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Hepatoprotective Activity of *Flueggea virosa* Against d-Galactosamine Induced Liver Damage in Rats

G. Dayanand Reddy[1*], R. Ganesan[2], J. Kowsalya[1], Shahana Ahamed[1], A. Arshad Ali[1] and Sunil Kumar Podh[1]

[1]Department of Pharmacology, Siddha Central Research Institute, Chennai - 600106, Tamil Nadu, India; dayanandscri@gmail.com

[2]Department of Biochemistry, Siddha Central Research Institute, Chennai - 600106, Tamil Nadu, India

Abstract

Flueggea virosa belonging to the family Phyllanthaceae, commonly known as White berry bush was traditionally used for the treatment of rheumatism, sterility, and rashes, and aninfusion of the root is taken to relieve malaria. The study was intended to evaluate the hepatoprotective effect of hydroethanolic extract of the roots of *Flueggea virosa* (200, 400, and 600 mg/kg) against d-Galactosamine-induced liver damage in rats. Silymarin (100 mg/kg) wasused as a reference drug. Blood samples were collected after 24 h for haematological and biochemical investigation before the rats were euthanized, and liver samples were taken for histopathology. Oral administration of the HEFV at a dose of 200 mg/kg displayed a significant hepatorenal protective effect against d-Galactosamine by lowering liver biomarkers (SGPT, SGOT, and ALP), kidney biomarker levels (urea and creatinine) and hematological parameters when compared with the disease control group. These findings were strongly supported by thehistopathological results of liver sections with fewer pathological changes in comparison withthe group treated by the standard drug silymarin and verified the protective effect of the plant extract. The LCMS report of the extract revealed the presence of hepatoprotective ingredients like Tocopherol, Fraxetin, Glaucine, Kaempferol, Methicillin, Capsaicin, and Austinol in the hydroethanolic extract of *Flueggea virosa* root. The results show that the selected dose of *Flueggea virosa* (200 and 400 mg/kg) showed dose-dependent hepatoprotective effects on d-Galactosamine-induced hepatotoxicity in rats. The protection of *Flueggea virosa* against d-Galactosamine-induced liver damage and restoration of biochemical values could result from the content of tocopherols and tetrahydroxy flavones.

Keywords: Antioxidant, d-Galactosamine, *Flueggea virosa*, Hepatotoxicity

Abbreviations: HEFV - Hydroethanolic Extract of *Flueggea virosa,* d-GAL - d-Galactosamine, SGPT- Serum Glutamate Pyruvate Transaminase, SGOT – Serum Glutamate Oxaloacetate Transaminase, ALP - Alkaline Phosphatase, GGT – Gamma Glutamyl Transferase, SOD - Superoxide Dismutase, CAT - Catalase, LPO - lipid peroxidase, GSH – Glutathione.

**Author for correspondence*

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1. Introduction

Herbal drugs play an important role in human health care. Most of the world's population relies on the use of traditional medicine which is predominantly based on plant materials[1]. These medical practices originated from time immemorial and developed gradually, to a large extent, by relying on practical experiences without significant references to modern scientific principles[2,3]. They were passed from one generation to another by oral tradition and guarded literature which were incorporated from ancient beliefs[4]. Most herbal drugs are unscientifically exploited and/or improperly used although they are effective in the treatment of various diseases[5]. Therefore, these plant drugs must be investigated properly with the aid of modern scientific technology.

The liver is the largest solid organ in the body and plays a very important role in the body owing to its multibiological functions in protein, lipid, and carbohydrate metabolism[6,7]. It also removes toxins from the body's blood supply, maintains healthy blood sugar levels, regulates blood clotting, and performs hundreds of other vital functions. Hepatic diseases constitute a global concern, and medical treatments for these diseases are often difficult to achieve and may have limited efficiency[8]. Liver diseases may be a great concern to international public health as they are fatal and posing a serious challenge of treatment. Due to the belief that herbal medicines are harmless and free from serious adverse reactions, the use of herbal medicines to treat liver diseases have been increased worldwide. Further the limited therapeutic choices and sometimes the unsatisfactory therapeutic failure of modern medicine have increased the usage of alternative medicine including herbal preparations.

Flueggea virosa (Roxb. ex Willd.) belonging the family Euphorbiaceae is usually a shrub of 1-9 m tall with glabrous obovate leaves grown in tropical areas. The plant contains a variety of phytochemical constituents like bergenin, flueggenoids, dinorditerpenoids, triterpenoids, kaempferol, quercetin, glycosides, anthraquinones, steroids, resins and various other alkaloids and flavonoids[9]. These phytoconstituents possesses vital properties of anti-oxidant, anti-hepatitis, anti-malarial, anti-arrhythmic, analgesic, anti-inflammatory and various other medicinally effective properties[10]. Whole plant of *Flueggea virosa* is traditionally claimed to possess various medicinal properties with evidence of its phytochemical and ethnopharmacological validation. This emphasized the need to carry out the evaluation of hepatoprotective activity of *Flueggea virosa* on d-Galactosamine induced liver damage in rats. Production of these herbal formulations with standards of efficacy, purity and safety can revitalise the treatment of liver ailments.

