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Hepatoprotective activity of *Spilanthes paniculata* flower extracts on liver damage induced by paracetamol in rats

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ABSTRACT

The present study was undertaken to investigate the antioxidant and hepatoprotective effect of Spilanthes paniculata flower extracts against paracetamol-induced liver damage. The study was conducted in 36 male Wistar rats of either sex, and six groups were established. While the first group was maintained as normal control (NC, distilled water), Groups 2–6 were administered 3gm/kg Paracetamol (PAR) for 2 day, 100 mg/kg Silymarin (SMR), 500 mg/kg Methanolic extract (MESP), Petroleum ether extract (PEESP), Ethyl acetate extract of S. paniculata (EAESP) suspended in 0.5% tween 80 plus PAR respectively, PAR was administered in the same schedule as in group 2, the treatment with silymarin and extracts was given for 10 days orally, respectively. It was shown that PAR significantly increased serum ALT, AST, ALP, liver MDA levels (P<0.01) and significantly decreased liver GSH, CAT, SOD activity (P<0.01), when compared with the normal control group (NC). On the other hand, statistical significant (P<0.01) changes were observed in the biochemical parameters of the group which was administered SMR, PEESP and EAESP. Compared with the pathological changes observed in the liver congested sinusoids and centrilobular necrosis, in the group which was administered paracetamol alone (PAR), lesions were determined to be less severe particularly in the group (PEESP and EAESP). The study had revealed that administration of PEESP & EAESP offered a therapeutic potential for the treatment of hepatotoxicity induced by paracetamol via regulation of endogenous antioxidant system in liver.

Key Words: *Spilanthes paniculata*, Paracetamol, Biochemical parameters, Oxidative stress, Rats

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INTRODUCTION

Liver diseases have become one of the major causes of morbidity and mortality all over world. From among, drug induced liver injury (DILI) is one of the most common causative factor that poses a major clinical and regulatory challenge [1]. The manifestations of drug-induced hepatotoxicity are highly variable, ranging from asymptomatic elevation of liver enzymes to fulminant hepatic failure. Paracetamol (PAR) also known as Acetaminophen, taken in overdose can cause severe hepatotoxicity and nephrotoxicity [2]. PAR is activated and converted by cytochrome P450 enzymes to toxic metabolite NAPQI (N-acetyl-p-benzoquinoneimine) that causes oxidative stress and glutathione (GSH) depletion [2, 3]. In spite of tremendous advances in modem medicine, there are hardly any reliable drugs that protect the liver from damage and/or help in regeneration of hepatic cell. Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease [4]. Therefore, searching for effective and safe drugs for liver disorders is still considered as an area of interest. *Spilanthes paniculata*, belongs to family Asteraceae is commonly known as toothache plant. It exhibits analgesic, strong larvicidal activity on *Anopheles stephensi Liston, Anopheles culicifacies*, antimicrobial and cytotoxic activity [5]. *S. paniculata* plant have been used in the traditional system of medicine for the treatment of various disease complications including relieve toothache, infections of throat and gums, paralysis of tongue, a popular remedy for stammering in children and as diuretic [6]. Various pharmacological experimental studies have been carried out with

S. acmmella another species of same genus. The chloroform, ethyl acetate, and methanol extracts, prepared from the aerial part of *S. acmmella* shows vasorelaxant and antioxidant activities [7].

Previous study has reported the presence of Stigmasterol, Sitosterol-o-β-D-glucoside *S. paniculata* plant [8]. Our phytochemical investigation has revealed the presence of flavonoids, tannins, phenolic compounds and Steroids in methanolic, petroleum ether, ethyl acetate extracts of *S. paniculata* flowers. Tannins, phenolic compounds were found to be absent in aqueous extract. Taking into consideration the antioxidant potential of flavonoids and phenolic compounds present in the flower extracts the present study was conducted to evaluate the hepatoprotective and antioxidant potential of *S. paniculata* another species of the same genus *spilanthes*, plant extracts against paracetamol-induced hepatotoxicity in rats.

MATERIALS AND METHODS

Drugs and Chemicals:
Bovin serum albumin, Folin's ciocalteau phenol reagent, Pyrogallol, Thiobarbituric acid (TBA), 5'-dithiobis 2-nitrobenzoic acid (DTNB), Tris buffer, was procured from Hi-Media, Bombay, India. Paracetamol drug (Ipca Pvt. Ltd.) purchased from local medical store. All reagents procured and used were of analytical grade.

