Hepatoprotective and Antioxidant Capacity of *Mallotus repandus* Ethyl Acetate Stem Extract against D-Galactosamine-Induced Hepatotoxicity in Rats

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**ABSTRACT:** *Mallotus repandus* (*M. repandus*) is traditionally used to treat muscle pain, itching, fever, rheumatic arthritis, and a variety of liver disorders. The aim of the present work was to evaluate the hepatoprotective activity and the antioxidant potential of the ethyl acetate stem extract of *M. repandus* (ESMR) against D-galactosamine (D-GalN)-induced hepatopathy, along with a possible mechanism of action in rats. In vivo hepatoprotective activity of ESMR was examined using D-galactosamine (D-GalN)-induced hepatotoxicity in Sprague–Dawley rats. For this purpose, levels of serum diagnostic markers, activity of hepatic antioxidant enzymes, and liver histo-architecture were employed to assess the protective efficacy of ESMR. Furthermore, the total phenolic, flavonoid, and tannin contents were quantitated, and the antioxidant capacity of the extract was evaluated using different methods such as 2,2’-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide (NO), hydrogen peroxide (H2O2), and hydroxyl radical (OH•) scavenging assays. Intraperitoneal D-GalN injection triggered hepatotoxicity, as shown by the noticeable increase in the serum hepatic marker enzymes, bilirubin content, γ-glutamyl transferase (GGT), total cholesterol (TC), triglycerides (TGs), and malondialdehyde (MDA), whereas glutathione, superoxide dismutase, and catalase levels were significantly lower compared with that of the control. Pretreatment with ESMR reduced the hepatic enzyme levels along with bilirubin, GGT, and MDA compared to the D-GalN-intoxicated group. These results were supported by histopathological studies, where D-galactosamine caused coagulative necrosis, hemorrhage, and inflammation. However, pretreatment with ESMR ameliorated the histo-architectural changes and brought them back to normal. Results also revealed that the total polyphenolic, flavonoid, and tannin content, and total antioxidant capacity of ESMR were 136.30 ± 0.78 mg GAE/g, 38.72 ± 0.85 mg QE/g, 75.88 ± 0.54 mg TAE/g, and 123.16 ± 0.24 mg AAE/g, respectively. In addition, ESMR inhibited free radicals with IC50 values of 94.47 ± 0.51, 127.33 ± 0.36, 164.12 ± 0.45, and 254.14 ± 0.35 μg/mL in DPPH, NO, H2O2, and OH• free radical scavenging assays, respectively. These findings highlight the protective role of ESMR against hepatic injury induced by D-GalN, which may be attributed to its higher antioxidant properties, thereby scientifically justifying its traditional use.

**INTRODUCTION**

Liver is one of the largest organs in the human body that regulates metabolism, secretion, storage, and detoxification in our body in which the hepatic damage is often linked with alterations of these functions.1 Most hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation (LPO) or by oxidative damage.2 Hepatotoxicity is a common disease, which leads to serious consequences ranging from metabolic disorders to even death.3 In this respect, different toxic agents may induce hepatic injury. D-Galactosamine (D-GalN) is a well-known hepatotoxic agent which induces liver injury with close resemblance to human viral hepatitis showing necrosis, inflammation, and regeneration.4 Noxiousness of D-GalN is mostly associated with the reduction of uridine pools that are linked to inadequate ribonucleic acid and protein synthesis, thus changing hepatocellular function.5 Subsequently, organ-elle damage and necrosis of hepatocytes take place. Along this line, research findings indicated that D-GalN directly triggers mast cells to release histamine and Kupffer cells to release tumor necrosis factor-alpha, which potentiates cell death in
numerous ways, including elevation of oxidative stress and inflammatory procedure.8