2. Material and Methods

2.1 Preparation of the Extract

The roots of *Flueggea virosa* were collected from Aralvaymozhi area of Kanyakumari district on August 2021. The plant material was identified and authenticated and the voucher specimen number is F16092101V. The roots were washed thoroughly in tap water, shade dried and powdered. The powder was successively extracted with ethanol and water (70:30) in a Soxhlet apparatus for 48 hrs. The filtrate was then concentrated and the solvent was evaporated under reduced pressure in a rotary evaporator. This crude extract was referred to as **HEFV (Hydroethanolic Extract of *Flueggea virosa*).** 2900 grams of dry root powder yielded 62grams of extract accounting to a yield of 2.14%. The extract obtained was further subjected to pharmacological investigation in rats. For administration, the crude extract was dissolved in normal saline to required concentrations.

2.2 Determination of Phenolic Compounds

The total phenols were estimated with the method of Noreen, *et al.* using gallic acid as a calibration standard. The results were expressed as mg of Gallic Acid Equivalents (GAE) per gram dry extract weight[11].

2.3 Determination of Total Flavonoids

The total flavonoid content was estimated according to Zhao, *et al.,* using quercetin dihydrate as a calibration standard. The results were expressed as mg of Quercetin Equivalents(QE) per gram dry extract weight[12,13].

2.4 LC-MS Analysis of HEFV

LC-MS was used for the determination of bioactive compounds in the root extract of *Flueggea virosa*. It was carried out at Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Bombay using Agilent Technologies TOF/Q-TOF Mass Spectrometer[14].

2.5 *In-Vitro* Antioxidant Activity

2.5.1 DPPH Method

The stable 1,1-Diphenyl-2-Picryl Hydrazyl Radical (DPPH) was used for the determination of free radical-scavenging activity of the extract[15]. To various concentrations ofthe root extract (10, 20, 30, 40 and 50 µ g/mL), 1 ml of 0.2 mM DPPH was added and incubated in dark for 30 min. The purple colour developed was read spectrophotometrically at 515 nm. The IC_{50} values was calculated and compared with the standard compound ascorbic acid.

2.5.2 Nitric Oxide Radical Scavenging Activity

The root extract of *Flueggea virosa* at graded concentrations of 10–50 µg/mL were added to 10 mM sodium nitroprusside and incubated for 2.5 h. After incubation, Griess reagent was added to the tubes and the absorbance of the chromophore formed was read at 540 nm. The IC_{50} values and per cent inhibition by various concentrations of extract were calculated by comparing the absorbance values of the control and test compounds[16].

2.5.3 FRAP Assay

The reducing power was determined based on the ability of the antioxidant to form a coloured complex with potassium ferricyanide, TCA and $FeCl_3$. Various concentrations of HEFV (10, 20, 30, 40 and 50 µ g/mL) were tested and the absorbance was then measured at 700 nm[17]. A higher absorbance of the reaction mixture indicates a higher reducing power.

2.6 Drugs and Chemicals

Silymarin was purchased from Sai Mirra Innophar, Chennai. The chemicals used in thestudy include Sodium Chloride, Formaldehyde, Sodium Citrate and EDTA of analytical grade were purchased from Theres scientific works, Chennai.

2.7 Animals

Healthy female albino rats of Wistar strain weighing about 150-200 g were used for the study. The rats were procured from Tamil Nadu Veterinary and Animal Sciences University (TANUVAS), Madhavaram, Chennai. They were kept in departmental animal house in well- ventilated room at 22 ± 2˚ C with 12 h light and 12 h dark cycles for 1 week before and during the experiments. Animals were provided with standard rodent pellet feed (Amrut, India)[18,19]. This study was conducted in compliance with the recommendations of the CCSEA guidelines for laboratory animal facility after approval of Institutional Animal Ethics Committee (IAEC) of Siddha Central Research Institute, Central Council for Research in Siddha (Ministry of AYUSH), Arumbakkam, Chennai-106. Approval No: **197/ Pharma- SCRI/2018.**

2.8 Experimental Design and Drug Administration

In this study 36 female rats of Wistar strain were used for a period of 3 weeks. The animals were divided into six groups initially and then randomized with Alanine Transferase levels (ALT) of the animals. (Effect size = 0.65, α = 0.05, Power(1-β) = 0.95, F = 4.22, calculated using G*power 3.1.9.4)

Group 1 was administered with 0.9%Na Cl (Normal Saline) served as solvent control(**Control**).

Group 2 was administered with d-Galactosamine 400 mg/kg i.p. on 21st day served as disease- induced control (**Disease control**).

Group 3 was administered with 100mg/kg of Silymarin orally along with d-Galactosamine 400mg/kg i.p. on 21st day served as disease-induced control (**Standard**).

Group 4 was administered with 200 mg/kg of HEFV orally along with d-Galactosamine 400mg/kg i.p. on 21st day served as low dose treatment group (**HEFV 200**)[20].

Group 5 was administered with 400 mg/kg of HEFV orally along with d-Galactosamine 400mg/kg i.p. on 21st day served as mid dose treatment group (**HEFV 400**).

Group 6 was administered with 600 mg/kg of HEFV orally along with d-Galactosamine 400mg/kg i.p. on 21st day served as high dose treatment group (**HEFV 600**).