Plant Material:
The fresh flowers of *S. paniculata* plant were collected from Mazalgaon, district Beed (Maharashtra). The plant was identified, confirmed and authenticated by Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad. The flowers were air dried indoors under subdued light with good ventilation and powdered with the help of grinder and stored in an airtight container. A methanolic extract was prepared by soxhlet extraction method. The dried powdered flowers of *S. paniculata* were extracted with methanol for 36 hrs using soxhlet extractor. The combined extracts were concentrated at 60°C under reduced pressure, to obtain dark brownish residue the remaining extract was freeze-dried. The yield obtained from the above process was found to be 2.53% w/w. The extracts were preserved in a refrigerator for further use. The marc obtained from methanolic extract was dried and used further, similarly for the petroleum ether extraction for 36 hrs. The yield obtained from the petroleum ether extraction process was found to be 2.76% w/w. The marc obtained from petroleum ether extract was dried and used further, similarly for the ethyl acetate extraction was carried out for 36 hrs. The yield obtained from the petroleum ether extraction process was found to be 2.60% w/w.

Phytochemical screening of *S. paniculata* flowers:
The Methanolic, Petroleum ether and Ethyl acetate extracts of *S. paniculata* flowers has shown presence of flavonoids, tannins phenolic compounds and steroids. The aqueous extract of *S. paniculata* flowers has shown presence of flavonoids and steroids but tannins, phenolic compounds were found to be absent.

Animals:
Male Wistar rats weighing 150 ± 15g were used for this study. The animals were kept in polypropylene cages and maintained at 25 ± 5ºC under 12 h light/dark cycle. The animals were allowed free access standard pellet diet (Amrut Laboratory Rat feed, Navmaharashtra Chakan Oil Mills Ltd., Pune, India) and water ad libitum. The animal experiment was approved by Institutional Animal Ethics Committee of Y.B.Chavan College of Pharmacy, Aurangabad, Maharashtra and performed according to the guidelines laid by Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Study design:
Animals were divided into six groups of six rats each and treated orally as below for 10 days.

Group1: Normal control and received 0.5% tween 80 suspended in distilled water, 2.5ml/kg *po.*
Group II: Animals were administered a single dose of 3gm/kg PAR *po.*
Group III: Animals were administered 100 mg/kg/day Silymarin for 10 days plus 3 gm/kg PAR *po.* for 2 day.
Group IV: Animals were administered 500 mg/kg/day Methanolic extract of *S. paniculata* flowers (MESP) suspended in 0.5% tween 80 *po.* for 10 days plus 3 gm/kg PAR *po.* for 2 day.
Group V: Animals were administered 500 mg/kg/day Petroleum ether extract of *S. paniculata* flowers (PEESP) suspended in 0.5% tween 80 *po.* for 10 days plus 3 gm/kg PAR *po.* for 2 day.
Group VI: Animals were administered 500 mg/kg/day Ethyl acetate extract of *S. paniculata* flowers (EAESP) suspended in 0.5% tween 80 *po.* for 10 days plus 3 gm/kg PAR *po.* for 2 day.
The rats included in the groups that were given paracetamol, silymarin and three extracts of *S. paniculata* (Groups 2–6) were weighed daily and accordingly dosed on body weight basis for assuring more accuracy and safety.

Collection and processing of samples:
Fourty-eight hours after the administration blood samples were collected by cardiac puncture in plain dry tubes from all animals of six groups. The rats, from which blood samples were collected, were euthanized under light ether anesthesia, and their liver tissue was excised. Blood samples were centrifuged at 3,000 rpm for 10 min for the separation of sera. The serum samples obtained were transferred into eppendorf tubes and were preserved in a deep freezer at - 80ºC. The liver tissues were used for the analyses of oxidative stress parameters. Liver tissue samples were homogenized in chilled 50 mM potassium phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatants obtained were transferred into eppendorf tubes, and preserved at -80 ºC in a deep freezer until used for analysis for various biochemical assays.

Biochemical analysis:
Serum ALT, AST, ALP, were measured using Prietest clinical chemistry label kits and a Biochemical autoanalyser [9-11]. Protein levels in liver homogenates were measured as described by Lowry et al. [12]. MDA analyses were performed in accordance with the method described by Ohkawa et al. [13]. GSH activity was measured as described by Ellman [14]. CAT activity was determined in accordance with the method described by Clairborne [15], SOD activity was determined as described by Marklund [16].