Medicinal plants play a vital role in the human health care system. Approximately 80% of the total world population largely depends on traditional medicine, which is mainly based on plant materials. In this context, Mallotus repandus (Willd.) Muell.-Arg., belonging to the Euphorbiaceae family, is locally known as “Gunti”, “Jhante”, or “Bon natai”. It is a wild species that grows in different regions of Bangladesh, especially in the Sundarbans, and has been traditionally used in the treatment of inflammation, liver toxicity, ulcer, and tumor.7−9 The plant has also been used as antioxidant, antiviral, and as a uterus muscle stimulant.10 In addition, the aqueous stem extract of M. repandus exhibited significant activity against carbon tetrachloride (CCL4)-induced hepatotoxicity in animal models.8 To the best of our knowledge, very little is known about the hepatoprotective role of the ethyl acetate stem extract of ESMR in animals. Accordingly, the present study was undertaken to evaluate the hepatoprotective role of ESMR against d-GaIN-induced hepatotoxicity in male Sprague–Dawley rats with the intention of providing a pharmacological justification for its use in traditional medicine.

RESULTS

Antioxidant Constituents. Results from our study show that the total polyphenol, flavonoids, and tannin in ESMR were 136.30 ± 0.78 mg/g GAE (gallic acid equivalent), 38.72 ± 0.85 mg/g QE (quercetin equivalents), and 75.88 ± 0.54 mg/g TAE (tannic acid equivalent), respectively. On the other hand, the total antioxidant capacity of ESMR was 123.16 ± 0.24 mg/g AAE [ascorbic acid (AA) equivalent].

Radical Scavenging Capacity. The antioxidant activity of ESMR was evaluated according to its ability to scavenge free radicals in different assays. Shown in Table 1 are the results of different radical scavenging activity of ESMR. In the 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, results reveal that ESMR displays moderate free radical scavenging activity with an IC50 value of 94.47 ± 0.51 µg/mL, whereas the standard drug AA exhibited an IC50 value of 36.47 ± 0.60 µg/mL. In the NO radical scavenging assay, ESMR showed a scavenging activity with an IC50 value of 127.33 ± 0.36 µg/mL in comparison with AA (IC50 value 41.74 ± 0.73 µg/mL). Furthermore, the extract displayed moderate activity with an IC50 value of 164.12 ± 0.45 µg/mL in the H2O2 radical scavenging assay, while the reference compound, AA, exhibited an IC50 value of 254.14 ± 0.35 µg/mL. Finally, our extract displayed an activity with an IC50 value of 254.14 ± 0.35 µg/mL in the OH radical scavenging assay, while AA exerted an activity with an IC50 value 17.53 ± 0.34 µg/mL.

Figure 1. Effect of ESMR and silymarin on ALT, AST, and ALP against d-GaIN-induced hepatotoxicity (values are mean ± SEM, n = 7. p values: *<0.05, **<0.01 compared between Group I and Group II, p values: #<0.05, ##<0.01 compared among Group II to Group VI).

Table 1. IC50 Value of ESMR in DPPH, NO, H2O2, and OH Scavenging Assays

| extracts/standard | DPPH [IC50 (µg/mL)] | NO [IC50 (µg/mL)] | H2O2 [IC50 (µg/mL)] | OH· [IC50 (µg/mL)] |
|------------------|---------------------|-------------------|---------------------|------------------|
| AA               | 36.47 ± 0.60#       | 41.74 ± 0.73#     | 11.19 ± 0.21*       | 17.53 ± 0.34*    |
| ESMR             | 94.47 ± 0.51#       | 127.33 ± 0.36#    | 164.12 ± 0.45#      | 254.14 ± 0.35#   |

“Values are the mean ± SD. Values in the same column with different superscripts are significantly different (p < 0.05). Experiments were performed in triplicate (n = 3).
GGT, whereas pretreatment of d-GalN-administered rats with ESMR (500 and 1000 mg/kg) significantly reduced GGT levels as was also observed with silymarin, a standard drug used to treat liver injuries.

Results related to the effect of ESMR on total protein, albumin, and globulin in d-GalN-induced rats are shown in Figure 4. No significant differences were observed in these parameters among different experimental groups. However, a noticeable increase in the levels of circulating total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and very low-density lipoprotein cholesterol (VLDL-C) was observed, whereas the high-density lipoprotein cholesterol (HDL-C) level was significantly lowered by d-GalN injection (p < 0.01). Interestingly, pre-administration of rats with ESMR led to significant dose-dependent (p < 0.05 and 0.001) amelioration of these abnormalities, as shown in Table 2.

