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THE BIOMOLECULES PRESENT IN FRUIT-SEEDS OF *GLYCOSMIS PENTAPHYLLA* L ACT AS POTENTIAL HEPATOPROTECTIVE AGENT

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Abstract:

The main aim and objective of the present research work was to extract the potential biomolecules present in the fruit seeds of Glycosmis pentaphylla L and to evaluate the in vitro and in vivo Hepatoprotective activity. The in vitro Hepatoprotective activity was carried out by ***DPPH assay.*** *The IC_{50} value (50% inhibition) of the EEFSGP was found to be 212.96 µg/ml. The in-vivo Hepatoprotective activity was carried out by using albino rats. The results displayed that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin were mainly due to CCl_4 intoxication, reduced significantly (*P<0.05) in rats, after treatment with ethanolic extract of fruit seeds of Glycosmis pentaphylla (EEFSGP). Treatment with EEFSGP at a dose of 250 mg/kg b.w. decreased the SGOT, SGPT, ALP, Serum Bilirubin levels by 6.23% ns(non significantly), 28.96, 8.81, and 11.11%^{ns} (non significantly) respectively, while a higher dose of 500 mg/kg b. wt. was more effective, causing a reduction of 25.02, 47.65, 24.09, and 27.35%. Silymarin used as standard showed a reduction of 55.09, 68.98, 57.46 and 35.04% receiving CCl_4 alone. So depending upon the experimental data it was confirmed that the biochemical parameters of the group treated with ethanolic extract was significantly lower than the CCl_4 treated group. Moreover the treatment with the extract significantly reduced the previously raised levels of AST, ALT, ALP and bilirubin in hepatotoxic rats. Histopathological investigation displayed that at both doses (250 mg/kg b.w. and 500 mg/kg b.w.), the EEFSGP was possessed moderate to good hepatoprotective activity, but at 500 mg/kg b.w. executed excellent hepatoprotective activity against CCl4 induced damaged hepatocytes.*

Key words : *Biomolecules, DPPH assay, Intoxication, Hepatocyte, SGOT, SGPT, SALP.*

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INTRODUCTION:

Glycosmis pentaphylla is a species of flowering plant in the citrus family, Rutaceae, known commonly as orangeberry and gin berry. It occurs in Southeast Asia and northern Australia and in India. It is cultivated for its edible pink fruits. In temperate zones, it can be cultivated indoors as a houseplant [1].

Morphology [2]:

Glycosmis pentaphylla (Retz.) Correa is a shrub or a small tree. **Leaves**: 3-5 foliolate,leaflets entire to sub-dentate to sub-crenate,lateral nerves to 12 pairs. **Flower**: 5-merous, in axillary, elongate ,dense racemes or cymes. **Fruit**: Globose to ellipsoid with glandular pericarp.

Chemical Constituents [2**]:**

Leaves: Contains quinolone alkaloid-glycolone. **Flowers:** Contains alkaloids and an amide, alkaloids-arborine, arborinine, skimmianine, glycorine, glycosmicine benzamide-2-methylamino. Also contains carbazole alkaloid-mupamine **Roots:** Contains Dictamine, γ-fagarine, skimmianine, β-sitosterol, coumarin, stigmasterol, myricylalcohol, base glyborine, triterpenes-arborinolA, arborinol B, arborine, arborinine,carbazolealkaloid-Glycozolinol,Glycozolicine, 3-formylcarbazole and glycosinine,glycozolidol. Root bark contains Acridone alkaloids-Noracronycine, de-methylacronycine and e-N-methylnoracronycine, quinazoline alkaloid-Glycophymine,glycosolone,glycolone,amide-Glycomide.

Traditional uses [3]: The plant is used in indigenous medicine for cough, rheumatism, anaemia and jaundice. The juice of the leaves, which is bitter, is used in fever, liver complaints and as vermifuge. A paste of the leaves with ginger is applied in eczema and skin infections. A decoction of the root is given for facial inflammations.

MATERIALS AND METHOD:

Chemicals and Drugs

The all chemicals used for the extraction and phytochemical screening were of LR and AR grade. The standard drug Silymarin was purchased from Local Retail Pharmacy Shop and solvents and other chemicals were used from Institutional Store and were of AR grade.

Hepatotoxin

In the present study the *in-vivo* hepatoprotective activity was evaluated by CCl_4 induced hepatotoxicity model in rats.

