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Hepatoprotective and antioxidant effect of *Actinodaphne hookeri* Meissn. leaf extracts against CCl_4-induced liver injury in rats

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The petroleum ether, chloroform and alcoholic extracts of *A. hookeri* Meissn. leaves were studied to evaluate the hepatoprotective and antioxidant activities in CCl_4-induced hepatotoxicity in rats. Oral administration of the extracts at doses of 200 and 400 mg/Kg once daily for 10 days significantly restored normalization of serum enzyme levels, viz. glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and markers, viz. total bilirubin and direct bilirubin and the results were comparable to the effects of Liv.-52. The chloroform and alcoholic extract at the dose of 400 mg/kg was found to be more potent when compared to petroleum ether extract at similar dose. The hepatoprotection is also supported by histopathology of treated animals. In regard to antioxidant activity, chloroform and ethanolic extract exhibited a significant effect showing increased levels of enzymatic and non-enzymatic parameters, viz. catalase, GSH, SOD and decreased level of malondialdhyde (MDA). The results of this study strongly indicate that *A. hookeri* leaves have potent antioxidant and hepatoprotective action against CCl_4-induced hepatic damage in rats which may be due to the presence of phytoconstituents such as flavonoids and triterpenoids.

Keywords: Hepatoprotective, Antioxidant, *Actinodaphne hookeri,* Flavonoids.
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Introduction

Liver diseases are a major worldwide health problem, with high endemicity in developing countries. They are mainly caused by chemicals and some drugs when taken in very high doses. Despite advances in modern medicine, there is no effective drug available that stimulates liver function, offers protection to the liver from damage or help to regenerate hepatic cells. It is therefore, necessary to search for alternative drugs to replace/supplement those in current use of doubtful efficacy and safety for the treatment of liver disease.

Today, human beings are exposed to certain environmental pollutants and foreign chemicals which are collectively referred to as xenobiotics, causing serious health problems. The liver is the major organ involved in the metabolism, detoxification and excretion of various endogenous and exogenous substances such as xenobiotics. Oxidative stress plays an important role in many diseases including liver diseases[1]. The over production of oxidative stress can lead to damage in DNA, cell membrane, protein and cellular membranes and consequently induces degeneration, destruction and toxicity of various molecules[2].The production of oxidative stress can be controlled by the antioxidant systems in the living organisms. Currently, many synthetic antioxidant drugs (Butylated hydroxyl toluene, Tertiary butyl hydroxyquinone) have been used in drug composition. However, these synthetic drugs can cause many side effects and lead to many potential health problems. Management of liver diseases is still a challenge to the modern medicine. The modern medicines have little role in alleviation of hepatic ailments whereas most of the important representatives are from phytoconstituents. In Ayurveda a number of medicinal preparations have been employed for treating liver disorders and there are no rational drug therapies. The herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness[3]. The plants containing flavonoids, tannins and some phenolic components possess broad biological properties to exert beneficial effects on some liver diseases involving

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uncontrolled lipid peroxidation and free radical scavenging activity. Inspite of tremendous advances made in allopathic medicine, effective hepatoprotective medicine is still wanting. About 80% of world population relies on folklore medicine for curing ailments related to liver. However, only a small number of these medicinal plants as well as formulations used are scientifically evaluated for their activity. In the context of our ongoing search for new natural substances possessing hepatoprotective efficacy, the present investigation was undertaken by utilizing the leaves of plant the *Actinodaphne hookeri* Meissn., belonging to the family Lauraceae. It is a widespread medium sized tree, sometimes a shrub, usually up to 6 m in height. The plant is found in Coorg, North Kanara, Shimoga, Peninsular and North East India including Sikkim, Western ghats and Satara in Maharashtra. Its leaves are coriaceous, in 2 whorls of 3 each, 10-18 by 4.5-6.3 cm, penninerved, elliptic lanceolate. Finely acuminate, young leaves densely silky with long tawny hairs, acute base, main nerves 6-10 pairs, petioles 1.3-2.5 cm long, silky pubescent. Leaves contain an amorphous alkaloid, β-sitosterol, rutin, hentriacontanol and quercetin-3-rhamnoside. The phytochemical investigations of the leaves revealed the presence of alkaloids, flavonoids, tannins, sterols, glycosides and carbohydrates[4-6].