Effects of ESMR on Biochemical Parameters in Liver Homogenate against d-GalN Induced Hepatotoxicity. Figure 5 illustrates the significant (p < 0.01) upsurge in the malondialdehyde (MDA) level as an outcome of cellular LPO in the d-GalN-treated rats. However, ESMR supplementation meaningfully (p < 0.05 and 0.001) neutralized dose-dependent LPO than the d-GalN control rats. Silymarin had exhibited significant (p < 0.001) reduction of the MDA level.

The hepatic glutathione (GSH) level was noticeably smaller in the d-GalN-treated rats (p < 0.01) than that in the control group. Pre-supplementation with ESMR significantly (p < 0.01) augmented the GSH-declined level triggered by a d-GalN injection which was similar to the level of the standard silymarin group (Figure 6).

The effect of ESMR on the hepatic antioxidant enzyme superoxide dismutase (SOD) content is shown in Figure 7. Pre-administration with ESMR to d-GalN-treated rats significantly (p < 0.05 to p < 0.001) improved this enzyme level, whereas d-GalN treatment markedly decreased (p < 0.001) this enzyme content compared to the control group. The outcomes of extract-treated groups were almost comparable to those of the groups treated with the standard hepatoprotective drug silymarin.

Significantly lower content of the endogenous antioxidant enzyme catalase (CAT) was detected in the d-GalN-injected rats (p < 0.01), where ESMR supplementation noticeably (p < 0.05, p < 0.01) augmented the content of CAT in a dose-dependent manner. In the case of silymarin, CAT content pointedly (p < 0.01) returned to that in the normal rats. Results are exhibited in Figure 8.

Histopathological Analysis. Histopathological examination revealed that Group I had a normal arrangement of hepatocytes (Figure 9A), whereas group II showed massive coagulative necrosis, hemorrhage, and inflammation (Figure 9B). The liver section of group III animals (100 mg/kg dose of silymarin) showed preservation in all of the histopathological features (Figure 9C). Moderate tissue necrosis and inflammation were observed in group IV, where animals were pretreated with 250 mg/kg ESMR followed by d-GalN (Figure 9D). Histoarchitecture of liver sections of groups V and VI showed an improvement in the pathological features, in which there was mild inflammation (Figure 9E,F). All of these findings indicate that ESMR provides defensive measures if pretreatment was administered to rats before d-GalN injection.

DISCUSSION

Because the liver controls numerous important functions in the body, it can cause serious illness when it becomes diseased or injured. Liver diseases can result from drugs, poisons, or consumption of lots of alcohol. Because of lack of dependable hepatoprotective drugs in contemporary medicine, there is an urgent need for the development of potent as well as nontoxic hepatoprotective agents for the treatment of hepatitis. In traditional medicine, medicinal plants have been used in many parts of the world, especially in underdeveloped countries, for the treatment of hepatic diseases. These plants have been recommended for appropriate in vivo pharmacological evaluation to find good candidates. Moreover, medicinal herbs which possess anti-inflammatory activity and antioxidant...
Table 2. Effect of ESMR on the Lipid Profile against \(\alpha\)-GalN-Induced Hepatotoxicity

| groups     | TC      | TG       | HDL-C   | LDL-C   | VLDL-C   |
|------------|---------|----------|---------|---------|----------|
| Group I    | 74.46 ± 1.22 | 61.96 ± 0.47 | 44.01 ± 0.48 | 18.05 ± 0.53 | 12.39 ± 0.09 |
| Group II   | 150.41 ± 2.63** | 121.58 ± 1.24** | 32.20 ± 0.23** | 93.89 ± 0.34** | 24.32 ± 0.25** |
| Group III  | 80.46 ± 0.89** | 70.40 ± 0.43** | 43.78 ± 0.50** | 22.60 ± 0.47** | 14.08 ± 0.08** |
| Group IV   | 130.82 ± 1.24** | 118.18 ± 0.65 | 33.61 ± 0.25 | 73.57 ± 0.35** | 23.64 ± 0.13 |
| Group V    | 120.68 ± 2.19** | 98.38 ± 1.10** | 34.62 ± 0.44* | 66.38 ± 0.56** | 19.68 ± 0.22** |
| Group VI   | 87.87 ± 1.33** | 81.27 ± 0.84** | 40.24 ± 0.40** | 31.38 ± 0.39** | 16.25 ± 0.17** |