After one week of randomization, all the animals were administered with the respective doses on daily basis at the same time for entire period of the study (21 days). 0.9% Sodium chloride (Normal saline) was used as vehicle. The volume of the drug given was not more than 1 ml/100 g body weight. d-Galactosamine (400mg/kg) was injected as a single dose through intraperitoneal route on day 21. The health condition of the animals was recorded at least twice daily and observed for morbidity and mortality. Body weight and feed consumption were

measured once in a week during the study. All the blood samples were collected from retro orbital plexus from the animals under anaesthesia. Haematological and biochemical examination was made on the 22nd and 23rd day to confirm the induction of hepatotoxicity and liver damage.

2.9 Preparation of Liver Tissue Samples

On the 22nd day, *i.e.*, 24 h after the last treatment, all rats were weighed and euthanized under proper anaesthesia using 1.5% isoflurane as proposed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The blood was collected by retro-orbital puncture in an EDTA-containing tube from all the experimental rats. The liver of each rat was removed and weighed and cryo-freezed at -80˚C. Left lobe of the liver was preserved in 10% formaldehyde solution for histopathological evaluation and the right lobe was subjected to homogenisation, which was prepared by centrifuging at 1000 × g for 10 min at 4°C. The supernatant was collected after centrifugation and was used for enzymatic assays.

2.10 Estimation of Haematological Parameters

Blood samples were obtained through the retro-orbital sinus from isoflurane anesthetized albino rats. These samples were subjected to hematology analysis. Hematology analysis of Packed cell volume, WBC, Lymphocytes, Granulocytes, hemoglobin, erythrocyte indices including MCV, MCH and MCHC and platelet count were determined using the hematology analyzer[21].

2.11 Estimation of Biochemical Parameters

Serum was analysed for various biochemical parameters like total bilirubin, Serum Glutamic Pyruvate Transaminase (SGPT), Serum Glutamic Oxaloacetic Transaminase (SGOT), Alkaline Phosphatase (ALP), total protein, albumin and GGT levels. The total protein concentration and total bilirubin were also measured by the method of Lowry, *et al.* and Mallay and Evelyn respectively[22]. Liver damage was assessed by estimating the enzymatic activities of serum AST, ALT, and ALP, as well as albumin levels using the corresponding commercialkits[23].

2.12 Evaluation of Antioxidant Properties

For assessment of antioxidant activities, immediately after collection of blood the rats were sacrificed and livers were dissected out and washed in ice cold normal saline, blotted dry and weighed. Required quantity of the tissue was weighed and 25% (w/v) of each tissue homogenate was then prepared using KCl solution (1.15% w/v) and centrifuged at 3000 g at 4˚C for 1 hr. The supernatant was used for the determination of Lipid Peroxidation (LPO) and endogenous antioxidant systems such as reduced Glutathione (GSH), Superoxide dismutase (SOD) and Catalase (CAT)[24].

2.13 Histological Observation

For histopathological study, the liver tissues were collected and immediately fixed in 10% formalin, dehydrated in gradual ethanol (50-100 %), cleared in xylene and embedded in paraffin[25]. Sections (4-5 mm) were prepared and then stained with hematoxylin-eosin dye for photo microscopic observations[26]. An experienced pathologist evaluated the sections and histological severity of liver damage was graded using Modified Histological Classification of Suzuki's score[27]. It comprised four components (Congestion, Necrosis, Bile duct hyperplasia, Mononuclear cell infiltration) which was graded from 0 to 4 (Table 1).

2.14 Statistical Analysis

Defined statistics were expressed as mean and standard deviation. Statistical significance between more than two groups was tested using one-way ANOVA followed by Dunnett's multiple comparison test by Graph pad prism software version-928. The significance level was set at P value<0.05 for all tests.

Table 1. Modified histological classification of suzuki's score

Score	Congestion	Necrosis	Bile duct hyper plasia	Mononuclear cell infiltration
0	None	None	None	None
1	Minimal	0-10 %	Minimal	Minimal
2	Mild	10-30 %	Mild	Mild
3	Moderate	30-60 %	Moderate	Moderate
4	Severe	> 60%	Severe	Severe

3. Results

3.1 Determination of Phenolic Compounds

The total phenols were estimated according to the method of Noreen *et al*[11]. The total phenolic content was expressed as 6.057 ± 0.133 mg/g gallic acid equivalents of the extract.

3.2 Determination of Total Flavonoids

The total flavonoid content was estimated according to Kosalec *et al*[13]. The totalflavonoid content was expressed as 3.217 ± 0.032 mg/g quercetin equivalents of the extract.

3.3 *In-Vitro* Antioxidant Activity

3.3.1 DPPH Method

The Results of the DPPH extract reveals that the composition of the extract has influenced the activity of DPPH. Figure 1(a) shows the percentage of the scavenging activity of the extract which was compared with the ascorbic acid as standard. The maximum scavenging activity can be observed at a concentration of 50 µg/ml which produced 92% inhibition.