Experimental Animals

White male albino rats weighing about 200-250 g were used. They were obtained from the animal house of C.L. Baid Metha College of Pharmacy, Chennai, **IAEC of CPCSEA reference number: IAEC/XXIX/10/2010.** They were kept under observation for about 7 days before the onset of the experiment to exclude any intercurrent infection, had free access to normal diet and water. The animals were housed in plastic well aerated cages at normal atmospheric temperature (25±5 °C) and normal 12- hour light/dark cycle under hygienic conditions.

Method of Extraction (Soxhlet Extraction)

Introduction

When an organic substance is to be reserved from a solid, it is extracted by means of an organic solvent in which impurities are insoluble. In actual practice the extraction from solids is often tedious and requires through contact and heating with the solvent. This is done in a special apparatus, the Soxhlet Extractor. It consists of a glass cylinder having a side tube and siphon. The cylinder carries a water condenser at the top and is fitted below into the neck of a boiling round bottom flask [4].

Methodology

First the dried fruits and seeds are triturate to make fine powder and the powered material is placed into the thimble made of stout filter paper and the apparatus is fitted up. The flask containing suitable solvent like ethanol is heated on a water bath or on a heating mental. As the solvent boil, its vapors rise through the side tube up into the water condenser. The condensed liquid drops on the solid in the thimble, dissolves the organic substances present in the powdered material and filters out into the space between the thimble and the glass cylinder. As the level of liquid here rises, the solution flows through the siphon back into the boiling flask. The solvent is once again vaporized, leaving behind the extracted substance in the flask. In this way a continuous stream of pure solvent drops on the solid material, extract the soluble substance and returns to the flask. At the end of the operation the solvent in the boiling flask is distilled off, leaving the organic substance behind [4]. Afterwards the ethanolic extract transfer in a clean and dried beaker and is concentrated by placing on a water bath and then cool, keep it in a freeze. From this concentrated extract that is Ethanolic Extract of Fruit-Seeds of Glycosmis pentaphylla (EEFSGP) the preliminary phytochemical screening has to be carried out.

Preliminary Phytochemical Screening [5, 6, 7, 8]

Preliminary Phytochemical screening of EEFSGP had shown the presence of various biologically active molecules such as carbohydrates, aminoacids and peptides, phytosterols, carotenoids, alkaloidsds (higher concentration), terpenoids especially diterpenoids, tri and tetra terpinoids di and aromatic acids and alcohols etc.

Evaluation of Acute Toxicity [9]

In the present study the acute oral toxicity of the EEFSGP was performed by acute toxic class method. In this method the toxicity of the extract was planned to test using step wise procedure, each step using three Wister rats. The rats were fasted prior to dosing (food but not water should be withheld) for three to four hrs. Following the period of fasting the animals were weighed and the extract was administered orally at a dose of 2000 mg/Kg b.w. Animals were observed individually after dosing at least once during the first 30 min; periodically the surveillance was carried out for the first 24 hrs with special attention given during the first 4 hrs and daily thereafter, for a total of 14 days.

Evaluation of In vitro Antioxidant Activity by DPPH Assay (Free Radical Scavenging Activity) [10, 11]

Principle

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay evaluates free radical scavenging activity by measuring the color change that occurs when a DPPH radical is quenched by a free radical scavenger that donates a hydrogen atom.

Method

The ethanolic plant extract was tested for the DPPH free radical scavenging activity according to the method of Pan et al. **[10]** with minor modification. 0.2 mL of the extract solution in ethanol (95 %) at different concentrations was added to 8 mL of 0.004 % (w/v) stock solution of DPPH in ethanol (95 %). The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm until the reaction reached the steady state, using a UV–Visible spectrophotometer. As a positive control, synthetic antioxidant gallic acid was used. All determinations were performed in triplicate. The DPPH radical scavenging activity (S%) was calculated using the following equation:

$$S\% = [(A_{control} – A_{sample}) / A_{control}] \times 100$$

Where Acontrol = absorbance of the blank control (containing all reagents except the extract solution) and Asample = absorbance of the test sample.

Experimental Protocol for the Evaluation of *In vivo* Hepatoprotetive Activity [12]

A total of 30 rats were taken and divided into 5 groups of 6 rats each

Group I: Normal Control Group (only the vehicle (1 mL/kg/day of 1% CMC; p.o.)

Group II: Negative Control CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p.

Group III: Positive Control/Standard Group [CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p.+ Standard Silymarin 100 mg/kg orally (p.o.) for 7 days]

Treatment Groups

Group IV: High Dose Group [CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p + EEFSGP (500 mg/ kg b. w., p.o.)]