*Values are mean ± SEM, \(n = 7\). \(p\) values: * <0.05, ** <0.01 compared between Groups I and II, \(p\) values: * <0.05, ** <0.01 compared among Groups II to VI.

Figure 5. Effect of ESMR and silymarin on the liver LPO level against \(\alpha\)-GalN-induced hepatotoxicity (Values are mean ± SEM, \(n = 7\). \(p\) values: * <0.05, ** <0.01 compared between Group I and Group II, \(p\) values: * <0.05, ** <0.01 compared among Group II to Group VI).

Figure 6. Effect of ESMR and silymarin on the liver GSH level against \(\alpha\)-GalN-induced hepatotoxicity (Values are mean ± SEM, \(n = 7\). \(p\) values: * <0.05, ** <0.01 compared between Group I and Group II, \(p\) values: * <0.05, ** <0.01 compared among Group II to Group VI).

Figure 7. Effect of ESMR and silymarin on SOD content against \(\alpha\)-GalN-induced hepatotoxicity (Values are mean ± SEM, \(n = 7\). \(p\) values: * <0.05, ** <0.01 compared between Group I and Group II, \(p\) values: * <0.05, ** <0.01 compared among Group II to Group VI).

capacity have become good candidates for investigation to alleviate tissue damage. In Taiwan, *M. repandus* has been used as a remedy for hepatitis and liver cirrhosis, as an antioxidant against active oxygen species, and as analgesic and anti-inflammatory drugs. Moreover, the aqueous ESMR showed significant liver protective effects against (CCl\(_4\))-induced hepatotoxicity model. To the best of our knowledge, there were no reports or studies pertaining to the hepatoprotective effect of ESMR against \(\alpha\)-GalN-induced hepatotoxicity in animals.

\(\alpha\)-GalN is a well-known hepatotoxic agent with a mechanism of action that closely resembles human viral hepatitis. \(\alpha\)-GalN produces excessive reactive free radicals which alter the antioxidant status of certain organs and make them more susceptible to oxidative stress. Therefore, the current in vivo investigation could be suitable for evaluation of hepatoprotective agents. Reactive free radicals oxidize polyunsaturated fatty acids in cell membranes, leading to their damage. \(\alpha\)-GalN modifies the redox status that makes certain organs more vulnerable to oxidative stress. \(\alpha\)-GalN injection interrupts permeability of the hepatic cell membrane, which initiates leakage of the hepatic enzymes from the cell, leading to elevation in the levels of enzymes in serum.

Enzymes such as ALT, AST, ALP, LDH, and GGT are important biomarkers that are directly involved in the hepatic injury and signify the severity of damage caused by \(\alpha\)-GalN. Our findings from this study showed that administration of \(\alpha\)-GalN in rats causes a significant increase in levels of ALT, AST, ALP, and GGT in serum which is consistent with earlier findings. These elevated enzyme levels indicate necrotic and inflammatory disorder in the hepatic cell. However, pretreatment with ESMR at doses of 250, 500, and 1000 mg/kg significantly attenuated these enzyme levels in a dose-dependent manner, which was quite similar to that seen by...
the standard hepatoprotective drug, silymarin. On the other hand, serum total bilirubin is a notable hepatic functional marker that is associated with hepatic and biliary disorder along with acute disruption of hepatocellular architecture and function; higher levels of serum bilirubin are usually found in liver injuries caused by toxicants.24 Our findings indicate that ESMR restored this level to normal. Thus, normal serum hepatic enzyme levels suggest that ESMR helps to regenerate hepatic parenchymal cells by its protective action on membrane fragility. Total protein, albumin, and globulin levels can be employed to evaluate the noxious effects of toxicants. Results from this study showed no significant change in the serum albumin level between the test and control groups. This may be ascribed to the short time period of the experiment.25