3.3.2 Nitric Oxide Radical Scavenging Activity

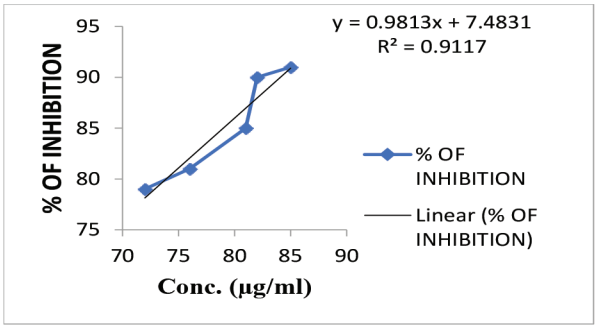
The amount of nitrous acid decreases with HEFV and the extent of decrease reflects the extent of scavenging. The extract had shown an IC_{50} values of 29.64 µg/ml as compared to the standard. The percentage inhibition of the extract showed the nitric oxide radical scavenging activity in a dose dependent manner.

3.3.3 FRAP Assay

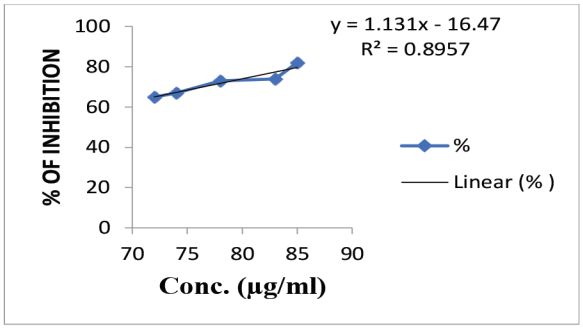
Figure 1(c) shows the percentage of ferric iron reducing activity. The capacity of reducing activity was determined by IC-50 value, which is 3.24 µg/ml for the HEFV extract. The maximum reduction activity achieved by the extract is 86% at the concentration of 50 µg/ml.

3.4 LC-MS Analysis of HEFV

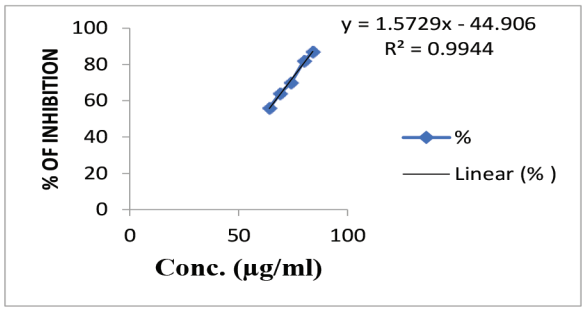
The LC-MS data of HEFV showed 12 prominent peaks with different retention time in the chromatogram which indicated the presence of various secondary bioactive metabolites inthe extract. Some of the principal bioactive compounds corresponding to various peaks were identified to belong to different groups namely



a) DPPH radical scavenging assay



b) Nitric oxide radical scavenging activity



c) Ferric reducing antioxidant power assay

Figure 1. *In vitro* antioxidant property of Hydroethanolic extract of *Flueggea virosa*: **(a)** DPPH radical scavenging assay, **(b)** Nitric oxide radical scavenging activity and **(c)** Ferric reducing antioxidant power assay.

Buformin, Tocopherol, Methyl benzaldehyde, Fraxetin, Glaucine, Trenbolone, Dolichyl phosphate D-mannose, Dobutamine, Kaempferol, Methicillin, Capsaicin and Austinol. Various other compounds such as Sylvatine, Carbosulfan, 8-Hydroxyadenine, 6-Mercaptopurine ribonucleotide, Cordeauxione and Feruloyl putrescine were also found to be present.

3.5 Estimation of Haematological Parameters

Pre-treatment with the root extract of *Flueggea virosa* retained the normal haematology characteristics in the rats as shown in Table 2 which revealed that there were no compound related changes in the hematological parameters.

3.6 Estimation of Biochemical Parameters

The rats treated with hydroethanolic extract of *Flueggea virosa* showed a significant decrease ($p<0.05$) in the elevated serum marker levels although there was not a much decrease in the Silymarin treated group as shown in Figure 2. A significant dose dependent reduction was observed with low dose of HEFV having the maximum reducing effect on SGOT and SGPT levels. Treatment with the root extract of *Flueggea virosa* showed a significant reduction in ALP levels of HEFV 200 mg/kg group. No significant changes were observed in Total bilirubin, total protein, albumin and GGT levels. A slight reduction of Urea and creatinine levels were observed in HEFV treated groups but the difference was not statistically significant when compared with the disease control group.

3.7 Evaluation of Antioxidant Properties

In the disease control group, the SOD activity decreased and no significant changes were observed when compared with normal control group. When treated with HEFV, SOD activity increased significantly in the 200 and 400mg/kg respectively (Figure 3(a)). LPO is a measurement for lipid peroxidation of cell membranes. A highest LPO was observed in d-GalN which gradually decreased in HEFV treated rats. (Figure 3(b)). In contrast, CAT activity was significantly increased in the HEFV treated group ($p<0.05$) when compared with the d-Gal N group (Figure 3(c)). GSH activity significantly increased in the HEFV group ($p<0.05$) in a dose dependent when compared with the control group (Figure 3(d)).

