each containing six rats.

The animals were kept fasting overnight and provided only with water, after which the extracts were administered orally at 5 mg/kg body weight by gastric intubations and observed for 14 days. According to the toxicity procedure, if mortality occurred, the dose administered is considered as toxic since there was no mortality observed, the procedure was again repeated for the higher doses of EF18 such as 50, 100 up to the range of 2000 mg/kg of the body weight.

Hepatotoxicity Studies: Hepatoprotective studies were carried out using standard procedure [16]. The animals were divided into 6 groups of 5 animals each. Silymarin was taken as a positive control in this study. The entire study was conducted for 14 days.

TABLE 1: EXPERIMENTAL DESIGN OF HEPATO-TOXICITY STUDIES

Groups	Treatments
Group I	Rats receiving saline only (Normal)
Group II	CCl_4 induced control groups
Group III	CCl_4 induced animals receiving silymarin (100 mg/kg) body weight
Group IV	CCl_4 induced animals receiving EF18 at the dose of 250 mg/kg body weight
Group V	CCl_4 induced animals receiving EF18 at the dose of 500 mg/kg bodyweight

Determination of Liver Antioxidant Status: At the end of the 14th day, all the animals were sacrificed; their blood and liver tissue was taken for analysis. Liver tissue homogenates (10% w/v) were prepared in ice-cold 10 mM Tris buffer of pH 7.4. This was then subjected to centrifugation at 12000 rpm for 30 min at 4 °C.

The supernatant obtained after the centrifugation was taken for the analysis of antioxidant status [17]. Superoxide dismutase (SOD) [18], glutathione peroxidase (GPx) activity [19], reduced glutathione (GSH) content [20], and lipid per-oxidation [21] were analyzed.

Determination of Serum Biochemical Parameters: The blood for biochemical analysis was collected, and serum was separated by centrifugation at 3000 rpm for 10 min. Serum obtained was transferred to fresh tubes and stored at 20 °C until use.

Serum was then used for evaluating biochemical markers of liver function, including serum glutamate oxaloacetic acid transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and total bilirubin.

This was carried out using diagnostic kits (Span Diagnostic Limited, Surat, India). The assay was performed according to the manufacture's procedure. The absorbance was read using a UV-Visible spectrophotometer (Hitachi U-5100).

Histopathological Analysis: Hepatic tissue was removed, washed in ice-cold saline, and then fixed in 10% neutral formaldehyde solution. For histopathological analysis, the paraffin-embedded paw tissue sections were stained with Hematoxylin-eosin (H&E) followed by the examination and photographed under the light microscope for observation of structural abnormality [22, 23].

Statistical Analysis: All the data were represented as mean ± SEM and analyzed by one-way ANOVA followed by Dunnett's test for the probable significant identification between the various groups. Differences were considered significant at $P<0.05$. Statistical analysis was carried out using graph pad prism 5 (GraphPad Software, San Diego, CA).

RESULTS:

Effect of EF18 on Acute Toxicity Studies: *Eugenia uniflora* active fraction EF18 did not cause any mortality up to the dosage of 2000 mg/kg. Therefore 1/8th and 1/4th of the maximum dose (2000 mg/kg) that was 250 mg/kg and 500 mg/kg were selected for the current *in-vivo* studies.

Hepatoprotective Studies: In hepatotoxicity studies, the effect of EF18 was examined on the CCl_4 induced animals. GSH, GPX, and SOD were evaluated. Serum parameters like SGOT, SGPT, and Bilirubin were also analyzed in the hepato-toxicity studies.

Effect of EF18 on Glutathione (GSH): GSH level was evaluated in the CCl_4 treated groups. Results revealed that upon treatment with EF18, especially the higher dose restored the GSH value to normal range when compared to the CCl_4 control group. The effect of EF18 higher dose can be comparable with that of Silymarin treated groups. The results are depicted in **Fig. 1**.