Increased serum TG and TC levels and reduced levels of HDL-C are common features of liver damage, which is in agreement with our findings.26 Our results demonstrated an increase in serum TC, TG, LDL-C, and VLDL-C levels along with a decrease in the HDL-C level in D-Ga1N-injected rats compared to the control; this is in agreement with the findings of Almajwal and Elsadek.27 Severe hepatocellular damage accumulates large amounts of TG in the parenchymal cells, which release a high quantity of TG in the blood.28 However, reduction in levels of serum TG and TC, coupled with an increase in the HDL-C level in the experimental groups, indicates that ESMR exerts a hepatoprotective effect against D-GalN toxicity.

LPO is a well-known mechanistic pathway for oxidative stress and cell damage.29 D-GalN enhances oxidative stress by forming unnecessary free radicals and by damaging macromolecules. Our results showed that intraperitoneal D-GalN injection increases the MDA level, a vital parameter of LPO, which is in agreement with the findings of Najmi et al.30 MDA is formed through oxidative degradation of polyunsaturated fatty acids. In addition, peroxidation suppresses the inbuilt defensive antioxidant system by excessive formation of reactive free radicals.31 Results from this investigation indicated that pretreatment with ESMR lowers the MDA level (p < 0.001) in the hepatic tissues compared to the D-GalN group, which supports the protective role of the extract against oxidative impairment. This hepatoprotective effect could be ascribed to the presence of bio-active compounds in ESMR with high radical scavenging activity, which may justify the traditional depurative activity of this plant against hepatocellular damage.

GSH is an important strong antioxidant that protects cells from oxidative damage, as this enzyme scavenges reactive oxygen species (ROS) and plays a significant role in defensive processes.32 A homeostatic decrease in the hepatic GSH level makes cells more vulnerable to oxidative stress and leads to additional injury by toxic substances. In this respect, D-GalN injection reduces the GSH content in experimental rats;33 however, treatment with ESMR significantly increased the GSH level and brought it back to normal. Similarly, cellular antioxidant enzymes (e.g., SOD, CAT) are required for the peroxidation of macromolecules such as carbohydrates, proteins, lipids, and genetic materials by removing ROS from the cell. SOD scavenges ROS and converts them into H2O2 and thus reduces their toxic effects.34 On the other hand, CAT decomposes these H2O2 molecules to water and molecular oxygen.35 Toxic substances induce liver damage indicated by the reduced levels of these enzymes in the rat liver. Our findings revealed that D-GalN intoxication caused depletion of SOD and CAT levels. However, supplementation with ESMR increased the levels of these anti-oxidative enzymes and brought their activities back to normal.

D-GalD produced intensive inflammatory infiltration with massive coagulative necrosis and hemorrhage in the hepatic parenchyma and outlying areas. These observations are consistent with other studies conducted on D-GalN.13 Meanwhile, pretreatment with ESMR and silymarin amended the histological changes induced by D-GalN. These results are in line with the traditional use of this plant and its liver protective effect.8 In addition, results from this study revealed that ESMR exhibits good radical scavenging activity and exerts anti-oxidative effect against ROS-mediated oxidative stress.

On the other hand, over production of free radicals such as O2, NO, and OH in addition to H2O2 can cause cell damage, including damage to cellular constituents and the cell membrane.36 Phenolics, flavonoids, and tannins, largely found in medicinal plants, are outstanding phytoconstituents that act as potent antioxidants and exhibit hepatoprotective activity. These compounds inhibit the generation of these free radicals by donating hydrogen atoms or electrons.37 Moreover, polyphenols and flavonoids support increasing endogenous