3.8 Histopathology of Liver Samples

The liver damage was graded according the Modified Histological Classification of Suzuki score which is shown in Table 3. Histopathological examination of liver sections of normal control group showed normal cellular architecture with distinct hepatic cells, sinusoidal

Table 2. Effect of HEFV on haematology parameters

Parameters	Control	d-Gal N	Silymarin	HEFV 200	HEFV 400	HEFV 600
WBC (109/L)	6.06 ± 3.11	8.02 ± 2.57	9.38 ± 4.81	8.46 ± 5.08	6.12 ± 3.03	6.51 ± 3.85
LYM (109/L)	4.38 ± 2.12	5.55 ± 2.08	6.58 ± 3.34	6.23 ± 3.68	4.38 ± 2.26	4.12 ± 2.69
MON (109/L)	0.25 ± 0.18	0.3 ± 0.11	0.41 ± 0.22	0.32 ± 0.26	0.18 ± 0.13	0.37 ± 0.21
GRA (109/L)	1.43 ± 0.90	2.17 ± 0.53	2.39 ± 1.61	1.91 ± 1.19	1.56 ± 0.73	2.02 ± 1.26
HGB (g/dL)	7.93 ± 3.59	10.91 ± 3.16	7.78 ± 4.51	9.9 ± 4.93	11.14 ± 2.52	10.18 ± 4.84
MCH (pg)	18.52 ± 0.66	18.47 ± 0.66	18.28 ± 0.44	18.78 ± 0.88	18.36 ± 0.50	18.73 ± 0.37
MCHC(g/dL)	36.25 ± 1.33	36.25 ± 1.33	36.72 ± 0.75	37.05 ± 0.58	36.82 ± 1.05	36.8 ± 0.94
RBC (1012/L)	4.33 ± 1.97	5.95 ± 1.80	4.26 ± 2.52	5.35 ± 2.66	6.10 ± 1.50	5.48 ± 2.61
MCV (fl)	51.18 ± 2.07	50.05 ± 2.81	50.98 ± 3.25	50.77 ± 2.73	49.88 ± 2.70	50.93 ± 2.19
HCT (%)	21.78 ± 9.37	29.42 ± 7.92	21.07 ± 12.06	26.63 ± 13.00	30.12 ± 6.09	27.47 ± 12.67
RDW (%)	15.47 ± 0.92	15.62 ± 0.43	15.62 ± 0.63	15.7 ± 0.55	15.12 ± 0.25	15.02 ± 0.62
RDWa (fl)	32.75 ± 1.56	33.88 ± 1.38	32.08 ± 0.82	33.82 ± 1.38	33.26 ± 0.57	33.65 ± 2.27
PLT (109/L)	672.5 ± 344.44	576.5 ± 149.56	627.33 ± 282.8	525.83 ± 242.29	458.2 ± 291.24	368.33 ± 209.54
MPV (fl)	5.08 ± 0.194	5.25 ± 0.39	5.35 ± 0.40	5.25 ± 0.18	5.06 ± 0.34	5 ± 0.24

Values are expressed as Mean ± SD of respective group of animals, where n=6 *p < 0.05, **p < 0.01 when compared with the d- Gal N group and normal control where thesignificance was performed by One way ANOVA followed by post hoc Dunnett's multiple comparison test. There were no significant changes in the HEFV treated animals when compared with both normal and disease *control groups.*