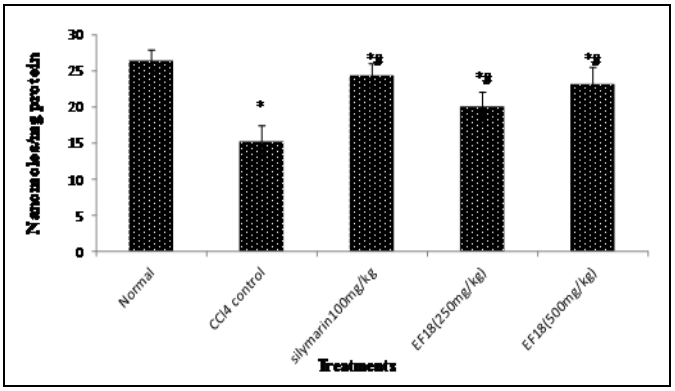

FIG. 1: EFFECT OF EF18 AND SILYMARIN ON GSH IN CCl_4 INDUCED LIVER TOXICITY. Values are the Mean ± SD (n=6). *denotes $p<0.05$ when compared with normal control, #denotes $p<0.05$ when compared with toxic control

Effect of EF18 on Glutathione Peroxidase (GP_X): The results of the study revealed that the GP_X level, which was reduced on CCl_4 treatment,

was restored by the EF18 treatment. The higher dose of EF18 was better than its lower dose in normalizing the GP_X value.

The GP_X values obtained by the EF18 treated group can be comparable with that of Silymarin drug-treated groups. The CCl_4 administration showed decreased GP_X enzyme level and thereby increased oxidative stress. The results are depicted in **Fig. 2**.

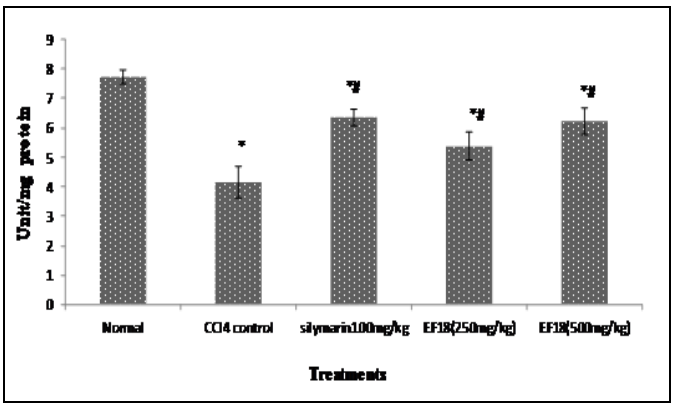

FIG. 2: EFFECT OF EF18 AND SILYMARIN ON GPX IN CCl_4 INDUCED LIVER TOXICITY. *denotes p< 0.05 when compared with normal control, #denotes p< 0.05 when compared with toxic control. Values are the Mean ± SD (n=6)

Effect of EF18 on Superoxide Dismutase (SOD): SOD level was evaluated in the CCl_4 induced hepatotoxicity studies. Results showed that initially, a significant decrease in the level of SOD was observed in CCl_4 administered groups when compared to normal.

This decrease was restored in groups treated with EF18, especially its higher dose. The effect of EF18 can be comparable with that of Silymarin. The results are shown in **Fig. 3**.

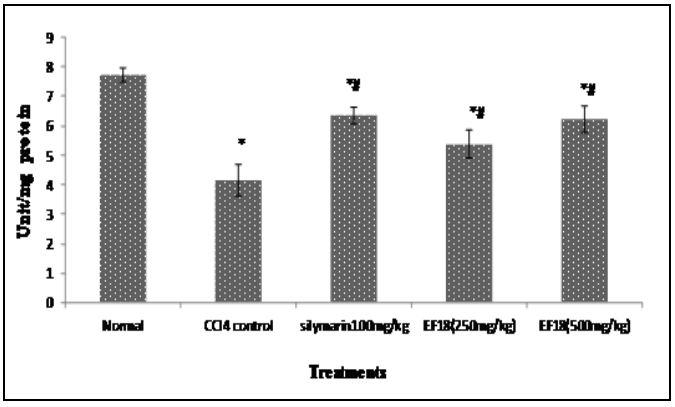

FIG. 3: EFFECT OF EF18 AND SILYMARIN ON SOD IN CCl_4 INDUCED LIVER TOXICITY. *denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n=6)

Effect of EF18 on Lipid peroxidation: Lipid peroxidation was assessed in the CCl_4 induced hepatotoxicity studies. Results revealed that CCl_4 administration significantly affected the level of lipid peroxidation. On CCl_4 administration, the lipid peroxidation was increased dramatically; these levels were restored to normal range upon the treatment of EF18.