Materials and Methods

Collection and authentication

The leaves of *A. hookeri* were collected from Jog falls and local areas of Shimoga district and were authenticated by Dr. B. D. Huddar, Head, Department of Botany, Kadasiddheshwar Arts College and H.S. Kotambari Science Institute, Hubli.

Preparation of extracts

The leaves were shade dried at room temperature, pulverized into coarse powder and the powder was successively extracted by continuous hot percolation (Soxhlation) with petroleum ether (40-60°C), chloroform and alcohol with increasing order of polarity. After the exhaustive extraction, the solvent was removed under reduced pressure (Buchi) using rotary flash evaporator then finally dried in dessicator.

Animal selection

The experiments were carried out using Swiss albino mice weighing between 20-30 g for acute toxicity study and Wister albino rats weighing around 150-250 g for the hepatoprotective and antioxidant activity. The animals were maintained *ad libitum* at normal laboratory conditions and were given standard animal feed.

Acute toxicity studies

The albino mice of either sex weighing between 20-30 g were used for the investigation. The animals were fasted over night prior to experiment. An acute toxicity test was carried out as per OECD[7] guidelines and accordingly doses of extracts were studied. As per OECD guidelines the safest dose for all the extracts is 2000 mg/Kg body weight, hence $1/10^{th}$ and $1/5^{th}$ of the dose was taken as therapeutic dose.

Extracts used

The petroleum ether, chloroform and alcoholic extracts of leaves were screened for hepatoprotective and *in vivo* antioxidant activity. The extracts were suspended in distilled water using *Tween* 80 and were employed to assess the above activity. The dose was given orally.

Phytochemical analysis

Phytochemical tests[8, 9] were carried out to detect the presence of phytoconstituents, viz. alkaloids, carbohydrates, flavonoids, tannins, triterpenoids, saponins, etc.

Hepatoprotective activity[10]

Chronic administration of carbon tetrachloride to rats induces severe disturbances of hepatic function together with histological observable liver disturbances. Hepatoprotective and *in vivo* antioxidant activity was carried out using Albino rats. The animals were divided into nine groups of six animals each as follows and maintained on standard diet and water *ad libitum.*

Group-I : Normal control (Vehicle treated Tween 80 (1%))
Group-II : Positive control (Untreated)
Group-III : Standard control (Liv. 52)
Group-IV : Extract-I (200 mg/Kg pet ether extract AH)
Group-V : Extract-II (200 mg/Kg chloroform extract AH)
Group-VI : Extract-III (200 mg/Kg alcoholic extract AH)
Group-VII : Extract-I (400 mg/Kg pet ether extract AH)
Group-VIII : Extract-II (400 mg/Kg chloroform extract AH)
Group-IX : Extract-III (400mg/Kg alcoholic extract AH)

All the groups were treated for 10 days. CCl_4 was used as a hepatotoxin to induce hepapatotoxicity to animals of groups II - IX on 3rd, 6th and 10th day by intraperitonial route. After 1 hour of the last dose of carbon tetrachloride injection, animals were sacrificed by cervical dislocation and the blood was collected from carotid artery and used for estimation of various biochemical parameters. The Biochemical parameters estimated includes serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloactetate transaminase (SGOT), serum alkaline phosphatase (SALP) and serum bilirubin with semiautoanalyser using diagnostic reagent kit.

Table 1—Qualitative chemical analysis of various solvent extracts of *A. hookeri* leaf

Phytoconstituents	Successive extracts			
	Petroleum ether (40-60°C)	Chloro form	Alcoholic	Aqueous
Carbohydrates	-	-	+	+
Molisch Test				
Reducing sugar test				
Proteins	-	-	-	-
Biurets test				
Millons test				
Xanthoproteic test				
Precipitation test				
Saponin Glycosides	-	-	-	-
Foam test				
Haemolytic test				
Flavonoid Glycosides	-	-	+	-
Shinoda test				
Lead acetate test				
Steroids and Triterpenoids	+	+	-	-
Liebermann - Burchard test				
Salkaowski test				
Tannins and Phenol	-	-	+	+
Neutral ferric chloride test				
Lead acetate test				
Alkaloids	-	+	-	-
Mayer's test, Dragendroff's test, Wagner's test				
Hager's test				

Antioxidant activity

The isolated liver was rinsed with water and washed with ice cold saline and blotted to dry immediately. A liver homogenate was prepared with ice cold phosphate buffer. After centrifugation, the clear supernatant was used for the assay of various endogenous antioxidant parameters, viz. Reduced glutathione (GSH), Malondialdehyde (MDA), Superoxide dismutase (SOD) and Catalase (CAT) by standard methods.