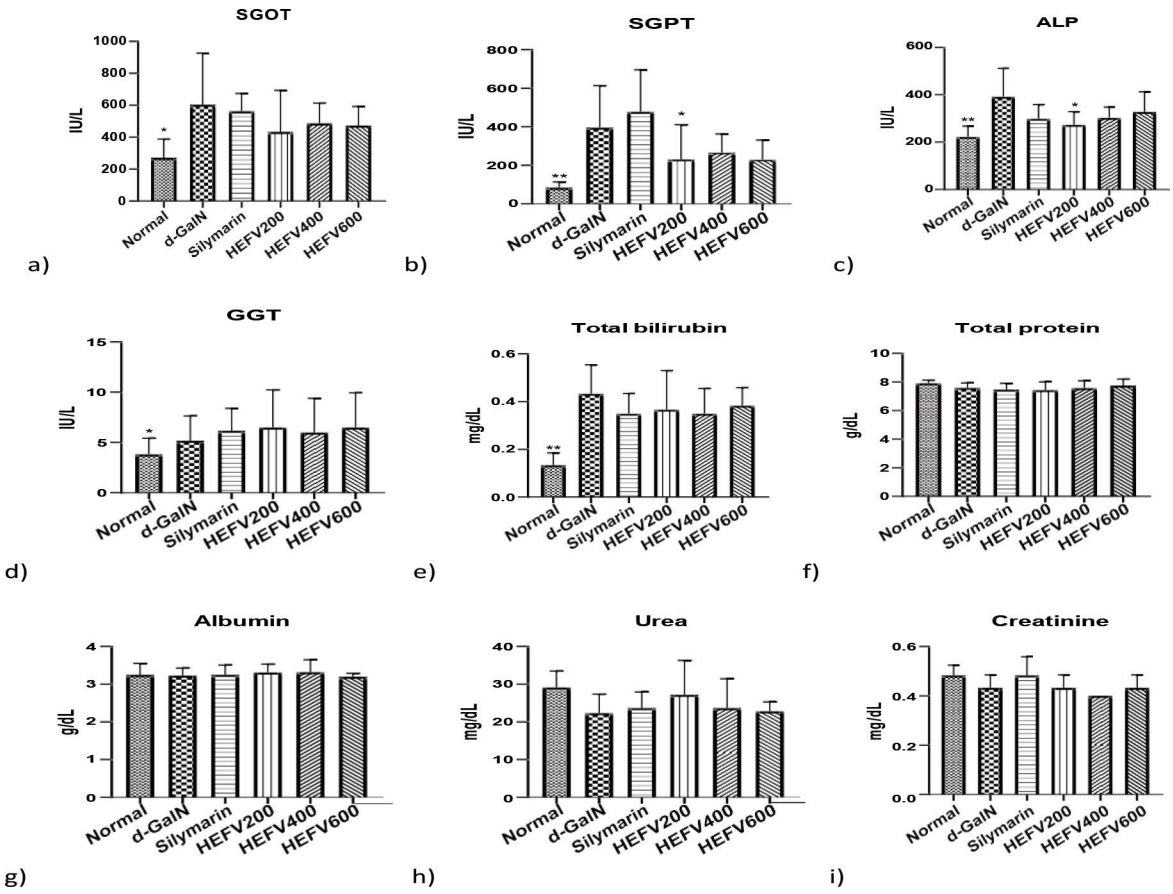


Figure 2. Effect of hydroethanolic extract of *Flueggea virosa* on serum enzyme levels of d- Gal N intoxicated rats: Values are Mean ± SD; n = 6 in each group. Drug treatment was donefor 21 days. **(a)** SGOT, **(b)** SGPT, **(c)** ALP, **(d)** GGT, **(e)** Total bilirubin, **(f)** Total protein, **(g)**Albumin, **(h)** Urea, **(i)** Creatinine *p < 0.05, **p < 0.01 when compared with the d- Gal N group where the significance was performed by One way ANOVA followed by post hoc Dunnett's multiple comparison test.

spaces and central vein (Figure 4(a)). Treatment with d-Galactosamine showed centrilobular necrosis and vacuolization (Figure 4(b)). The rats treated with Silymarin showed slight protection of liver with degeneration and necrosis intoxicated by d- Gal N (Figure 4(c)). The liver sections of the rats treated with HEFV low and mid dose showed a sign of protection against d-Galactosamine intoxication as evident by presence of normal hepatic cords and absence of necrosis with minimal inflammatory conditions around the central vein as shown inthe (Figures 4(d)-(f)).

4. Discussion

Liver damage induced by d-Galactosamine is a widely used model for the screening ofhepatoprotective drugs. d-Gal N can decrease the uracil nucleotides in the liver and heart[29]. This suggests that d-Gal predominantly inhibits hepatic glucuronidation leading to progressivedamage of cellular membranes[30,31]. In this study, administration of 400mg/kg of d-Galactosamine to rats on 21st day markedly increased the plasma levels of ALT confirming that liver injury occurred within 24 hours of treatment. ALT is the most reliable, sensitive and specific marker of liver injury[32]. It is abundant in hepatocytes and is released into serum as a result of hepatocellular damage, so its level in plasma approximates the extent of liverdamage[33].

Phenols and flavonoids are the most important plant secondary metabolites and wildly spread throughout the plant kingdom[34]. It is universally accepted that many biological activities and health benefits of plants may be attributed to the antioxidant activity of flavonoids and phenolic compounds they contained. Therefore, the total phenolic and flavonoids content in plant extracts were investigated. The result represented that hydroethanolic extract of *Flueggea virosa* had higher content of phenols