The higher dose of EF18 was better than the lower dose in reducing the lipid peroxidation level. A dose-dependent depletion of lipid peroxidation was observed in the study. The values obtained by the EF18 treated groups can be comparable with that of Silymarin. The results are depicted in **Fig. 4**.

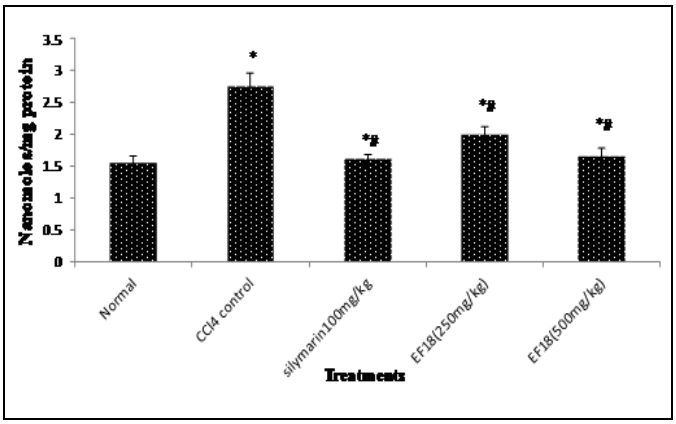

FIG. 4: EFFECT OF EF18 AND SILYMARIN ON LIPID PEROXIDATION IN CCL_4 INDUCED HEPATO-TOXICITY STUDIES. *denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n=6)

Effect of EF18 on Serum Parameters: In the present study, the serum parameters such as SGOT, SGPT, ALP, and bilirubin levels were evaluated.

Effect of EF18 on Aspartate Transaminase (AST or SGOT): Aspartate transaminase level in serum was measured in CCl_4 induced liver toxicity study.

The level of SGOT was dramatically increased in groups treated with CCl_4 when compared to the normal level. SGOT level was restored to the normal range by the treatment of EF18.

The higher dose of EF18 was efficient in reducing the SGOT level than its lower dose. The values obtained by the EF18 can be comparable with that of Silymarin drug-treated groups. The results are depicted in **Fig. 5**.

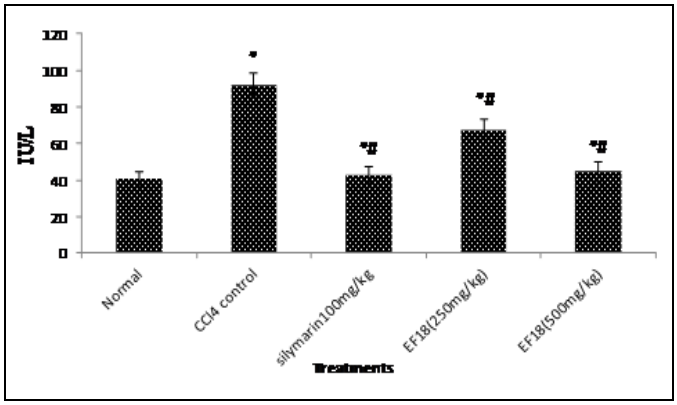

FIG .5: EFFECT OF EF18 AND SILYMARIN ON SGOT IN CCl_4 INDUCED LIVER TOXICITY. Values are the mean ± SD (n=6). *denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n = 6).

Effect of EF18 on Alanine transaminase (ALT or SGPT): Alanine transaminase level in CCl_4 induced toxicity study was assessed. Results showed that the increased SGPT level upon the CCl_4 administration was reduced to the normal range by the EF18 treatment, especially its higher dose. A dose-dependent depletion of SGPT was observed in the case of groups treated with EF18. The effect of EF18 higher dose can be comparable with that of the Silymarin drug. The results are shown in **Fig. 6**.