Histopathological examinations:
Tissue samples were taken from the liver of the necropsied animals and fixed in10% formalin neutral buffer solution. The trimmed tissues were first washed with tap water followed by dehydration through a graded alcohol series and then passed though xylol and paraffin series before finally blocked in paraffin. The paraffin blocks were cut into5–6 mm sections using a microtome stained using hematoxylin and eosin and examined under a light microscope.

Statistical analysis:
The results are presented as mean ± standard error of mean (SEM). Significance of difference among the groups was determined by one-way ANOVA using Instat Graphpad and then differences among means were analyzed using Dunnett's test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Biochemical findings:
A statistically significant difference was observed in serum ALT, AST and ALP levels in Group 2 ($P < 0.01$) as compared with Group 1 (Table 1). Also a statistically significant difference was observed in serum ALT, AST and ALP levels in Group 3, 5 and 6 ($P< 0.01$) as compared with Group 2 (Table 1). No statistically significant difference was observed in serum ALT, AST and ALP levels in Group 4 ($P<0.01$) as compared with Group 2 (Table 1). Also a statistically significant difference was observed in serum ALT, levels in Group 1 ($P<0.05$), Group 2 ($P<0.01$), decrease was observed as compared with Group 3 (Table 1). Group 4 ($P<0.01$) i.e. with MESP no decrease was observed as compared with Group 3 (Table 1). A statistically significant difference was observed in serum AST, levels in Group 2 ($P<0.01$), Group 5 ($P<0.05$) decrease was observed as compared with Group 3 (Table 1), Group 4 ($P<0.01$) i.e. with MESP no decrease was observed as compared with Group 3 (Table 1). A statistically significant difference was observed in serum ALP, levels in Group 2 ($P<0.01$), Group 2 ($P<0.05$) decrease was observed as compared with Group 3 (Table 1). Group 4 ($P<0.01$) i.e. with MESP no decrease was observed as compared with Group 3 (Table 1).

A statistically significant difference was observed in liver MDA level and GSH, CAT, SOD (Table 2) in Groups 2 ($P<0.01$) as compared with Group 1 (Table 2). Also a statistically significant difference was observed in MDA level and GSH, CAT, SOD in Group 3, 5 and 6 ($P < 0.01$) as compared with Group 2 (Table 2). No statistically significant difference was observed in MDA level and GSH, CAT, SOD in Group 4 ($P< 0.01$) as compared with Group 2 (Table 2). Also a statistically significant difference was observed in GSH levels in Group 1 ($P<0.05$), Group 2 ($P<0.01$), Group 5 ($P<0.05$), Group 6 ($P<0.05$) decrease was observed as compared with Group 3 (Table 1). Group 4 ($P<0.01$) i.e. with MESP no increase in GSH levels was observed as compared with Group 3 (Table 2). A statistically significant difference was observed in MDA levels in Group 2 ($P<0.01$) increased, Group 5 ($P<0.05$) decreased MDA levels was observed as compared with Group 3 (Table 2). Group 4 ($P<0.01$) i.e. with MESP no decrease was observed as compared with Group 3 (Table 2). Also a statistically significant difference was observed in catalase levels in Group 2 ($P<0.01$) decreased, Group 5 ($P<0.05$), Group 6 ($P<0.05$) increased catalase activity was observed as compared with Group 3 (Table 2). Group 4 ($P<0.01$) i.e. with MESP no increase in catalase levels was observed as compared with Group 3 (Table 2). A statistically significant difference was observed in SOD levels in Group 1 ($P<0.05$) increase and Group 2 ($P<0.01$) decreased SOD levels was observed as compared with Group 3 (Table 2). Group 4 ($P<0.05$) i.e. with MESP no increase was observed as compared with Group 3 (Table 2).

Histopathological findings

Group NC: Liver shows normal architecture. The central vein, portal tract and sinusoids appear normal (Fig. A). Group PAR: Liver shows congested liver tissue with congested sinusoids and centrilobular necrosis with sparse infiltrate of lymphocytes and neutrophils (Fig. B). Group SMR: Liver shows normal architecture with absence of fatty change and necrosis (Fig. C). Group MESP: Liver shows congested liver tissue. At some places congested sinusoids with necrosis of perivenular hepatocytes with sparse infiltrate of lymphocytes seen (Fig. D). Group PEESP: Liver shows normal hepatic architecture with regeneration of hepatocytes and absence of fatty changes and necrosis (Fig. E). Group EAESP: liver shows normal hepatic architecture with regeneration of hepatocytes and absence of fatty change and necrosis (Fig. F), (Table 3).

Table 1: Effect of Silymarin and *S. paniculata* extracts on Serum ALT, AST and ALP activities.

