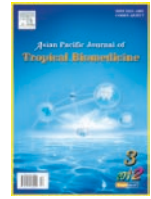




Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Document heading

doi:

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Hepatoprotective activity of *Sphaeranthus amaranthoides* on D–galactosamine induced hepatitis in albino rats

Swarnalatha.L.¹, P.Neelakanta Reddy^{2*}¹Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Chennai–600119,²Department of Bio–organic laboratory, Central Leather Research Institute, Adyar, Chennai.

ARTICLE INFO

Article history:

Received 2 June 2012

Received in revised form 6 July 2012

Accepted 9 October 2012

Available online 28 December 2012

Keywords:

Hepatoprotective activity
Sphaeranthus amaranthoides
 D–galactosamine
 Antioxidant enzymes

ABSTRACT

Objective: The plant *sphaeranthus amaranthoides* Burm.f is used in the district thriunaveli in the treatment of various liver disorders. **Methods:** in the present study the ethanol extract from *sphaeranthus amaranthoides* was studied against the D–galactosamine hepatotoxicity. **Results:** significant hepatoprotective effect was obtained against liver damage induced by the D–galactosamine as evident from changed antioxidant enzymes like CAT, SOD, GPx, GST, GSH, G6PD and GR and a normal architecture of liver and mitochondria compared to toxin controls. **Conclusions:** the results indicate that ethanol extract of the *sphaeranthus amaranthoides* could be useful in preventing D–galactosamine induced liver injury.

1. Introduction

Liver is the most important organ in the body. It plays a pivotal role in regulating various physiological processes. It is also involved in several vital functions, such as metabolism, secretion and storage. It has great capacity to detoxicate toxic substances and synthesize useful principles[1] is a frequent target of no of toxicants[2]. Reactive oxygen species may be generated during this process, these superoxide radicals are neutralized by the natural protective mechanisms of the liver and are over powered during the all such exposures, this will lead to hepatic injury. Liver diseases are worldwide problems, and conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious adverse effects. [3]

Toxicant or a drug induced liver injury can be prevented by using non toxic hepatoprotective herbs, which can possess membrane stabilizing, hepatoprotective and antioxidative activities. The most active and universal components among the body's free radical neutralising systems are the antioxidants and antioxidant enzymes, which are the major line of defenses. However these defenses may not be fulfilled or sufficient during the excessive generation of free radicals while metabolizing the harmful substances.

Therefore many natural agents possessing antioxidative activity have been proposed to prevent and cure hepatotoxicity induced by oxidative damage. No of plants have been shown to possess protective role by improving the antioxidant status because of presence of flavonoides, polyphenolic compounds, poly hydroxy organic compounds and triterpenoides.

In the present investigation *S.amaranthoides* was taken up for extensive biochemical and histopathological studies, to evaluate its protective effects against D–GalN induced experimental hepatitis in rats. D–GalN was chosen as the model for inducing hepatitis in the present study since the liver injury provoked by D–GalN resembles both chemical induced and viral hepatitis of humans.

2. Materials and methods

2.1. Chemicals

Thiobarbituric acid (TBA), 2,4, dinitrobenzene, reduced glutathione, glucose–6–phosphate, oxidised glutathione, hydrogen peroxide, EDTA, DTNB, Glutaraldehyde, Osmium tetroxide. All the chemicals used are analytical grade and obtained by sisco laboratories, Chennai, India.

2.2. Collection of plant material

*Corresponding author: Dr.Swarnalatha.L., Department of Biotechnology, Sathyabama University Jeppiaar nagar, OMR, Chennai 119.
 Email: lokiswarnalatha@gmail.com

The plant *Sphaeranthus amaranthoides* was collected from the Sengottai, Tirunelveli, Tamil Nadu, India. The plant material was identified and authenticated by Mr. V.Chelladurai, Retired Research officer–botany, Central Council For Research In Ayurveda and Sidha (C.C.R.A.S). Govt. of India, Tirunelveli. The Collected plant material was free from diseases and also free from contamination of other plants.

2.3. Preparation of plant extract

One Kg of powdered plant material was soaked in petroleum ether to remove the pigments. Then the plant material is transferred into 2.75lt of 95% ethanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture filtered by a piece of clean, white cotton material followed by a through Whatmann filter paper no.42 to get a filtrate (ethanol extract). Then this filtrate was evaporated using rotary evaporator under reduced pressure to get a powder. The extract obtained was 14 % (w/w) of dry powder. This extract is given to the rats by mixing in a Tween 80.