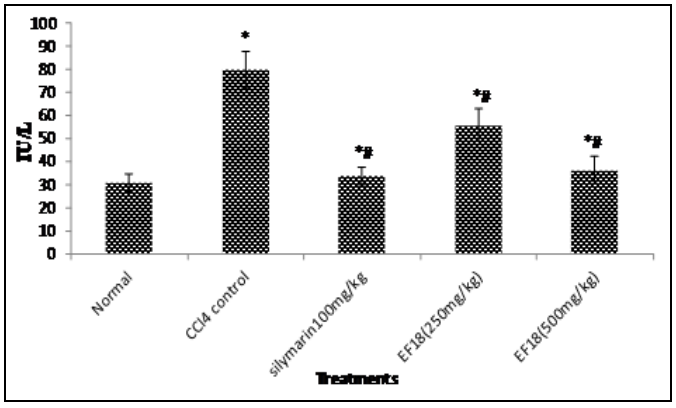

FIG. 6: EFFECT OF EF18 AND SILYMARIN ON SGPT IN CCl_4 INDUCED LIVER TOXICITY. Values are the mean ± SD (n=6). * denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n=6).

Effect of EF18 on Alkaline Phosphatase (ALP): Alkaline phosphatase level was measured in CCl_4 induced toxicity study. Results indicated that the ALP level was increased in the CCl_4 administration. This was reduced to the normal range by the EF18 treatment, especially its higher dose. A dose-dependent reduction of ALP was observed in the case of groups treated with EF18. The effect of EF18 higher dose can be comparable with that of the Silymarin drug. The results are shown in **Fig. 7**.

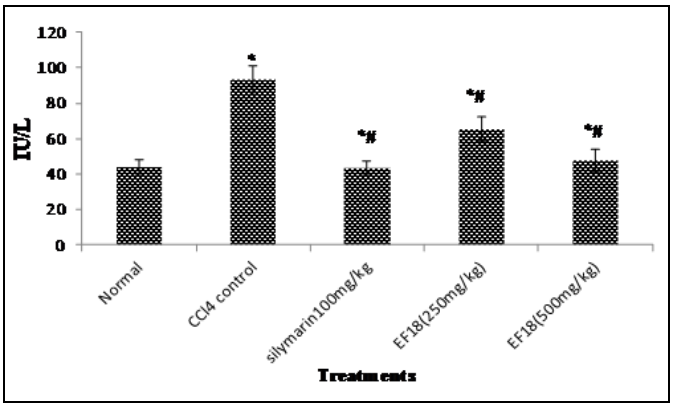

FIG. 7: EFFECT OF EF18 AND SILYMARIN ON ALKALINE PHOSPHATASE LEVEL IN CCl_4 INDUCED HEPATOTOXICITY STUDIES. *denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n=6).

Effect of EF18 on Total Bilirubin: Serum bilirubin level was evaluated in CCl_4 induced hepatotoxicity study. The results of the study revealed that the bilirubin level was significantly elevated in CCl_4 administration. This elevated bilirubin level was restored to normal upon EF18 treatment.

The higher dose of EF18 was better than its lower dose. A dose-dependent reduction of bilirubin was observed in the case of groups treated with EF18. The effect of EF18 higher dose treated groups can be comparable with that of standard drug Silymarin. The results are shown in **Fig. 8**.

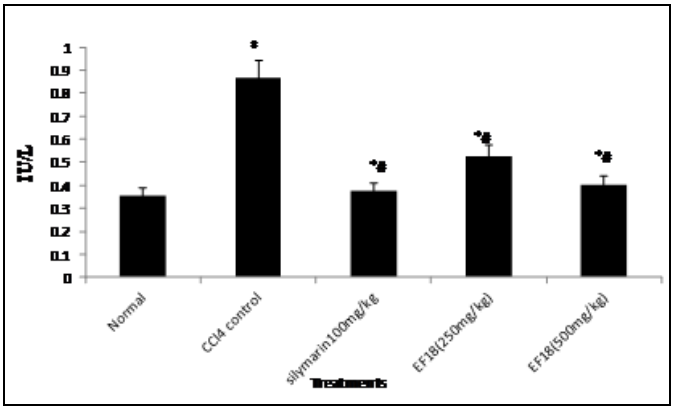

FIG. 8: EFFECT OF EF18 AND SILYMARIN ON TOTAL BILIRUBIN LEVEL IN CCl_4 INDUCED HEPATOTOXICITY STUDIES. * denotes p<0.05 when compared with normal control, #denotes p<0.05 when compared with toxic control. Values are the Mean ± SD (n=6)

Histopathological Analysis of Liver Tissue: Histopathological analysis of CCl_4 induced liver tissue was conducted. Histology of liver tissue showed signs of acute liver damage such as necrosis and fatty degeneration. Normal liver showed normal hepatocytes, intact central vein, and no fatty degeneration. In CCl_4 control group damaged hepatocytes, enlarged central vein and fatty degeneration were observed. In EF18 lower dose treated groups damaged hepatocytes, enlarged central vein, and mild improvement in fatty degeneration was observed. Where in EF18 higher dose (500 mg/kg) treated groups showed normal hepatocytes, retrieval from fatty degeneration and the central vein that was not enlarged.