Statistical analysis

The results were expressed as mean ± SEM and evaluated using one way ANOVA followed by Dunnette multiple comparison test.

Results and Discussion

The qualitative chemical investigations of various extracts of *A. hookeri* revealed the presence of triterpenoids and steroids in (40-60°C) extract. The chloroform extract was found to contain triterpenoids and alkaloids, while alcoholic extract contained carbohydrates, glycosides, flavonoids and tannins. Further thin layer chromatographic studies were done to confirm the above phytoconstituents present in the various extracts and fractions. The results are as depicted in Table 1.

An attempt has been made to evaluate the hepatoprotective and *in vivo* antioxidant activity by carbon tetrachloride induced hepatotoxicity model. The hepatoprotective results are reported in Table 2.

Table 2—Effect of extracts of *A. hookeri* on biochemical parameters in carbon tetrachloride induced hepatotoxicity

Treatment	SGOT IU/L	SGPT IU/L	ALP IU/L	Total bilirubin mg/dl	Direct bilirubin mg/dl
Normal	62.5 ± 1.21	58.15 ± 1.48	157.8 ± 6.60	0.138 ± 0.01	0.075±0.01
Diabetes control	203.2 ± 8.73	176.6 ± 1.71	380.2 ± 19.72	3.94 ± 0.37	0.498 ±0.06
Standard (Liv-52)	73.80 ± 1.98***	73.38 ± 2.89***	174.2 ± 3.90***	1.13 ± 0.04***	0.116 ± 0.10***
Pet ether (200 mg)	121.3 ± 3.26***	112.7 ± 16.10***	233 ± 10.66***	1.758 ± 0.05***	0.302 ± 0.03***
Chloroform (200 mg)	98.9 ± 4.25***	88.14 ± 4.23***	215 ± 11.06***	1.492 ± 0.08***	0.230 ± 0.02***
Alcoholic (200 mg)	95.85 ± 4.72***	86.40 ± 9.05***	196.5 ± 5.75***	1.344 ± 0.10***	0.183 ± 0.02***
Pet ether (400 mg)	106.6 ± 3.26***	100.5 ± 2.55***	204.8 ± 20.81***	1.64 ± 0.04***	0.293 ± 0.03***
Chloroform (400mg)	86.29 ± 4.29***	86.98 ± 3.40***	191.7 ± 5.46***	1.23 ± 0.025***	0.188 ± 0.011***
Alcoholic (400 mg)	84.07 ± 3.66***	84.57±3.45***	179±6.31***	1.20 ± 0.08***	0.14 ± 0.02***

Data were analysed by ANOVA followed by Dunnett's test
Values are represented as mean ± S.E.M. (n=6); NS=non significant, ***P < 0.001

The same results have been graphically represented in Fig.1 and 2.

In the present study, the capability of the above extracts to protect against CCl_4 induced hepatotoxicity and oxidative stress was investigated. Carbon tetrachloride is a potent hepatotoxin producing centrilobular hepatic necrosis which causes liver injury. CCl_4 induced liver injury depends on a toxic agent that has to be metabolized by the liver NAPDH-cytochrome P450 enzyme system to a highly reactive intermediate. It has been suggested that this toxic intermediate is the trichloromethyl radical (CCl_3) producing maximum damage to liver[11-12]. The free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events, which eventually leads to membrane lipid peroxidation and finally cell necrosis[13-15] The liver damage was assessed by biochemical studies and histopathological examinations. SGOT and SGPT are well known diagnostic indicators of liver disease. ALT activity is considered to be a sensitive biomarker of hepatotoxicity, as it is primarily localized in liver. In the cases of liver damage with hepatocellular lesions and parenchymal cell necrosis, these marker enzymes are released from the damaged tissues into the blood stream[16]. Increased levels of SGOT and SGPT in serum of CCl_4 treated animals indicate that the integrity of hepatocytes was abnormal, resulting in the release of intracellular enzymes into the systemic circulation[17]. In present study, pre-treatment with *A. hookeri* extracts caused a decrease in the activities of the above enzymes when compared with CCl_4 treatment groups which may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue. Similar observations have been reported by Pal *et al*[18].