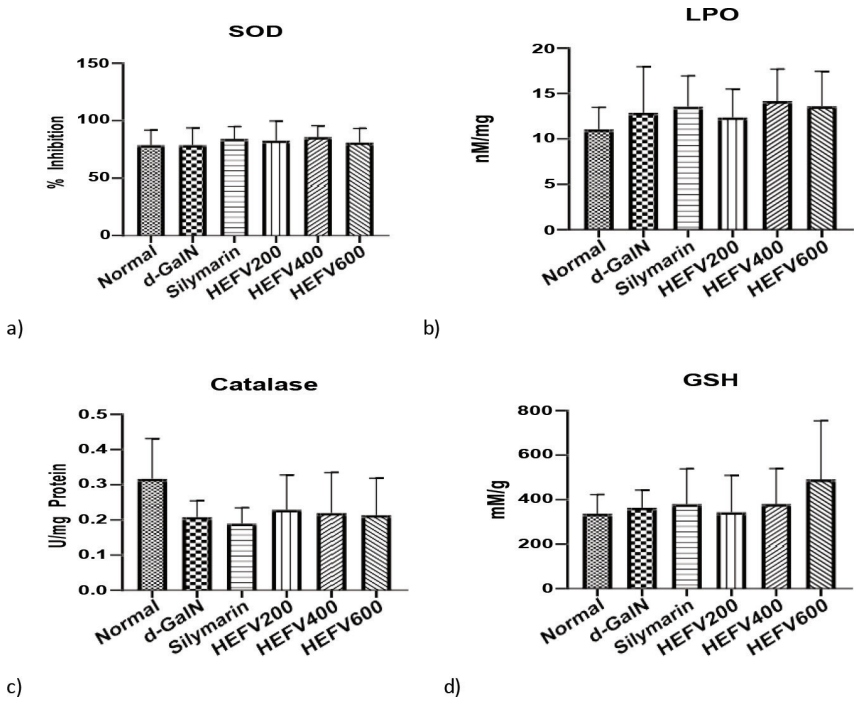


Figure 3. Effect of hydroethanolic extract of *Flueggea virosa* on antioxidant liver enzymes of d-Gal-N intoxicated rats: **(a)** Superoxide Dismutase (SOD), **(b)** Lipid Peroxidase (LPO), **(c)** Catalase, **(d)** Glutathione (GSH) *p < 0.05, **p < 0.01 when compared with the d- Gal N group where the significance was performed by One way ANOVA followed by post hoc Dunnett's multiple comparison test.

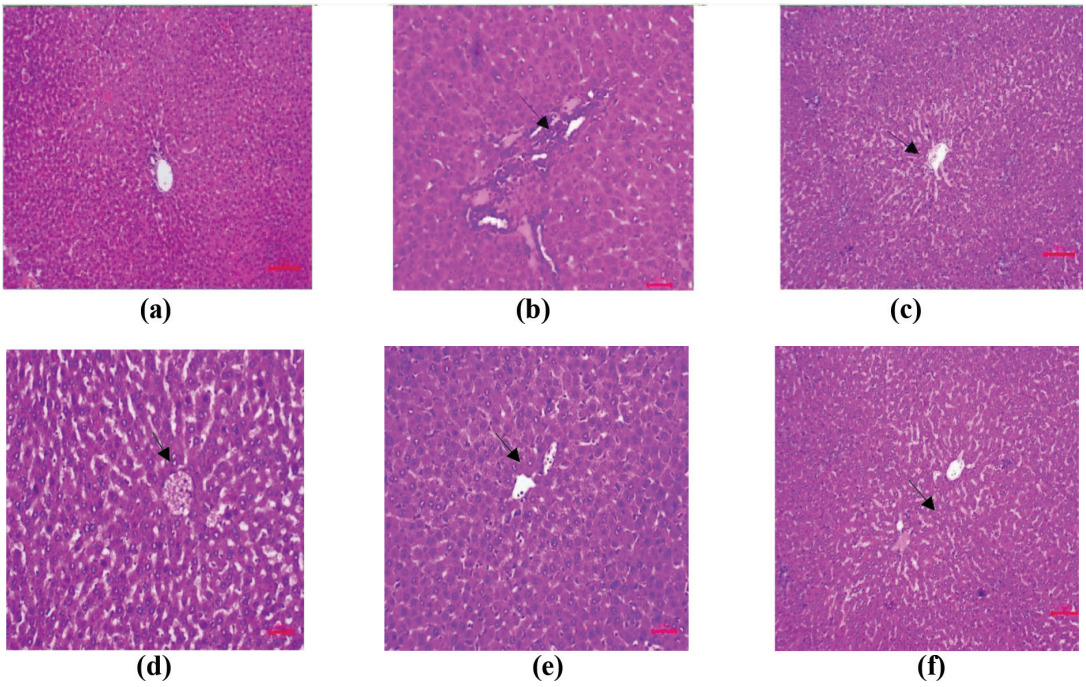


Figure 4. Effect of hydroethanolic extract of *Flueggea virosa* on histopathology of liver in d- Gal N intoxicated rats: Histological liver sections of rats of different study groups were taken under 100X magnification with 50 µm. **(a)** Histologic sections of the liver treated with Normalsaline, **(b)** Liver intoxicated with d-Galactosamine, **(c)** Liver treated with Silymarin (100 mg/kg), **(d)** Liver treated with HEFV 200 mg/kg, **(e)** HEFV 400mg/kg, **(f)** HEFV 600 mg/kg

Table 3. Modified Histopathological Classification of Suzuki (MHCS) scoring of liver

Group	Congestion	Necrosis	Bile duct hyperplasia	Mononuclear cell infiltration	Score
Group I (Normal control)	None	None	None	None	**0**
Group II (Disease control)	Moderate	Severe	Severe	Severe	**4**
Group III (Silymarin)	Mild	None	Mild	Mild	**2**
Group IV (HEFV 200mg/kg)	Mild	None	Mild	Mild	**2**
Group V (HEFV 400mg/kg)	Moderate	None	Moderate	Moderate	**3**
Group VI (HEFV 600mg/kg)	Moderate	Minimal	Moderate	Moderate	**3**

and flavonoids which contributes to its antioxidant property.

The *in vitro* antioxidant assays like DPPH, Nitric oxide radical scavenging activity andferric reducing antioxidant power assay were performed in the hydroethanolic extract of the *Flueggea virosa* root which unveiled a strong antioxidant property. From the results it is postulated that the extract has a promising antioxidant activity which was significantly comparable to its free radical scavenging activity.