Figure 9. Histological examination of liver sections from different groups [(A) Group I: normal arrangement of hepatocytes. (B) Group II: section of liver tissue of d-galactosamine-treated group showing massive coagulative necrosis, hemorrhage, and inflammation. (C) Group III: section of 100 mg/kg silymarin liver tissue pretreated in the liver followed by d-galactosamine, showing preservation of normal hepatocytes. (D) Group IV: section of liver tissue pretreated with 250 mg/kg ESMR followed by d-galactosamine, showing tissue necrosis and inflammation. (E) Group V: section of liver tissue pretreated with 500 mg/kg ESMR followed by d-galactosamine, showing mild inflammation. (F) Group VI: section of liver tissue pretreated with 1000 mg/kg ESMR followed by d-galactosamine, showing normal histology with mild inflammation (100X magnification)].
GSH content by upregulating the expression of glutamylcysteine synthetase.38 Along this line, it is worth mentioning that food supplementation with natural antioxidant-containing herbs may be effective in the fight against potential harmful effects of free radicals and may be used as a defensive mechanism against these extremely reactive oxidants. Our study revealed that ESMR is a good source of natural antioxidants (e.g., phenolics, flavonoids, and tannins) which scavenge radical species and reduce molecular damage of the cell. Results from the DPPH, NO, OH\(^*\), and H\(_2\)O\(_2\) radical scavenging assays further confirmed the high natural antioxidant content of ESMR validated by the scavenging capacity of the extract against free radicals and ROS.15

In earlier studies, some biochemicals such as mallotinic acid, furosin, lupeol, amyrin, usoric acid, and bergenin were identified as constituents of the M. repandus stem.39,40 In this context, Rivi\`ere et al. found that flavonoids and phenolic compounds are mainly present in the ethyl acetate extract and that the ethyl acetate fraction of M. repandus stems exhibited the greatest superoxide-scavenging activity.41 On the other hand, Tabata and co-workers demonstrated the antioxidant effects of mallotinic acid, which is a strong free-radical scavenger and can inhibit ROS-mediated protein degradation of bovine serum albumin.42 Similarly, furosin exerts protective effects which reduce glutamate-induced oxidative stress and inhibit apoptotic cell death.43 Sunitha et al. demonstrated the hepatoprotective effect of lupeol against cadmium-induced toxicity in rats.44 Lupeol prevents carcinogenic-induced liver injury in animal models where dietary lupeol supplementation induced Bcl-2 and suppressed Bax and caspase-3 that constrains injury in animal models where dietary lupeol supplementation induced hepatotoxicity.47 Findings by Lim and colleagues indicated that bergenin inhibits LPO and preserves su peroxide dismutase which reduce glutamate-induced oxidative stress and toxic radical species formation and exhibited a protective effect against hepatic damage, whereas usoric acid displayed protective effect against ethanol-induced liver impairment in experimental rats. Additionally, usoric acid was able to inhibit the steatosis process induced by the anti-tubercular drugs and showed a noticeable protective action against paracetamol and d-GalN-induced hepatotoxicity.45 Findings by Lim and colleagues indicated that bergenin inhibits LPO and preserves sufficient levels of GSH for the removal of toxic substances.46 On the other hand, Raish et al. recommended that d-GalN showed necrosis and inflammation in the parenchymal cells and portal track of the liver.47 However, pre-administration with the M. repandus extract attenuated dose-dependent LPO and down-regulated d-GalN-induced inflammation in hepatic toxicity. Our previous study concluded that the M. repandus stem possess anti-inflammatory activities.16

**CONCLUSIONS**

In summary, findings from this investigation suggest that ESMR displays remarkable hepatoprotective effect against d-GalN-induced hepatotoxicity in experimental rats. These findings may explain the traditional medicinal use of the M. repandus stem as a hepatoprotective, possibly because of the presence of bio-active compounds which reduce oxidative stress by scavenging toxic radicals produced by d-GalN. Our findings also suggest that M. repandus has a cellular protective role against d-GalN-induced hepatotoxicity. However, more detailed studies are still required to establish the safety, efficacy, and active constituents of this plant to achieve better outcomes in clinical treatments.