Group V: Low Dose Group [CCl4 1 mL/kg (1:1 of CCl4 in olive oil) i.p + EEFSGP (250 mg/ kg b. w., p.o.)] Treatment was given daily for seven days orally.

Collection of Blood: On the 8th day, blood was collected by retro orbital puncture, under mild ether anesthesia after 8 hr fasting. Blood samples were centrifuged at 3000 rpm for 20 mins. Serum was separated and stored at – 200 C until biochemical estimations were carried out.

Biochemical Analysis

The Serum samples were analyzed for

(I) Alanine Aminotransferase (ALT) (SGPT)
(II) Aspartate Aminotransferase (AST) (SGOT)
(III) Alkaline Phosphatase (ALP)
(IV) Serum Bilirubin

Histopathological Analysis

The liver tissue was dissected out and fixed in 10% formalin solution. It was then dehydrated in ethanol (50%-100%), cleared in xylene and embedded in paraffin wax. Afterwards thick sections (5-6 mm) were made and then stained with hematoxylin and eosin dye for photo microscopic observation. The whole biochemical and histopathological analysis was carried out at V.H.S Hospital in Chennai.

RESULT AND DISCUSSION:

Table 1: Results of DPPH Scavenging Activity

Sl. No.	Concentration	Absorbance	S% = [(A0-A)÷A0]	IC50
	(ug/mL)	(A)	X 100	(µg/ml)
Control (DPPH Sol.)				
1.	0.1mM in ethanol	1.174 (A_0)		-
Std (Ascorbic Acid)				
1	4	0.983	16.26	
2	6	0.954	18.73	
3	8	0.917	21.89	
4	10	0.870	25.89	39.87
5	25	0.565	51.87	
6	50	0.037	96.84	
EEFSGP				
1	10	1.044	11.07	
2	25	0.936	20.01	
3	50	0.850	27.59	212.96
4	75	0.786	33.04	
5	100	0.702	40.20	
6	250	0.568	51.61	