Alkaline phosphatase is excreted normally via bile by the liver. Its activity on endothelial cell surfaces is responsible for the conversion of adenosine nucleotides to adenosine, a potent vasodilator and anti-inflammatory mediator that results from injury. So, following the injury, accumulation of interleukin-6 can lead to production of adenosine by alkaline phosphatase and subsequent protection from ischemic injury. This may be the reason for the increment in ALP in intoxicated rats, which have cell necrosis. The treatment with extracts caused a decrease in the activity of ALP when compared with CCl_4 treatment

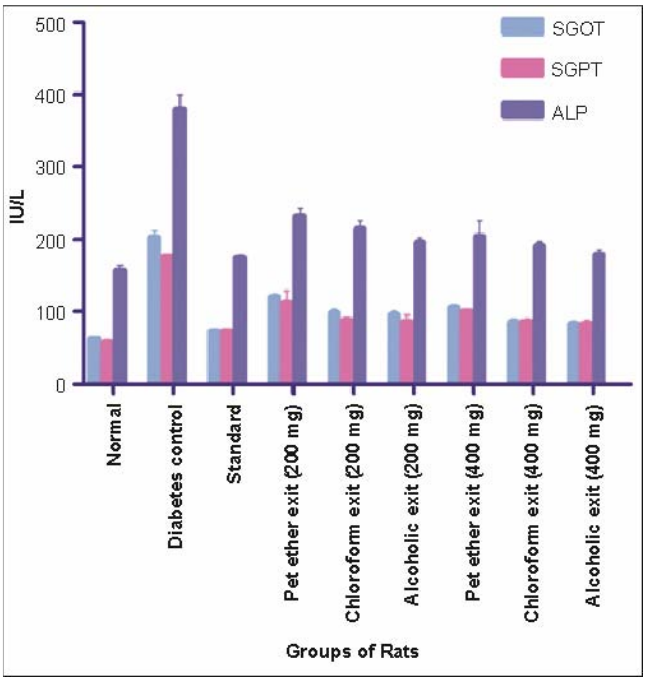

Fig. 1—Graph showing effect of extracts of *A. hookeri* on SGOT, SGPT and ALP in carbon tetrachloride induced hepatotoxicity

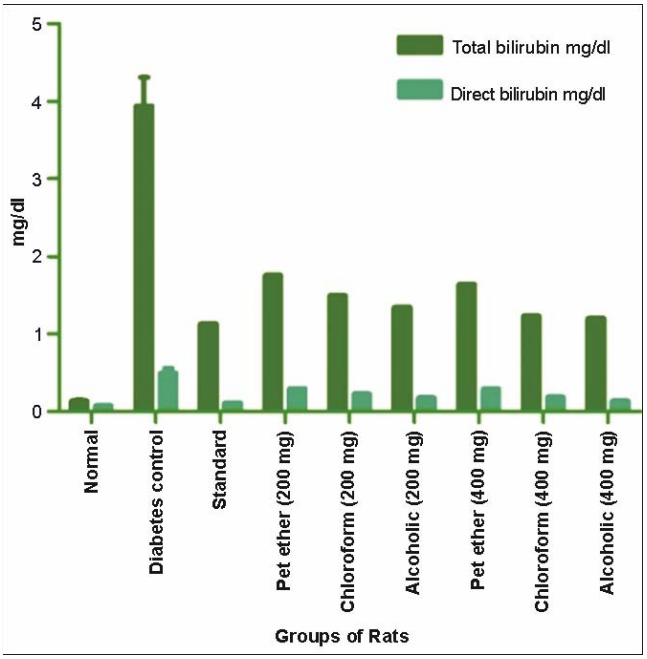

Fig. 2—Graph showing effect of extracts of *A. hookeri* on total bilirubin and direct bilirubin in carbon tetrachloride induced hepatotoxicity

group, respectively, showing its hepatoprotective potential.