**EXPERIMENTAL SECTION**

**Chemicals and Drugs.** The following compounds and reagents used throughout this investigation were purchased from different companies such as Merck (Germany), Sigma Chemicals, Merck (India), and SD Fine Chem. Ltd.: Ranbaxy Lab Folin–Ciocalteu reagent, sodium carbonate, methanol, gallic acid, aluminum chloride, potassium acetate, quercetin, concentrated H\(_2\)SO\(_4\) (98%), sodium phosphate (Na\(_2\)PO\(_4\)), ammonium molybdate, AA, ethyl acetate, tannic acid, DPPH, sodium nitroprusside, sulfurilamide, naphthyl ethylenediamine dihydrochloride, hydrogen peroxide (H\(_2\)O\(_2\)), phosphate buffer, and phosphoric acid (H\(_3\)PO\(_4\)). We obtained silimarin capsules from Square Pharmaceuticals Ltd., (Dhaka, Bangladesh), whereas d-galactosamine (d-GalN) was procured from Atomax Chemicals Co. Ltd., (Shenzhen, China).

**Plant Material.** Stems of M. repandus (M. repandus) were collected from Savar, Dhaka, Bangladesh, during the dry season between November and December. The plant was identified and authenticated, and a voucher specimen was deposited at the Bangladesh National Herbarium (DACB accession no. 38733).

**Extract Preparation.** Collected stems were cleaned and properly washed under running tap water. They were then partially dried by fan aeration followed by complete drying in an oven at 40 °C for two days. Approximately, 500 g of the powdered plant material was subjected to soxhlet extraction at 65 °C using ethyl acetate (500 mL). Extraction was completed when the solvent running through the soxhlet apparatus turned colorless. The solution was filtered using a fresh cotton bed, and the extract was concentrated and dried by means of rotary evaporation at 40 ± 2 °C to afford a gummy concentrate of the crude extract. Dried extracts thus obtained were kept in a Petri dish at 2–8 °C and were screened for their pharmacological properties.

**Determination of Total Phenol, Flavonoid, Tannin, and Antioxidant Capacity.** Total phenol, flavonoid, and antioxidant capacity were estimated according to the procedure outlined by Hossain et al., whereas the total tannin content was assessed by the method of Folin and Ciocalteu with slight modification. The total content of phenols, flavonoids, antioxidant capacity, and tannin of ESMR is expressed in mg/g GAE, mg/g QE, mg/g AA equivalent (AAE), and mg/g TAE, respectively.

**Determination of Free Radical Scavenging Capacity.** DPPH free radical scavenging assay was accomplished according to the method described by Braca et al., whereas the NO scavenging assay was performed following the method of Govindarajan and co-workers. On the other hand, the ability of the ESMR to scavenge H\(_2\)O\(_2\) was evaluated according to the procedure outlined by Nabavi et al., while the OH\(^*\) scavenging activity was assessed as per the method of Halliwell and colleagues. The percentage of radical scavenging capacity of the extracts and standard compound was calculated from the following equation

\[
\% \text{ of scavenged radical} = \left(\frac{A_0 - A_t}{A_0}\right) \times 100
\]

where \(A_0\) is the absorbance of the control and \(A_t\) is the absorbance in the presence of the sample of the extract and standard; IC\(_{50}\) was calculated by the linear regression method.
Animals for In Vivo Experiments. Male Sprague–Dawley rats (150–170 g) and Swiss albino male mice (25–30 g), obtained from Focused Research on Ayurvedic Medicine and Education (FRAME) Laboratory, Department of Pharmacy, Jahangirnagar University, Bangladesh, were used throughout this investigation. These animals were kept under normal laboratory conditions (temperature: 25 ± 3 °C, humidity: 50 ± 5%, and 12 h light/dark cycles) and were provided with a standard laboratory pellet diet and water ad libitum. Experiments were conducted according to the ethical guidelines approved by the Bangladesh Association for Laboratory Animal Science, whereas the experimental protocol was approved by the Biosafety, Biosecurity, and Ethical Committee of Faculty of Biological Sciences of Jahangirnagar University, Savar, Dhaka, Bangladesh [approval number: BBECJU/M2013(20)].