Groups	ALT (U/L)	AST (U/L)	ALP (U/L)
I	40.76 ±3.44†	143.22 ±8.20	195.08 ±5.53
II	144.88 ±8.14**††	61.32 ±10.52**††	513.36 ±9.17** ††
III	56.55 ±5.25**	154.56 ±3.02**	199.98 ±3.59**
IV	137.02 ±4.99$^{\#\dagger\dagger}$	339.10 ±5.58$^{\#\dagger\dagger}$	490.60 ±7.82$^{\#\dagger\dagger}$
V	62.62 ±3.83**	211.86 ±9.62** †	269.98 ±4.87**†
VI	60.76 ±6.65**	168.24 ±9.94**	210.96 ±9.18**

*Values are expressed as mean ±S.E.M for six rats in each group. Comparisons were made with toxic group (PAR) vs. all treated groups and control group, ** represents statistical significance at P<0.01 and # indicates non significance. † represents comparison made with group (SMR) vs. all treated groups, †† represents statistical significance at P<0.01 and † represents statistical significance at P<0.05.*

Table 2: Effect of Silymarin and *S. paniculata* extracts on Liver GSH, MDA levels, CAT and SOD activities.

Groups	GSH (µmol/g liver)	MDA (nmol/g liver)	Catalase (nmol/mg Protein)	SOD (U/mg protein)
I	298.00±7.92†	2.28±0.41	295.30±5.20	21.59±1.07†
II	66.64±9.16**††	6.70±0.23**††	109.59±1.92**††	9.16±1.36**††
III	210.06±6.76**	2.77±0.23**	253.82±5.66**	16.40±0.84**
IV	82.10±7.64$^{\#\dagger\dagger}$	5.76±0.08$^{\#\dagger\dagger}$	120.78±1.82$^{\#\dagger\dagger}$	12.95±0.50$^{\#\dagger}$
V	130.58±3.27**†	3.27±0.16**†	176.33±2.69**†	18.24±1.05**
VI	159.26±5.73**†	2.91±0.23**	217.15±3.51** †	16.94±0.94**

*Values are expressed as mean ±S.E.M for six rats in each group. Comparisons were made with toxic group (PAR) vs. all treated groups and control group, ** represents statistical significance at P<0.01 and # indicates non significance. † represents comparison made with group (SMR) vs. all treated groups, †† represents statistical significance at P<0.01 and † represents statistical significance at P<0.05.Catalase (CAT), nmol of H_2O_2 consumed per min per mg protein. Superoxide dismutase (SOD) amount of enzyme required to give 50% inhibition of pyrogallol auto oxidation, U per mg protein.*

Table 3: Effect of Silymarin and *S. paniculata* extracts on Liver histopathological changes.

Groups	Sinusoids	Centrilobular Necrosis	lymphocytes and neutrophils
I	---	---	---
II	+++	+++	+++
III	---	---	---
IV	+++	+++	+++
V	---	---	---
VI	---	---	---

DISCUSSION

Paracetamol is a common analgesic and antipyretic drug. Liver injury induced by paracetamol is the best characterized system of xenobiotic induced hepatotoxicity and is commonly used model for screening hepatoprotective drugs. Several studies have demonstrated the induction of hepatocellular damage or necrosis by paracetamol in higher doses in experimental animals and human [17]. For screening of hepatoprotective agents, paracetamol-induced hepatotoxicity has been used as a reliable method. Paracetamol is metabolized primarily in the liver and eliminated by conjugation with sulfate and glucuronide, and then excreted by the kidney. Moreover, paracetamol hepatotoxicity has been attributed to the formation of toxic metabolites, when a part of paracetamol is activated by hepatic cytochrome P-450 to a highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI) [18]. N-acetyl-p-benzoquineimine can alkylate and oxidise intracellular GSH, which results in liver GSH depletion subsequently leads to increased lipid peroxidation by abstracting hydrogen from a polyunsaturated fatty acid and ultimately, liver damage due to higher doses of paracetamol. Reactive metabolites can exert initial cell stress through a wide range of mechanisms including depletion of glutathione (GSH) or binding to enzymes, lipids, nucleic acids and other cell structures [19].