2.4. Animals during experimental period

The laboratory animal protocol used for this study was approved by Institutional animal ethic committee (IAEC), CLRI, Chennai. And the work was supported by CLRI with the IAEC NO–03–002–07. Albino rats of Wistar strain were used in this study. They were obtained from King’s Institute of Preventive Medicine, Chennai. They were maintained in clean, sterile, polypropylene cage and fed with pelleted rat diet (M/s. Hindustan Lever Ltd., Bangalore, India), water ad libitum and kept in a well ventilated room with 12 hrs light / dark cycles throughout the experimental period.

2.5. D–galactosamine induced hepatotoxicity:

The rats were divided into four major groups. Each group consisted of six rats weighing 150–200gms. Group I are control rats received the normal diet; Group II rats are induced with hepatitis by giving intraperitoneal injections of D–Galactosamine (500 mg/kg body weight) 18 hours before the experiment; Group III rats were given *S.amaranthoides* extract alone and Group IV rats were given *S.amaranthoides* extract for 21 days (500 mg/kg body weight) +

D–galactosamine on the 21st day.^[4] Extract of *S.amaranthoides* (500 mg/kg body weight/day) was dissolved in Tween 80 and administered through oral gavages. Control rats received physiological saline alone. Hepatitis is induced by the administration of D– GalN (500mg/kg body weight) intra peritoneally 24hr before sacrificing. On completion of experimental period, rats were sacrificed by cervical decapitation.

The blood and liver tissues were used to assess the antioxidant enzymes. Liver was excised immediately; liver regions were separated and immersed in ice–cold physiological saline, the tissue was homogenized using 0.01 M Tris–HCl buffer (pH 7.4) and used for further analysis.

2.6. Determination of antioxidant activity:

Assay of Superoxide Dismutase (SOD) (EC 1.15.1.1):Superoxide dismutase was assayed following the method of Misra and Pridovich,^[5] Assay of Catalase (CAT) (EC 1.11.1.6)The activity of catalase was assayed by the method of Bergmeyer et al,^[6] Assay of glutathione peroxidase (GPx) (EC 1.11.1.9)The activity of glutathione peroxidase was assayed by the method of Rotruck et al,^[7] Assay of glutathione reductase (GR) (EC 1.6.4.2) The activity of glutathione reductase was measured by the method of Staal et al,^[8] Assay of glucose–6–phosphate dehydrogenase (G6PD) (EC 1.1.1.49)G6PD activity was assayed by the method of Baquer,^[9] Assay of glutathione S–transferase (GST) (EC 2.5.1.18)The activity of glutathione S–transferase was assayed by the method of Habig et al,^[10] Assay of lipid peroxidation (MDA): The level of lipid peroxides was assayed by the method of Ohkawa et al.^[11]

Histopathology

2.7. Light microscopic studies

Histological evaluation was performed on the livers of different groups, were removed and fixed in 10% formalin saline and embedded in Paraffin. Sections of 4 μ M thick were stained with hematoxylin and eosin by the method of Drury and Walligton,^[12] and viewed under light microscope for histological changes.

2.8. Transmission electron microscopy

Small blocks of liver from median lobe were fixed in 10% neutral buffered formalin, processed for histopathology by routine methods and Haematoxylin– Eosin (H & E) stained. For EM, pinhead sized blocks of distal end of median lobe of 1–2 mm was fixed in 3% glutaraldehyde in 0.1 M sodium–cacodylate buffer (pH 7.4) for 1 hr at 4°C. The sections were post–stained with 1% osmium tetroxide. Ultrathin sections of (100 nm) were cut on an LKB 2088 Ultramicrotome, mounted on 300 meshcopper grids and stained with uranyl acetate and lead citrate were examined under JEOL JEM–1010 transmission electron microscope operating at 80 kV.

2.9. Statistical analysis:

Data was presented as mean \pm standard deviation and analysed using students t–test

3. Results

Results for enzymatic antioxidative defense system are shown in the table 1. Rats intoxicated with DGalN alone (Group II) developed hepatocellular damage as evident from a significant decrease (< 0.01) in the level of CAT,SOD,GPx, GST,GSH,G6PDand GR when compared with control (Table–1). Pretreatment of *sphaeranthus amaranthoides* plant extract afforded a significant protection against D–galactosamine –induced liver injury by maintaining the levels to near normal. Treatment with alcoholic extracts of *sphaeranthus amaranthoides* plant at a dose of 500 mg/kg showed a significant increase of CAT,SOD,GPx, GST,GSH,G6PDand GR levels.