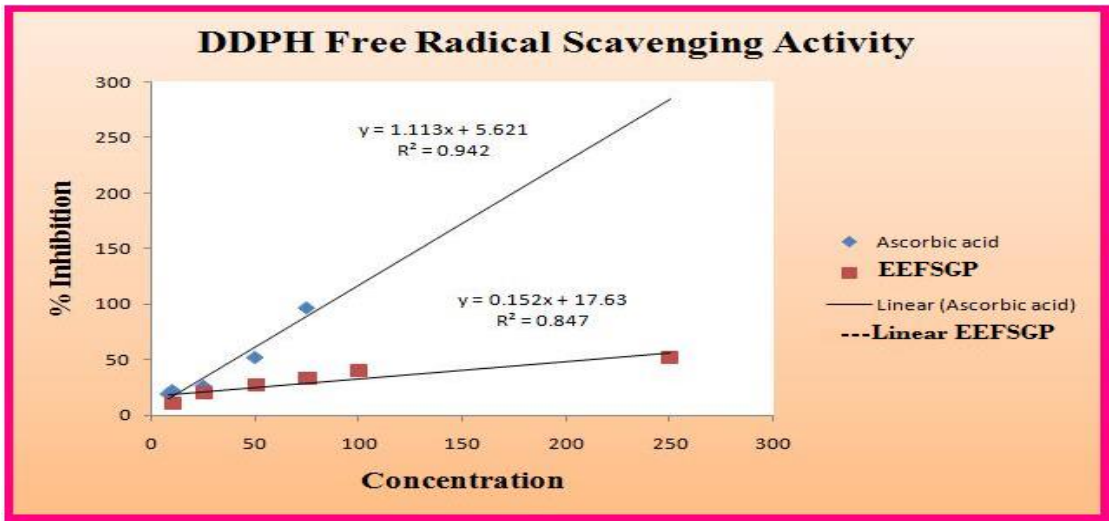

Fig 1: DDPH scavenging Activity of Ascorbic Acid and EEFSGP

Table 2: Results of Hepatoprotective Activity

Group	Treatment	AST(SGOT)	ALT(SGPT)	ALP(SALP)	Serum bilirubin
		IU/L	IU/L	IU/L	mg/dL
1	Normal Control Group (only the vehicle, 1% CMC; p.o.)	53.00±8.672***	46.60±11.95***	139.2±6.914***	0.58±0.08***
2	Negative Control (1:1 of CCl_4 in olive oil; i.p.)	202.2±30.45	204.4±47.74	399.2±16.18	1.17±0.16
3	Low dose [(1:1 of CCl_4 in olive oil) i.p + EEFSGP (250 mg/ kg b. w., p.o.)]	189.6±14.48ns	145.2±39.75*	364.0±16.52*	1.04± 0.15ns
4	High dose [(1:1 of CCl_4 in olive oil) i.p + EEFSGP (500 mg/ kg b. w., p.o.)]	151.6±13.52***	107.0±19.47***	303.0±38.78***	0.85±0.20**
5	Positive Control/Standard				
	Group[(1:1 of CCl_4 in olive oil)i.p.+ Silymarin 100 mg/kg orally (p.o.)]	90.80±17.61***	63.40±15.73***	169.8±8.55***	0.76±0.14***

Data are expressed as mean±SD (n = 6). One-way ANOVA followed by Dunnett's Multiple Comparison Test (P< 0.05) compared with group 2 ;(ns=non significant.*

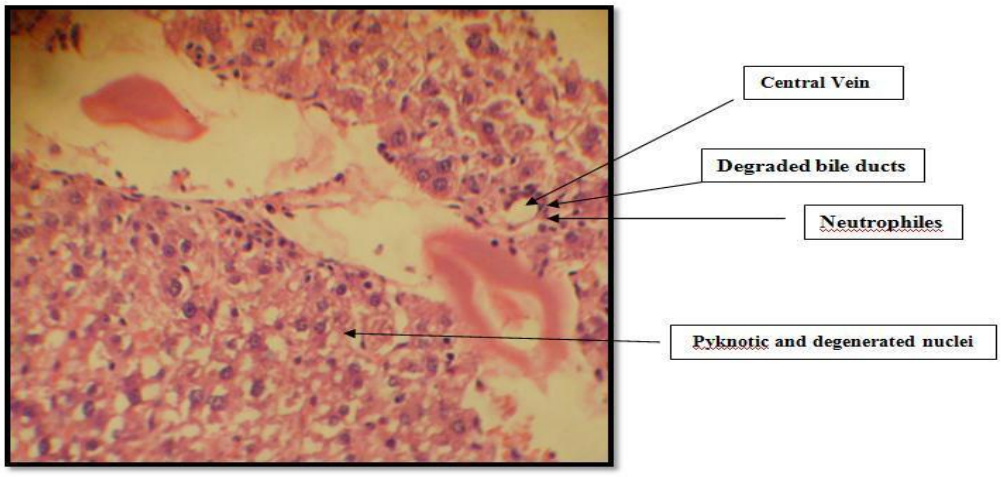

Fig 2: Liver Section of CCl4 Treated Rats.

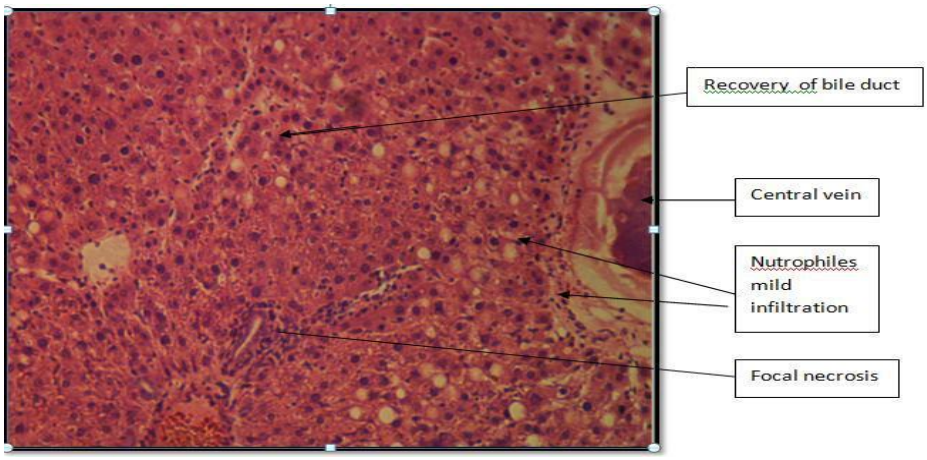

Fig 3: Liver Section of Rats Treated CCl_4 and 100 mg/kg of Silymarin.