Bilirubin is the breakdown product of heam in red blood cells and hyperbilirubinemia reflects the pathophysiology of liver. It is a most useful clinical

indicator of the severity of necrosis and its accumulation is a measure of the binding, conjugation and excretory capacity of liver cells. In liver injury due to hepatotoxin, there is a defective excretion of bile by the liver which is reflected in their increased levels in serum[19]. Hepatotoxicity is characterized by cirrhotic liver condition which in turn increased the bilirubin release[20]. The results shown that the serum bilirubin levels were elevated in CCl_4 treatment group. Depletion of elevated bilirubin level in the serum of rats treated with extracts suggest that there is possibility of extracts and fractions to stabilize biliary dysfunction of rat liver, which is a clear indication of the improvement of the functions of the liver cells and its cytoprotective action which may be due to the inhibitory effect on cytochrome P450. The restoration of serum enzyme levels to normal levels in CCl_4 treated rats after treatment indicates prevention of the leakage of intracellular enzymes by stabilizing the hepatic cell membrane. Restoration of increased hepatic serum enzyme level to normal level reflects protection by the extracts and fractions against the hepatic damage caused by hepatotoxins.

The *in vivo* antioxidant results of leaf extracts are reported in Table 3. The same results have been graphically represented in Fig. 3 and 4.

The increase in liver MDA levels induced by CCl_4 suggests enhanced lipid peroxidation, leading to hepatic tissue damage and failure of antioxidant defense mechanisms to prevent formation of excessive free radicals[21]. The free radical scavenging is one of the major antioxidation mechanisms inhibiting the chain reaction of lipid peroxidation. The treatment with alcoholic and chloroform extracts plays an important role in reducing the free radicals which resulted in the subsequent decrease in the membrane damage and MDA level.

Hence it may be possible that the mechanism of the hepatoprotective activity by the above extracts is due to its antioxidant effect indicating the free radical scavenging activity under *in vivo* conditions.

The non-enzymic antioxidant, glutathione is one of the most abundantly naturally occurring tripeptides present in the liver[22]. Its functions are mainly concerned with the removal of free radical species such as hydrogen peroxide, superoxide radical, alkoxy radical and maintenance of membrane protein thiols and as substrates for glutathione peroxidase and GSH. The results in the study indicate that the decrease level of GSH has been associated with an enhanced lipid peroxidation in CCl_4 treated rats. Administration of above extracts and fractions significantly increased the level of glutathione in dose-dependent manner.

Decrease in enzyme activity of SOD is sensitive index in hepatocellular damage and its the most sensitive enzymatic index in liver injury. SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system[23]. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. The above extracts showed significant increase in hepatic SOD activity and thus reducing free radical induced oxidative damage in liver.

CAT is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radicals. Administration of above extracts increases the level of CAT in induced liver damage in rats to prevent the accumulation of excessive free radical and protected the liver from CCl_4 intoxication.

Table 3—Effect of extracts of *A. hookeri* on antioxidant enzymes

Treatment	Antioxidant level			
	MDA nmol/mg wet tissue	GSH nmol/mg wet tissue	SOD U/mg protein	CAT U/mg protein
Normal	2.45 ± 0.11	6.45 ± 0.15	12.91 ± 0.24	47.07 ± 0.41
Diabetes control	6.40 ± 0.18	2.55 ± 0.27	4.58 ± 0.13	25.45 ± 1.80
Standard (Liv-52)	3.13 ± 0.15***	5.40 ± 0.40***	11.50 ± 0.12***	39.06 ± 0.42***
Pet ether (200mg)	4.53 ± 0.57**	4.50 ± 0.73**	6.25 ± 0.44*	32.25 ± 1.29**
Chloroform (200mg)	4.45 ± 0.23**	4.25 ± 0.32**	8.37 ± 0.37***	33.37 ± 1.96***
Alcoholic (200mg)	3.88 ± 0.54***	4.45 ± 0.41**	9.25 ± 0.21***	34.60 ± 1.74***
Pet ether (400mg)	4.25 ± 0.31**	4.64 ± 0.45**	7.81 ± 0.31**	34.86 ± 0.98***
Chloroform (400mg)	3.73 ± 0.44***	5.03 ± 0.35***	10.57 ± 0.36***	37.80 ±1.98***
Alcoholic (400mg)	3.35 ± 0.39***	5.25 ± 0.49***	11.15 ± 0.25***	38.40 ± 1.24***

Data were analysed by ANOVA followed by Dunnett's test.
Values are represented as mean ± S.E.M. (n=6); ns=non-significant, $^{***}P < 0.001$ and $^{**}P < 0.01$.