Acute Toxicity Study. An acute oral toxicity study was conducted using the limit test procedure according to the Organization for Economic Co-operation and Development (OECD) guidelines for evaluation of chemicals (Test Guideline 425). According to the procedure, Swiss albino mice were randomly divided into five groups of eight animals each. Different doses (250, 500, 1000, 2000, and 4000 mg/kg) of ESMR were administered by oral gavage. Then, animals were observed uninterruptedly for 1 h for any behavioral changes, signs of toxicity, and death, intermittently for the next 6 h, and then again at 24 h, after dosing ESMR.

\[ \text{d-GalN-Induced Hepatoprotective Studies.} \] Forty-two male Sprague–Dawley rats were randomly divided into six groups of 7 animals each:
- Group I: treated only with water (orally) and served as the normal control.
- Group II: d-GalN-treated control and received water. This group served as a negative control.
- Group III: animals in this group were treated orally with the standard drug silymarin at 100 mg/kg body weight, and this group is known as the standard group.
- Groups IV–VI: animals in these groups were orally treated with ESMR at doses of 250, 500, and 1000 mg/kg body weight and these were the treatment groups.

All these protocols were continued for 7 days. On the final day of the treatment, animals of groups II–VI received a single dose of d-GalN intraperitoneally at 300 mg/kg of rats’ body weight. On the next day, rats from each group were anaesthetized by administration (i.p.) of ketamine (500 mg/kg). Then, each rat was placed on a board and pins were driven through its paws, crucifixion-style. We looped a string over the front teeth to hold the head back and took shiny thin scissors and cut into the animal’s skin and right through the ribcage. Inside the ribcage was the dark-red, still-beating heart where we had to push the blood out while the animal was still alive. Blood samples (4 mL) were collected for further biochemical analyses. All analyses were accomplished within 24 h of sample collection. In addition, liver tissues were instantly removed from the surrounding tissues, washed with ice-cold phosphate-buffered saline, weighed, and stored at −20 °C for histopathological examinations.

Assessment of Serum Biochemical Parameters. For assessment of biochemical parameters related to liver function, serum samples were analyzed for ALT, AST, ALP, GGT, total protein, albumin, globulin, total bilirubin, TC, HDL-C, and TG following the standard procedures with the aid of a Humalyzer 3500 (Human Inc., Steinembronn, Germany) and using human commercial kits. Similarly, VLDL-C and LDL-C concentrations were calculated using the following Friedewald equations:

\[ \text{LDL-C} = \{\text{TC} - \text{HDL-C} - (\text{TG}/5)\} \]

\[ \text{VLDL-C} = \text{TG}/5 \]

Assessment of Hepatic Biochemical Parameters. MDA is known as an indicator of LPO. We determined MDA by following the procedure outlined by Ohkawa and co-workers. Levels of MDA were expressed in nmol of thiobarbituric acid reactive substances per mg of protein. On the other hand, we employed Ellman’s reagent method for the detection of the GSH level. GSH activity was expressed as nmol/mg of protein. In a similar fashion, we followed the procedure of the Chatterjee method to determine the level of SOD. On the other hand, the activity of catalase (CAT) was accomplished following the method of Aebi and using hydrogen peroxide as a substrate. Levels of SOD and CAT were expressed as units/mg of protein.

Histopathological Evaluation. After sacrificing rats, livers of the sacrificed rats were immediately removed, sliced, and washed with saline and fixed at 10% neutral buffered formalin for histopathological studies. Liver tissues were then trimmed (5 μm thicknesses) with the aid of a rotary microtome and embedded in paraffin wax. Afterward, tissue sections were stained with hematoxylin and eosin for histopathological studies using established protocols and photographed with an Olympus DP 72 microscope (Tokyo, Japan).

Statistical Analysis. Results from this investigation are expressed as the mean ± standard error of the mean (SEM). We employed SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, USA) and GraphPad Prism (version 6.02; GraphPad Software Inc., San Diego, CA, USA) to analyze data. Data were subjected to Student’s t-test and one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison to analyze data sets. Differences between means were considered significant at \( p < 0.01 \) and \( p < 0.05 \).

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