3.1. Histopathological observations

The histopathological examination of the liver sections included the examinations of four different group's. The microscopic examination of liver of group-I showed a normal portal triad, sinusoids, and cord arrangement of hepatocytes (Fig- 1A). The microscopic examination of liver of group-II showed marked to moderately severe fatty change of liver with presence of large fat vacuoles in the cytoplasm pushing the nuclei at the periphery. At places many fat vacuoles are seen united and are forming small fat cysts as well. Areas in this group are also showing degeneration and necrosis of hepatocytes . The microscopic examination of liver of group III showed almost normal appearing hepatocytes and no fatty change, or absence of fatty change in hepatocytes, this indicated that *sphaeranthus amaranthoides* provided significant Hepatoprotection from fatty change (Fig-1D). This observation revealed that the test drug when used in 500mg/kg body wt. was able to provide protection from fatty change in liver and no significant fatty necrosis or marked inflammation was seen.

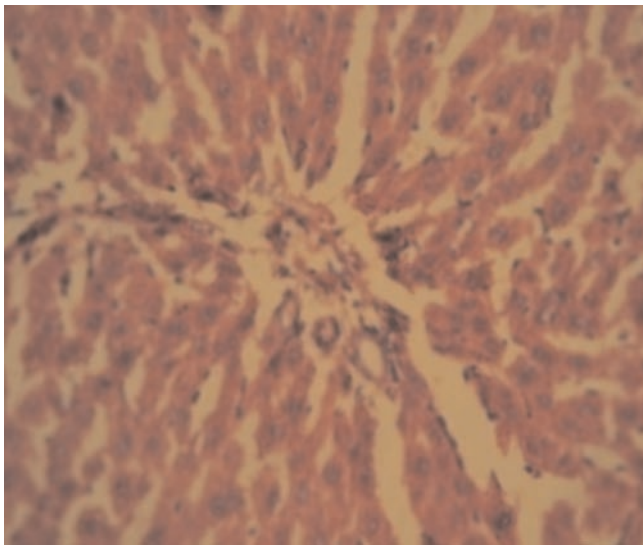


Fig:1 A Control rat liver showing normal architecture(H&E)

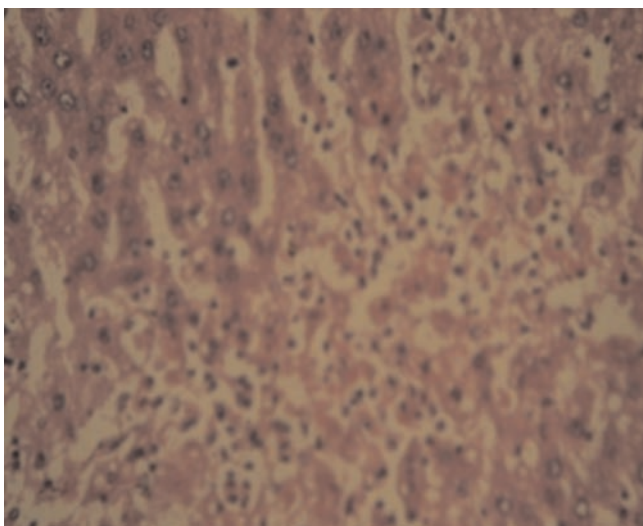


Fig: 1B *S. amaranthoides* treated rat liver showing absence of severe necrotic and inflammatory infiltration(H&E)

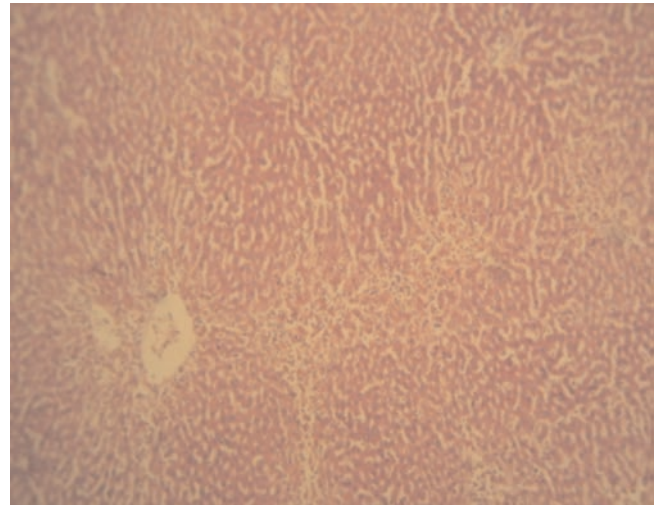


Fig: 1C D-galactosamine induced liver showing severe necrotic appearance (arrow shows inflammatory infiltration). (H&E)