EF18 treatment showed mild damages to the liver tissue structure. The results of the study proved that the higher dose (500 mg/kg) of EF18 ameliorated the CCl_4 toxic effects resulting in normal liver tissue with mild lesions and necrosis. The results are shown in **Fig. 9**.

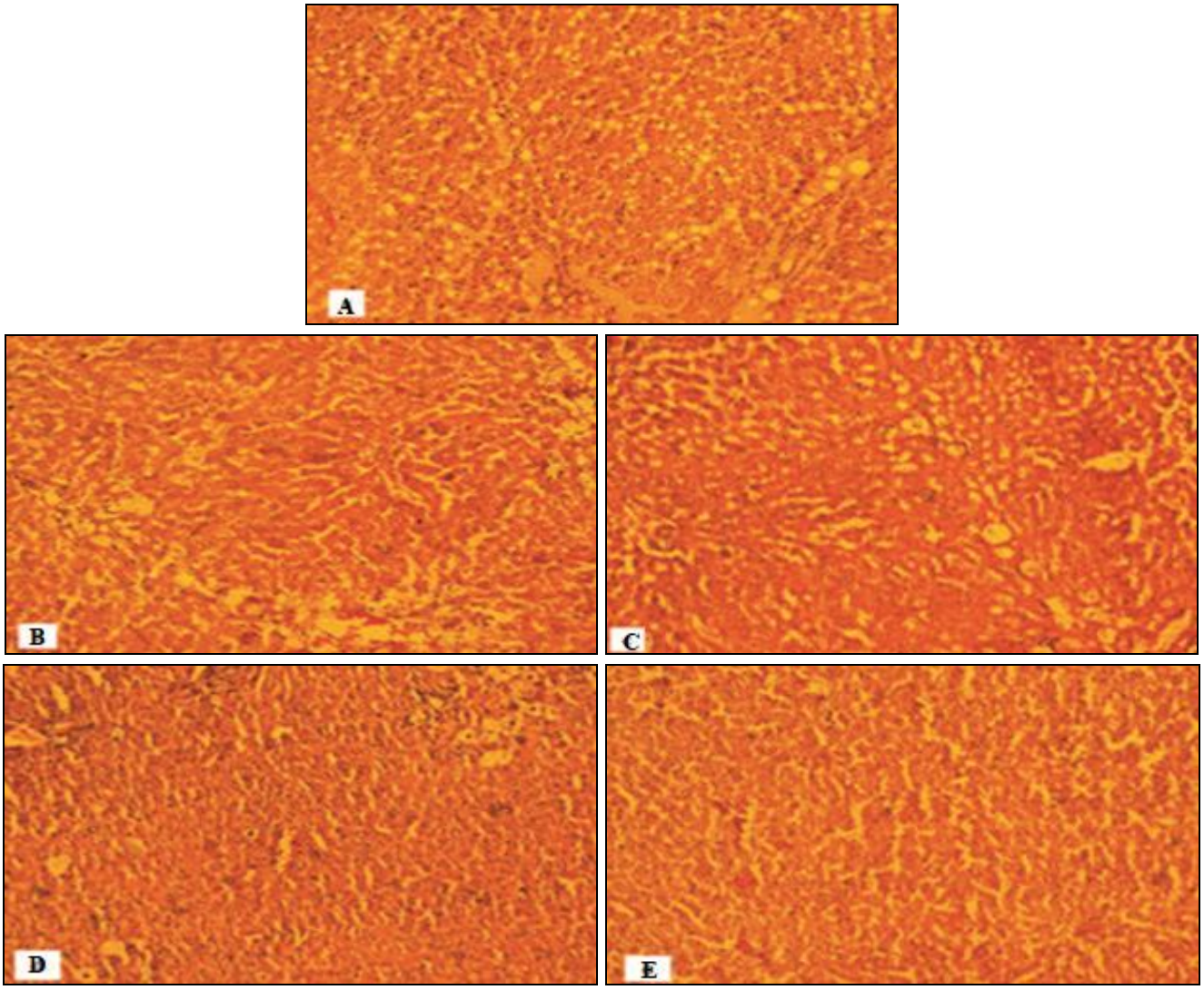

FIG. 9: HISTOPATHOLOGICAL ANALYSIS OF CCl_4 INDUCED LIVER TOXICITY (a) CCl_4 control (b) CCl_4 + EF18 (250 mg/kg) (c) CCl_4 +EF18 (500 mg/kg) (d) CCl_4+ Silymarin (e) Normal liver tissue

DISCUSSION: Oxidative stress occurs as a result of the imbalance between the free radicals and the antioxidants. Antioxidants protect the biomolecules from the attack of free radicals. Results of the acute toxicity studies revealed that EF18 was safe to a dosage of 2000 mg/kg since it did not cause any mortality or any other physical changes like changes in the behavior of animals. In hepato-toxicity studies, the effect of EF18 was examined on the CCl_4 induced animal models. Any damage that reduces the functioning of the liver can be referred to as liver diseases [24]. The present study demonstrated that EF18 exhibited a therapeutic effect on CCl_4 induced liver damage in rats.

CCl_4 inhibited the synthesis of proteins. CCl_4 is well known for the hepatic and renal toxic action [25]. CCl_4 administration is a commonly used experimental model that mimics the liver failure caused by the toxic chemicals [26]. Not only the liver but also the heart, testes, lungs, brain, and kidneys are also the targets of CCl_4 [27]. Silymarin, the drug used in the study, is a natural compound derived from Silybum marianum species, which is also known as milk thistle. Silymarin belongs to the aster family. It exhibits hepatoprotective effect [28].

This hepatoprotective effect of Silymarin is mainly because of its ability in controlling free radicals that are produced by toxic substances or hepatic metabolism [29]. Silymarin exhibits antioxidant, anti-proliferative, antiviral properties, anti-fibrotic and immune-modulatory properties. Silymarin maintains the structural integrity of hepatocytes [30] and