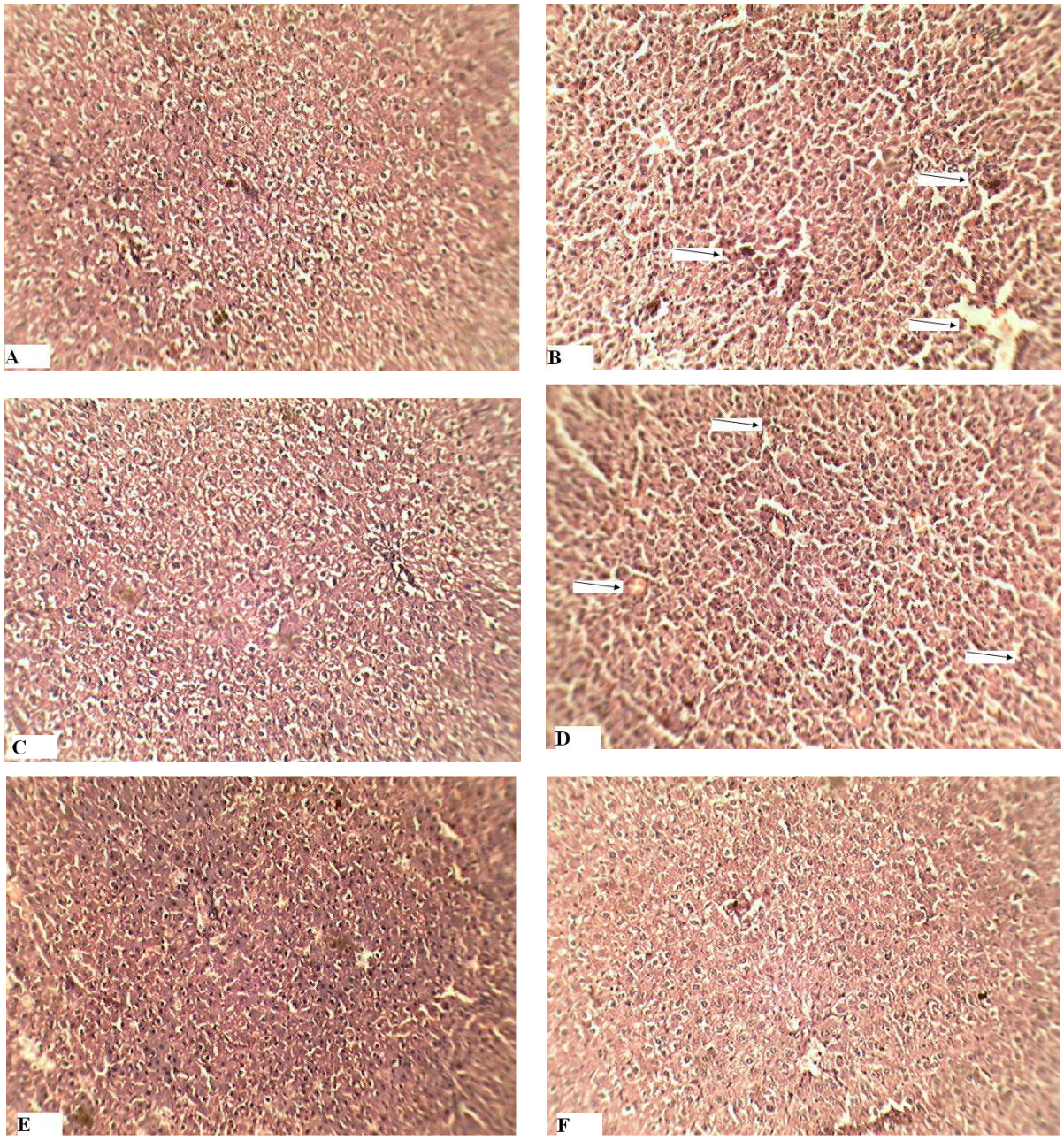

Figure 1. Effects of PAR, SMR, MESP, PEESP and EAESP on histopathological changes in liver of paracetamol induced hepatotoxicity in rats. Rats were administered with silymarin, methanolic, petroleum ether, ethyl acetate extracts of *S. paniculata* for 10 days and paracetamol for 2 days. Livers were harvested, fixed in formalin and stained with hematoxylin and eosin for evaluation of pathological changes due to feeding a vehicle (A) paracetamol (B), Silymarin plus 3gm/kg B.W. paracetamol (C) MESP plus 3gm/kg B.W. paracetamol (D) PEESP plus 3gm/kg B.W. paracetamol (E) EAESP plus 3gm/kg B.W. paracetamol (F). The photomicrographs show views of the liver sections, magnification ×40.