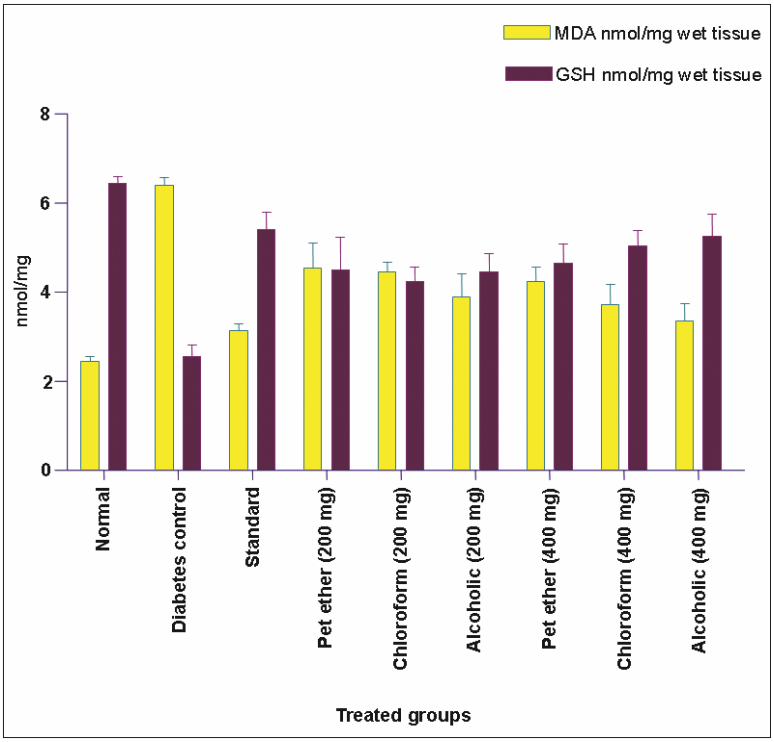

Fig. 3—Effect of extracts of *A. hookeri* on antioxidant enzymes (MDA and GSH)

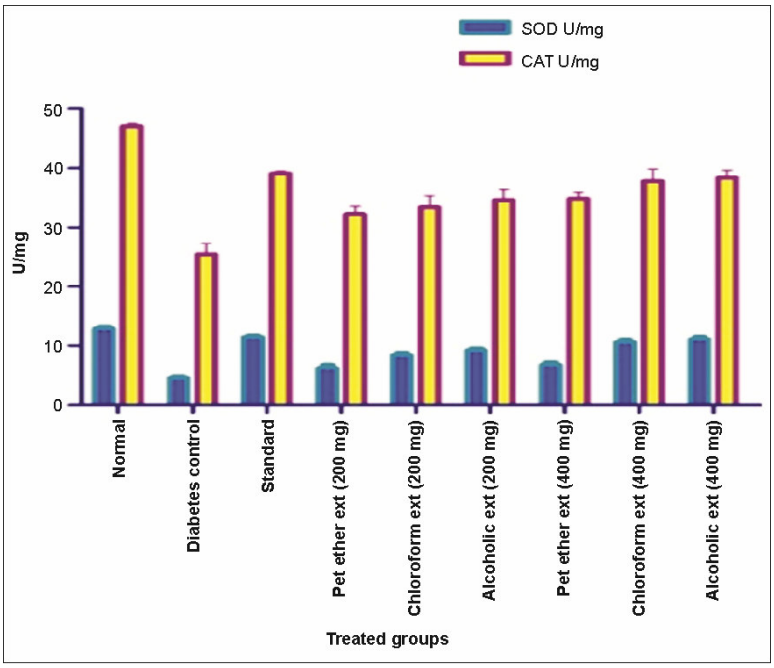

Fig. 4—Effect of extracts of *A. hookeri* on antioxidant enzymes (SOD and CAT)

The significant activity of chloroform extracts may be attributed due to the presence of triterpenes[24] which might be potentially useful in counteracting free radical mediated injuries and alcoholic extract may be due to the flavonoid[25] which is known to exhibit protection against paracetamol and CCl_4 induced liver injuries and is found to attenuate ethanol-induced oxidative stress[26].

Conclusion

From the above studies the alcoholic and chloroform extracts of *A. hookeri* showed significant hepatoprotective activity by decreasing the elevated levels of serum enzymes and significant antioxidant activity by increasing the decreased levels of antioxidant enzymes such as superoxide dismutase, catalase and reduced glutathione and decreasing the lipid peroxidation state. These parameters were also comparable with that of the standard. These results can thus be concluded that possible mechanism of hepatoprotection of leaves may be due to its antioxidant action.

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