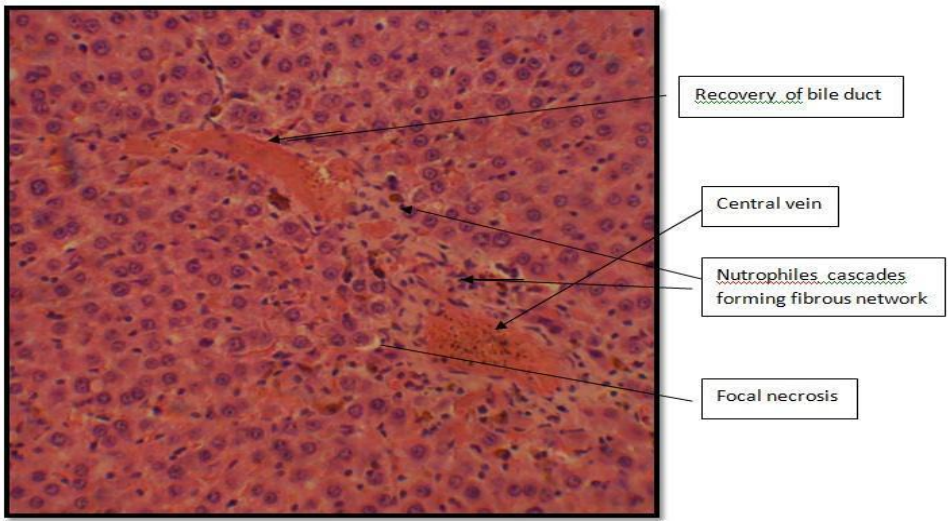

Fig 4: Liver Section of Rats Treated CCl_4 and 500 mg/kg of EEFSGP.

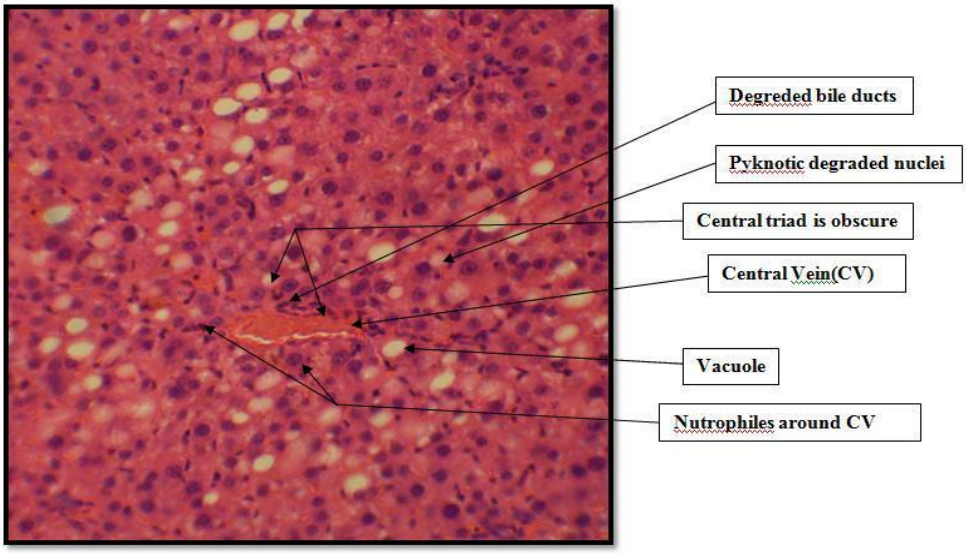

Fig 5: Liver Section of Rats Treated CCl_4 and 250 mg/kg of EEFSGP.

1. Phytochemical Screening

Preliminary Phytochemical screening of EEFSGP had shown the presence of various biologically active molecules such as carbohydrates, aminoacids and peptides, phytosterols, carotenoids, alkaloidsds (higher concentration), terpenoids especially diterpenoids, tri and tetra terpinoids di and aromatic acids and alcohols etc.

2. Acute Oral Toxicity Studies

In this study the acute oral toxicity was evaluated by "Acute toxic class methods (OECD guideline-423)". The extract was administered orally at a dose of 2000 mg/Kg b. wt. During the surveillance period no significant toxicity occurred along with minute non-considerable behavioral changes. After a statistical analysis by trial and error, the significant doses were chosen at 250 mg/kg b. wt p. o (LD) and 500 mg/kg b. wt p. o. (HD) considerably.

3. Hepatoprotective Activity

(i) Statistical analysis

The data were expressed as mean ± SD. Statistical differences at *P < 0.05 between the groups were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 5.04 Instate software package. The data's were compared with group 2 i.e. Negative Control group.