AST predominantly found in mitochondria of hepatocytes. ALT is more specific to liver, is one of the most sensitive tests employed in the diagnosis of hepatic diseases and thus is a better parameter for detecting liver injury. Serum ALP is also associated with liver cell damage. The ALT, AST and ALP activity are largely used as most common biochemical markers to evaluate liver injury [20]. Administration of paracetamol caused a significant elevation of enzymes level such as AST, ALT and ALP, it has been attributed to damage structural integrity of liver, because they are cytoplasmic in location and released into circulation after cellular damages indicating development of hepatotoxicity [21]. The administrations of Petroleum ether extract (500 mg/kg) and Ethyl acetate extract (500 mg/kg) of *S. paniculata* flowers has significantly prevented the increased liver marker enzymes AST, ALT and ALP level, whereas methanol extract (500 mg/kg) of *S. paniculata* flowers has not significantly reduced the increased serum marker enzymes AST, ALT and ALP level. This is in agreement with the commonly accepted view that serum levels of AST, ALT and ALP return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [22]. Among three different extracts of *S. paniculata* flowers, petroleum ether and ethyl acetate extracts exhibits the excellent hepatoprotective properties as indicated by maximum prevention of increased serum biochemical parameters on paracetamol induced toxicity. Catalase converts harmful hydrogen peroxide into water and oxygen and protects the tissues from highly reactive hydroxyl radicals. The reduction in the activity of this enzyme may results in number of deleterious effect due to accumulation of highly toxic metabolites and hydrogen peroxide on paracetamol administration, which has induced oxidative stress in the cells [15]. Administration of petroleum ether and ethyl acetate extracts of *S. paniculata* flowers significantly increased the activity of catalase thereby protecting the liver from paracetamol intoxication, whereas methanol extracts of *S. paniculata* flowers has not significantly increased the activity of catalase in rats liver, thus it has not protected the liver from paracetamol intoxication.

SOD is an extremely effective antioxidant enzyme, and is responsible for catalytic dismutation of highly reactive and potentially toxic superoxide radicals to H_2O_2 [16]. The activities of SOD in the paracetamol group were significantly decreased when compared with the control group. The results strongly suggest that the significant decrease of hepatic SOD activities observed in rats treated with paracetamol may be largely due to increased free radical production and all the SOD enzymes content were utilized. In rats treated with petroleum ether and ethyl acetate extracts of *S. paniculata* flowers, the activity of this antioxidant enzyme were significantly higher than in the rats exposed to paracetamol alone. Whereas, methanol extracts of *S. paniculata* flowers has not significantly increased the activity of SOD in rats. Reduced glutathione (GSH) is a substrate for glutathione related enzymes, and a regenerator for alpha-tocopherol, therefore it plays an important role in the antioxidant defense system. Reduced GSH removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. The reduced GSH depletion in hepatic mitochondria is considered the most important mechanism in the paracetamol induced hepatotoxicity [23]. Reduced GSH level was depleted in paracetamol treated group may be due to conjugation of reduced GSH with NAPQI to form mercapturic acid. In present study, administration of petroleum ether and ethyl acetate extracts of *S. paniculata* flowers has shown greatest ability to reduce oxidative stress by increasing the levels of reduced glutathione as compared to paracetamol intoxicated group.

The increase in MDA level in liver induced by paracetamol suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism [18]. A highly significant decrease in lipid peroxidation in liver tissue was observed in petroleum ether and ethyl acetate extracts of *S. paniculata* flowers treated groups indicates that petroleum ether and ethyl acetate extracts of flower possess antioxidant properties and has prevented the lipid peroxidation. The studied plant extracts contain antioxidants and hepatoprotective activity through regulatory action on cellular permeability, stability and suppressing oxidative stress. Hence, it can be concluded that the possible mechanism of hepatoprotective activity of *S. paniculata* flowers may be due to the reduction of oxidative stress and its ability to reduce elevated levels of serum marker enzymes. A number of scientific reports indicated that certain flavonoids, triterpenoids and steroids have protective effect on liver due to its antioxidant properties [24-29].

CONCLUSION

In the present study the phytochemical screening of the plant extracts was performed and it was found to contain flavonoids, tannins, phenolic compounds and Steroids. Therefore, it can be concluded that in *S. paniculata* flowers, flavonoids, sesquiterpene, steroids and bioactive phenolic compound might have prevented the liver injury induced by oxidative stress and the plant can play key role in hepatoprotective activity. Further work on *S. paniculata* flowers for its phytochemical constituents & their active principle is necessary for the complete evaluation of its antioxidant and hepatoprotective activity.

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