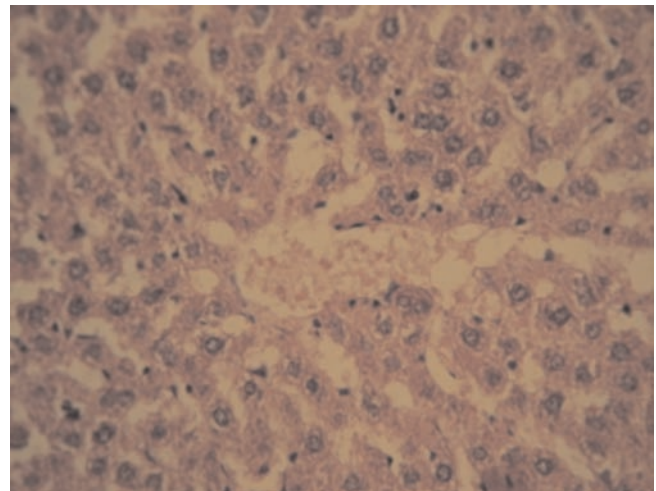


Fig: 1D *Sphaeranthus amaranthoides* & D-Galactosamine rat liver showing reduced necrosis(H&E)

The results for transmission electron microscopy of liver mitochondria are shown in the Fig. 2. Fig 2B shows the liver mitochondrial electron micrograph of DgalN induced toxicity and the disintegration of cristae and distorted shape of mitochondria indicates the extent of damage, when compared to the control group(Fig.2A). But in the fig 2C pretreated with *sphaeranthus amaranthoides* indicates the recovery of mitochondrial damage. This proves the protective role *sphaeranthus amaranthoides* on liver.

Transmission electron microscopic figures:

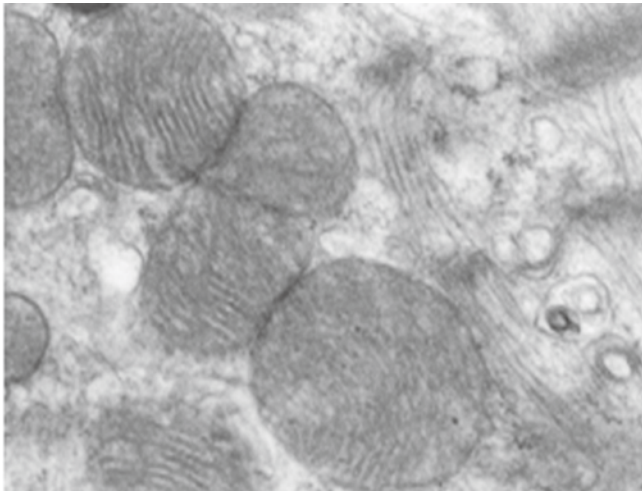


Fig 2A: Control :showing normal architecture Of liver mitochondria

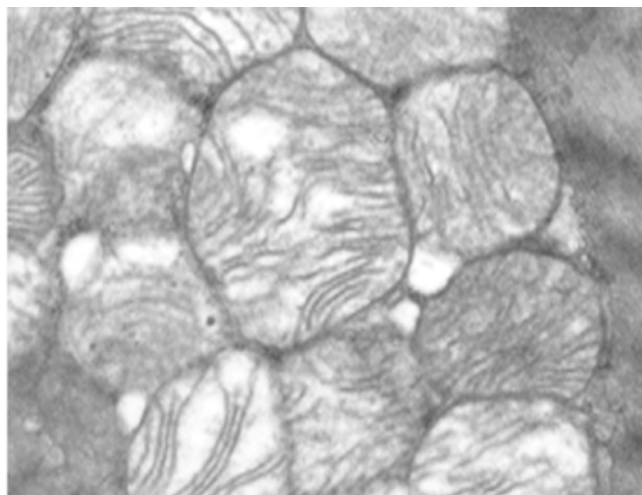


Fig 2B: D GalN Treated: showing disintigration of mitochondrial cristae

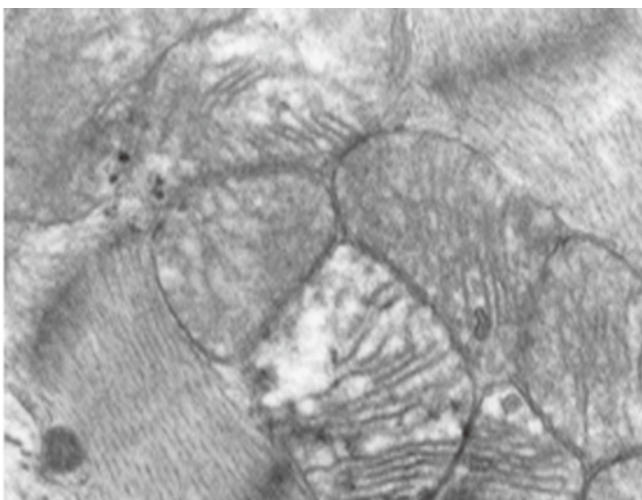


Fig 2 C: D- GalN and *S. amaranthoides* treated: less disintigration of mitochondrial cristae

4. Discussion

For understanding the exact mechanism of the xenobiotic hepatotoxicity is one of the major challenges hepatologists are faced with today. Recent advances in the studies of toxicology have been useful in elucidating several different pathways of hepatotoxicity. Further confirmation is needed on this study to gain a mechanistic understanding of toxic changes that occur in liver.

Intraperitoneal administration of GalN has been found to induce hepatitis which is closely resembles human viral hepatitis^[13]. The previous reports on the mechanism of GalN induced hepatotoxicity suggests that GalN inhibits the synthesis of RNA as well as protein via reduction of cellular UTP, a substrate for RNA polymerase. GalN shifts the equilibrium between the uridine phosphate consuming and producing reactions, in favor of the former via the rapid formation and accumulation of UDP amino sugars^[14]. Studies from Quintero et al^[15] showed that free radical dependent pathway is involved in GalN induced hepatic apoptosis and necrosis in rat hepatocytes. Mangeney-Andreani et al^[16] suggested that energy metabolism of hepatocytes was also inhibited by GalN application. In addition, report from Sire et al^[17] reveals that GalN damaged the enzymes involved in the transport of substrates to the mitochondria and consequently modified the phospholipid composition of the membrane this brings about the changes in the mitochondria also.

In addition, DGalN contributes to increased oxidative stress and formation of the reactive oxygen species, which are also fatal to the cell and result in hepatocyte necrosis^[18]. Further, the oxidative stress causes a misbalance in pro-oxidant/antioxidant steady state due to generation of increased amount of oxidants resulting in cellular damage as manifested by necrosis^[19]. oxidative stress can be induced by toxins, the source for these toxins may be virus, bacteria or a xenobiotic compound and it causes accumulation of reactive oxygen/nitrogen species, by the activation of nitric oxide synthase^[20]. SOD, CAT, Gpx and GSH are major antioxidant enzymes. Reduced glutathione (GSH) is a powerful antioxidant that protects cells from oxidative injury by scavenging reactive oxygen/nitrogen species and a homeostatic decrease in the GSH pool can make cells more vulnerable to further damage by toxins^[21]. In addition to antioxidant action of GSH, the antioxidant enzymes SOD, Gpx and catalase work together to counteract the oxidation of proteins, lipids and DNA, by removing ROS from the cell^[22]. SOD is a specific enzyme to reduce superoxide ion into hydrogen peroxide, which is further reduced to water by the action of catalase and glutathione peroxidase.^[23] Various response of these enzymes may be in indication to cellular needs in fight against increased levels of reactive oxygen species in induced oxidative stress states.^[24]

4.1. Light Microscopical studies on excised liver

The assessment of hepatoprotective potential of a drug is

Table 1Effect of *S. amaranthoides* on enzymatic antioxidative defense system in liver

Parameters	Group I Control	Group II D–GalN intoxicated	Group III <i>S. amaranthoides</i> treated	Group IV D–GalN+ <i>S. amaranthoides</i> treated
SOD	8.09±0.23	4.71±0.32***	7.65±0.91NS	7.28±0.55***
CAT	63.57±6.01	41.64±4.09***	65.54±7.40NS	67.77±6.33***
GPx	120.10±9.5	72.17±5.59***	121.19±13.7NS	109.6±1.9***
GST	1628±121	2208±157***	1633±147NS	1805±174***
GSH	9.10±0.76	5.16±0.37***	9.58±0.71NS	8.26±0.79***
G6PD	5.12±0.33	6.61±0.42***	5.48±0.64NS	4.63±0.27***
GR	51.2±3.7	35.54±3.7***	53.4±6.2NS	46.4±7.0***