(ii) Analysis of DPPH free Radical Scavenging Activity

The extract was planned to be evaluated for *in-vitro* antioxidant activity by DPPH free radical scavenging activity. The study was carried out taking ascorbic acid as the standard antioxidant which is also a natural antioxidant. The results of antioxidant activity by DPPH free radical scavenging activity was expressed in terms of % inhibition of generated free radicals respectively with respect to various concentrations. Concentration dependent effects were observed i.e.; higher concentrations were found to exhibit higher % inhibition. The graph was constructed by taking % inhibition along the X-axis and various concentrations were taken along the Y-axis **(Fig 1).** The IC_{50} value (50% inhibition) of the EEFSGP and the standard ascorbic acid were determined in all the studies.

The DPPH radical scavenging activity ethanolic extract of fruit-seeds of Glycosmis pentaphylla **(EEFSGP)** was evaluated and compared with Ascorbic acid and the results are given in **Table 1.** The % inhibition at various concentration (4-50 µg/ml) of ethanolic extract of fruit-seeds of Glycosmis pentaphylla as well as standard Ascorbic acid (10 -250 µg/ml) were calculated and plotted in Figure 6.3 using Microsoft Office Excel 2007. The IC_{50} values are calculated from graph and were found to be 39.87µg/ml (Ascorbic acid) and 212.96 ((**EEFSGP)**.

(iii) Biochemical Analysis

The effects of **EEFSGP** on liver marker enzymes and serum bilirubin content are displayed in **Table 2.** The data exhibited that Normal Control Group demonstrated a normal range of AST, ALT, and bilirubin levels while the CCl_4-treated group showed elevated levels of AST, ALT, and bilirubin, thus confirming that CCl_4 causes hepatocellular degeneration at higher doses. The elevation of cytoplasmic AST and ALT is considered an indicator for the release of enzymes from disrupted liver cells. Bilirubin concentration has been used to evaluate chemically induced hepatic injury.

The results displayed in **Table 2** indicate that the elevated levels of SGOT, SGPT, ALP and Serum bilirubin due to CCl_4 intoxication were reduced significantly (*P<0.05) in rats, after treatment with **EEFSGP**. Treatment with ethanolic extract at a **dose of 250 mg/kg b.w.** decreased the SGOT, SGPT, ALP, Serum Bilirubin levels by **6.23%ns(non significantly), 28.96, 8.81, and 11.11%ns (non significantly**) respectively, while a higher dose of **500mg/kg b. wt**. was more effective, causing a reduction of **25.02, 47.65, 24.09, and 27.35%. Silymarin used as standard showed a reduction of 55.09, 68.98, 57.46 and 35.04%** receiving CCl4 alone. So depending upon the data of **Table 2** it was confirmed that the biochemical parameters of the group treated with **EEFSGP** was significantly lower than the CCl_4-treated group. Moreover the treatment with the **EEFSGP** significantly reduced the previously raised levels of AST, ALT, ALP and bilirubin in hepatotoxic rats.

(iv) Histopathological Analysis

A. Fig 2: Liver section of CCl4 treated Rats showed massive fatty changes, necrosis, ballooning degeneration, and severe infiltration of the lymphocytes around central vein and the loss of cellular boundaries, pyknotic and degenerated nuclei.

B. Fig 3: Liver section of rats treated CCl_4 and 100 mg/kg of Silymarin displayed signs of inflammatory cascade around central vein indicating a mild degree of fatty change, and necrosis and focal necrosis (dilatation).

C. Fig 4: Liver section of rats treated CCl_4 and 500 mg/kg of EEFSGP demonstrated minimal inflammatory cellular infiltration around central vein, absence of necrosis, neutrophil cascades forming fibrous network, considerable protection and large septa of connective tissue flowing together and penetrating into the parenchyma, normal liver architecture.

D. Fig 5: Liver section of rats treated CCl_4 and 250 mg/kg of EEFSGP had shown the very less recovery, with obscure central triad and infiltration of neutrophils around central vein, degraded fatty change, and necrosis and focal necrosis (dilatation), loss of cellular boundaries.

CONCLUSION:

In conclusion, we report here that the The IC_{50} value (50% inhibition) of the **Ethanolic Extract of Fruit-seeds of *Glycosmis pentaphylla* (EEFSGP)** was found to be **212.96 µg/ml and EEFSGP** had ability to regenerate the hepatocytes ***in vivo*** and also possessed potential antiinflmmatory activity which was confirmed by **liver biopsy.** The hepatoprotective activity of the **EEFSGP** could be considered as excellent with regards to the standard drug silymarin.

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