The liver plays a key role in the biosynthesis of protein and lipid metabolism. So synthetic dysfunction in the liver can have adverse effects on both cellular and soluble components of blood[35,36]. Certain drugs including alkylating cytotoxic agents could also affect blood formation rate and the normal range of haematological parameters[37,38]. Treatment of rats with d-Galactosamine in this study, did not significantly affect the haematological parameters such as RBC, Hb, PCV, WBC, Granulocytes, Lymphocytes, Monocyte, Platelet count and neutrophils percentage. Pre-treatment with the root extract of *Flueggea virosa* retained the normal haematology characteristics and no compound related changes were noted in the hematological parameters.

The enhanced levels of the serum liver markers like total bilirubin, SGPT, SGOT, ALP and GGT levels were observed in d-Galactosamine treated rats which corresponds to the extensive liver damage induced by the toxin. Restoration of these enzymes to the near normal values in the selected dose of HEFV is a clear manifestation of hepatoprotective effect of the extract. Bilirubin, a metabolic product of haemoglobin, undergoes conjugation with glucuronic acid in hepatocytes to increase its water solubilithy[39]. Determination of serum bilirubin represents an index for assessment of hepatic function, and any abnormal increase in the levels of serum bilirubin indicates hepatobiliary diseases and severe disturbance of hepatocellular function[40]. Decreased serum bilirubin level following HEFV extract and Silymarin treatment indicated the effectiveness of the extract and the standard drug in restoring normal functional status of the liver. There was no significant change in the total protein and albumin level stating the comprehensive metabolic panel of liver. The estimation of the renal markers like serum urea and creatinine is a useful quantitative marker of the extent and type of hepatorenal damage[41,42]. The stabilization of these renal markers by the HEFV extract clearly indicates the improvement of the functional status of the kidney cells. The reversal of the increased serum enzymes in d-Galactosamine induced liver damage by the extract may be due to prevention of the leakage of intracellular enzymes by its membrane stabilizing activity.

The body has an effective defense mechanism, consisting of a set of endogenous antioxidant enzymes including SOD, CAT as well as non-enzymic antioxidants, such as reduced glutathione and lipid peroxidase[43]. In d-Galactosamine intoxicated hepatotoxicity, the balance between ROS production and these antioxidant defense may be lost, consequently oxidative stress may occur which may finally lead to hepatic necrosis[44,45]. Decrease in their activities may result in several deleterious effects in the regulation of physiological mechanisms like cell differentiation, apoptosis, regulation of redox-sensitive signalling pathways etc. Toxic dose of d-Galactosamine significantly reduced the activities of enzymic (CAT and SOD) and non-enzymic (GSH) antioxidant system and enhanced lipid peroxidation (LPO) level of liver tissue, as were found in normal control animals. It was observed that HEFV treatment significantly raised both enzymic and non-enzymic antioxidant systems as compared with the disease control group, while the elevated LPO level were found to be reduced back towards the normal level in HEFV treated animals. Silymarin treated groups did not significantly increase the antioxidant enzymes like SOD, CAT and GSH.

Liver damage and alteration in structure and function of cellular membranes was measured by the level of lipid peroxide[46]. Elevation of MDA level in liver indicates excessive free radical generation and consequently enhanced lipid peroxidation which leads to severe tissue damage[47]. Treatment with HEFV significantly reversed these changes whereas Silymarin treated group increased the LPO levels which indicates that the hepatic damage induced by d-Galactosamine was not reversed by the standard drug Silymarin.

Glutathione (GSH) is one of the tripeptide and non-enzymatic biological antioxidants present in high quantities in the liver[48]. It helps to remove free radical species such as hydrogen peroxide, superoxide radicals, alkoxy radicals and maintain membrane protein thiols, and serves as a substrate for glutathione peroxidase and glutathione transferase[49]. Reduced level of GSH is implicated in the enhancement of lipid peroxidation in d-Galactosamine treated rats[50]. Pre-treatment with HEFV significantly increased the level of GSH in a dose-dependent mannerportraying its ability to scavenge these free radicals.

Histological examination of the liver sections reveals that the normal liver architecture with central vein, hepatic sinusoids, hepatocytes, Kupffer cells and hepatic portal vein was disturbed and vacuolar degeneration was caused by hepatotoxin intoxication after d-Galactosamine administration. The normal hepatic cellular architecture was retained in silymarin treated and drug treated animals which was evident by reduction of necrosis, inflammation and fatty degeneration. HEFV 200 mg/kg group was found to be more protective in comparison to Silymarin treated group which illuminates its efficacy equal to the standard drug by protecting the liver against hepatic damage caused by d-Galactosamine. Hence, it was evident that similar to silymarin, the hepatoprotective action of HEFV might be due to the free radical scavenging and antioxidant effects and proving that *Flueggea virosa* root extract might be a potential drug in hepatoprotection.

5. Conclusion

The hepatoprotective activity of the root extract of *Flueggea virosa* against d-Galactosamine intoxication can be contributed to the strong antioxidant property of the extract. The amelioration of hepatotoxicity by improved levels of anti-oxidant enzymes adjacent to oxidative stress markers mentions that HEFV protects the histological changes of liver by its powerful defensive mechanism. The activities of antioxidant enzymes counteract the redox state precipitated intracellularly and hence ensure hepatoprotection against d-Galactosamine induced liver injury. This pampers more investigations on HEFV for scrutinising the precise mechanical action and needs further scientific validation for pharmacological and toxicological drug responses with respect to the dose regimen.

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