Values are expressed as mean±SD (six animals in each group). As compared with respective controls (comparisons are made between Group II and Group I; Group III and Group I, Group IV and Group II) statistical analysis by students t–test *** $P < 0.001$, ** $P < 0.01$, NS –Non significant.

incomplete without histological description of changes in the architecture of the liver a caused by its administration. Fig.1A shows the normal liver sections (control) wherein normal architectures of hepatic cells were seen in rats, which were fed with normal diet alone. The Fig.1C (D–GalN induced liver sections) illustrates the architecture of the liver of the rats induced with severe hepatocellular necrosis accompanied by a mixed inflammatory infiltration of leucocytes shows prominent cytoplasmic vacuolization suggesting lipid accumulation. Sinusoidal distension, kupffer cell hyperplasia and neutrophil accumulation. Whereas the Fig.1B. Shows (*S. amaranthoides* extract alone) the normal architecture of the liver tissue as compared to the control rats.

The beneficial effects of *Sphaeranthus amaranthoides* on antioxidant parameters is further supported by histological studies. The regeneration activity of *Sphaeranthus amaranthoides* liver cells of pretreated animals indicates the protective nature against the oxidative stress. However these observations are correlated with silymarin. Silymarin is a known hepatoprotective compound that has been reported to possess a protective effect on the plasma membrane of hepatocytes.[25, 26] This observation reveals that the rats pretreated with *S. amaranthoides* extract afforded a protective effect against the development of hepatic necrosis.

4.2. Transmission Electron Microscopy of Liver Mitochondria

Tissues and cells would be subjected to oxidative injuries when large quantities of free radicals are generated or the deterioration of activities of antioxidant systems. Mitochondria have membranes rich in electron transport system and are sensitive to attack of free radicals. Fig. 2 shows the liver mitochondrial electron micrograph of control, D–GalN induced and plant extracts treated rats. A result of the electron microscopic studies of the Fig.2B indicates the extent of hepatic injury inflicted by the administration of D–GalN. Hepatitis provoked by D–GalN was characterized by alterations in the histology of the liver such as distorted shape changes in mitochondria and endoplasmic reticulum, disruption and fragmentation of the mitochondrial cristae. In addition, the lipid peroxidation could impair the normal cell membrane construction of mitochondria and increases its permeability and thus swells it[27].

Rats pretreated with *S. amaranthoides* extracts for 21 days offered considerable hepatoprotection against the toxic effects of D–GalN as revealed by the absence of the degeneration changes in the cristae of mitochondria. This confirms the ability of *S. amaranthoides* extracts in the preservation of mitochondrial membrane integrity and hepatic function (Fig.2C). The present studies correlates well with the previous studies in the plant *Sargassum* in acetaminophen induced rat models[28] Polyunsaturated fatty acids are ready to oxidize free radicals to generate products for lipid peroxidation such as MDA; meanwhile, the quantity of PUFA reduces during the procedure of oxidation, thereby lowering membrane fluidity. Hence, it is pertinent to study the architecture of hepatitis liver mitochondria to know the severity of the disease.

Silymarin, a standardized extract of *Silybum marianum* (Compositae) is also a potent hepatoprotective agent. It reverses hepatotoxin–induced alterations of biochemical parameters and has so far been the most thoroughly investigated of all the plant substances in prevented liver damage induced by carbon tetra chloride, D–galN and paracetamol in rat models[29]. The phytochemical investigation of the plant extract demonstrated the presence of flavonoids, triterpenoids and phenol compounds. Flavonoids are well known for hepatoprotective and antioxidant activities[30]. Mangathayaru et al., (2005) [31] reported hepatoprotective activity of flavonoids present in *Leucas aspera* (Labiatae) in rats. Hence, the hepatoprotective activity of *Sphaeranthus amaranthoides* may be correlated to its containing phenol, triterpenoid and flavonoidal constituents. On the basis of results presented, *Sphaeranthus amaranthoides* protects the liver from severe damage caused by D–galN and may serve as a useful adjuvant in several clinical conditions associated with liver damage.

Conflict of interest statement

We declare that we have no conflict of interest.

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