protects the plasma membrane of hepatocytes [31]. When the rats were administered with the CCl_4, it produced toxicity in liver cells, maintaining a semi-normal metabolic function. CCl_4 induced rats showed decreased enzyme activities, which in turn results in the accumulation of free radicals that are very reactive. This results in deleterious effects like loss of integrity of cell membrane and function of the membrane [32].

Liver antioxidant enzymes status and serum markers were also evaluated in the study. Antioxidant enzymes like Superoxide dismutase (SOD), Glutathione (GSH), Glutathione peroxidase (GPX) were assessed. In the study, hepatic serum markers like SGPT, SGOT, ALP, and bilirubin were evaluated. Liver damage can be evaluated by both non-enzymatic and enzymatic biochemical markers. CCl_4 treatment results in a significant increase in serum parameters [33, 34,] and the level of lipid peroxidation [35]. Toxicity results in the changes in the endoplasmic reticulum and the loss of enzymes located in the intracellular membranes. Because of the toxic effect of CCl_4, a reduction in the level of GSH and SOD [36] was observed. GSH level is good for maintaining the structure and function of different organs. Glutathione (GSH) is a hydrophilic tripe tide that reacts directly with free radicals and has a role in detoxification [37]. GSH level was evaluated in the CCl_4 treated groups. Here a significant $p<0.05$ reduction in GSH level was found in animals treated with CCl_4 when compared to the normal group. Initially, when compared to normal, CCl_4 treatment decreased the GSH level. Oral administration of EF18 results in dose-dependent protection against the GSH level depletion. Results revealed that treatment with EF18, especially the higher dose, restored the GSH value to normal range when compared to CCl_4control. The effect of EF18 higher dose was compared to that of Silymarin treated groups. GP_X is one of the major antioxidant enzymes that breakdown the hydrogen peroxide into water. Glutathione peroxidase (GP_X) has a role in the prevention of lipid peroxidation [38].

The level of GP_X was evaluated in animal groups treated with CCl_4. CCl_4 induced hepatotoxicity results in the variation of antioxidant enzyme superoxide dismutase (SOD). SOD is one of the vital antioxidant enzymes found in all aerobic organisms having a role in scavenging free radicals [39]. SOD levels of the animals treated with the CCl_4 was significantly decreased. SOD is needed for the scavenging of free radicals. The decreased level of these enzymes may be due to the increased level of superoxide radicals or the enzyme inactivation. These superoxide radicals were formed as a result of lipid peroxidation.

Results showed that initially there was a significant decrease in the SOD level of the groups administered with CCl_4, when compared to normal groups. This decrease was restored in groups treated with EF18, especially its higher dose. Lipid peroxidation act as one of the major marker of oxidative stress. These increased levels of superoxide radicals were restored by the treatment of EF18. GSH reacts with the trichloromethyl radical formed from carbon tetrachloride that results in cellular lipid peroxidation. MDA, which is the end product of lipid peroxidation, gets accumulated in the case of CCl_4 toxicity. The major end product of lipid peroxidation is the MDA (Malondialdehyde). Its level in the liver tissue indicates the level of lipid peroxidation. Increased lipid peroxidation is due to the liver injury caused by the CCl_4 administration. This increased lipid peroxidation results in the dehydration of cells [40]. Lipid peroxidation was assessed in the CCl_4 induced liver toxicity. Results revealed that CCl_4 administration significantly affected the level of lipid peroxidation.

On CCl_4 administration the lipid peroxidation was increased dramatically and these levels were restored to normal range upon the treatment of EF18. EF18 treatment reduced lipid peroxidation, thereby preventing the accumulation of MDA. All these changes were noticeable in the case of a higher dose of EF18. The higher dose of EF18 was better than a lower dose in reducing the lipid peroxidation level. A dose-dependent depletion of lipid peroxidation was observed in the study. Bilirubin and Alkaline phosphatase levels act as biomarkers of liver function. Its elevated levels showed the dysfunction of hepatocytes. Aspartate transaminase (AST or SGOT), Alanine transaminase (ALT or SGPT) and alkaline phosphatase (ALP) are useful biomarkers in liver diseases [41].

SGOT and SGPT levels were increased after 48 hours of the liver damage, and these marker enzymes enter into the bloodstream due to membrane permeability. Because of this process, the hepatocytes' architecture gets damaged. Bilirubin is an endogenous anion derived from hemoglobin degradation from the RBC [42]. For the proper functioning of the liver, bilirubin is needed [43]. The major source of bilirubin is the senescent erythrocytes [44].

The bilirubin level was also restored with EF18 treatment. CCl_4 changes the level of serum parameters. EF18 restored the enzyme level and was capable of preserving the membrane integrity against the CCl_4 induced toxicity. EF18 administration caused tremendous changes in the liver histopathology in a dose-dependent manner. Histopathological observations support the early described biochemical observations. The histopathological analysis also proved that EF18 employs a positive effect on CCl_4 induced toxicity. Preliminary phytochemical screening of EF18 fraction revealed the presence of phytochemicals like flavonoids, phenolics, tannins and also showed free radical scavenging activity. Phytochemicals having antioxidant activity have a role in the prevention of various diseases caused by oxidative stress [45]. The hepatoprotective activity of EF18 may be due to the presence of these pharmacologically active compounds in it.

CONCLUSION: Herbal medicines have become prevalent due to the expense of synthetic medicine and its numerous harmful side effects.

The present study mainly focuses on the efficiency of *Eugenia uniflora* active fraction against liver toxicity. It can be hypothesized from the acute toxicity study that *Eugenia uniflora* active fraction (EF18) is safer to use as traditional medicine since there was no mortality or signs of toxicity. The EF18 fraction reversed the toxic effects of CCl_4 in a dose-dependent manner when compared to the standard drug Silymarin. The level of antioxidant status, evaluation of biochemical parameters, and histopathological analysis revealed the curative potential of the active fraction of *Eugenia uniflora* (EF18) against hepatic injury.

On the basis of the study, it can be concluded that the active fraction of *Eugenia uniflora* (EF18) can be used as an effective source for the development of therapeutic drugs against liver diseases.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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