Antioxidant and hepatoprotective action of *Asparagus racemosus* Willd. root extracts

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The antioxidant activities of the crude hydro-alcoholic extract (CE) and its four fractions viz. methanol (MF), ethyl acetate (EF), n-Butanol (BF), and precipitated aqueous (PAF) of *A.racemosus* roots tested decreased in the order of EF > MF > CE > BF > PAF when investigated by DPPH free radical scavenging assay. Under iron induced lipid peroxidation almost similar results were observed except that the activity was more in PAF than BF. Hepatoprotective activity of the extracts was also demonstrable *in vivo* by the inhibition of–CCl_4 induced formation of lipid peroxides in the liver of rats by pretreatment with the extracts. CCl_4–induced hepatotoxicity in rats, as judged by the raised serum enzymes viz. glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase and total and direct bilirubin as well as oxidant enzyme viz. malon dialdehyde were prevented, while antioxidant enzymes viz. superoxide dismutase, reduced glutathione and catalase were elevated by pretreatment with the extracts, demonstrating the potent hepatoprotective action of the roots of *A. racemosus*.

Reactive Oxygen Species (ROS) are mainly associated in the development of degenerative diseases including liver disorders, atherosclerosis, cancer, inflammatory bowel disease and lung disease[1-2]. Certain oxidants produce toxicity during its catalytic cycle by protein oxidation, enzyme inactivation and damage to cell membrane via lipid peroxidation and production of reactive lipid aldehydes such as malon dialdehyde (MDA). Antioxidants are believed to protect against certain diseases by preventing the deleterious effects of free radical-mediated processes in cell membranes and by reducing the susceptibility of tissues to oxidative stress. Several studies have shown that the hepatoprotective effect is well associated with antioxidant rich plant extracts[3-5].

Administration of antioxidant could reduce the hepatic injury. *Asparagus racemosus* Willd. (Family: Asparagaceae) commonly known as 'Shatavari' in India, is a well-known traditional rasayana drug used for variety of ailments. The roots of *A. racemosus* (AR), have been very widely used traditionally in 'amlapitta' (disease related to bile) since ancient time in India and also reported to possess adaptogenic activity[6]. In view of this information, the present study have been undertaken to find out the antioxidant and hepatoprotective effect of different extracts of roots of *A. racemosus* and to explore the responsible class of chemical constituents for the above said activity.

Materials and Methods

Chemicals— All chemicals and reagents used were of analytical grade. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Tris HCl, thio barbituric acid (TBA), CCl_4, glutathione (GSH), ascorbic acid and silymarin were purchased from Sigma Aldrich (St. Louis, USA). Biochemical kits for determining serum-glutamate pyruvate transaminase (SGPT), serum glutamate-oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), and bilirubin content were supplied by Span Diagnostics Ltd, Surat, India. Solvent used during the experiments were procured from Ranchem (New Delhi).

Plant material— The roots of AR were collected from Van Aushadhi Garden, Gandhinagar, Gujarat, India during December 2005. This plant was identified by comparing its morphological and

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microscopical characters with that in literature[7] and the voucher specimen no. [KB/PCOG/0401] was preserved at Department of Pharmacognosy, K. B. Institute of Pharmaceutical Education and Research, Gandhinagar, Gujarat, India. The roots were air-dried avoiding sun-light and pulverized for the experiment.

Extraction and fractionation—The powder of roots (1 kg) was extracted with methanol: water (70:30) in Soxhlet assembly for 48 h and crude extract was obtained. The crude extract [CE] was dried under the vacuum using rotary evaporator at temperature below 50 °C and weighed (200 g). The yield of 70 % methanolic extract of roots was 25 % (w/w). The major sub fractions prepared were: methanolic fraction [MF], ethyl acetate fraction [EF], n-butanol (n-BuOH) fraction [BF] and precipitated aqueous fraction [PAF] as per the extraction scheme given below. All the above sub-fractions were dried under the vacuum below 50 °C using rotary evaporator.

Phytochemical screening—Preliminary chemical tests were done with individual extract for the presence of different group of chemicals i.e. saponins, flavonoids, polysaccharides, free sugars and sterols[7].

Animals—Wistar rats weighing between 200-300 g of either sex were procured from Zydus Cadila Healthcare Ltd., Ahmedabad, India. All animals were housed in well ventilated polypropylene cages at 12:12 h light/dark schedule at 25±2 °C and 55-65 % RH. The rats were fed with commercial pelleted rats chow (M/S Pranav Agro Private Limited, Vaghodiya, Vadodara) and water *ad libitum* as a standard diet. All the animal experiments were approved by Institutional Animal Ethics Committee of K. B. Institute of Pharmaceutical Education & Research, Gandhinagar, and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). [Project No: KBIPER/0541].

Preparation of test drug material— Suspension (1 %) of CE and its sub-fractionated extracts (MF, EF, BF and PAF) were prepared freshly using carboxy methyl cellulose (0.5% w/w) as suspending agent.

DPPH radical scavenging activity—The antioxidant activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical, was determined as per Braca *et al*[8]. Percent inhibition (I) was calculated by the following equation:

$$I = (A_{control} - A_{sample})/A_{control} \times 100 \%$$

where $A_{control}$ is the absorbance of the ethanol containing control, and A_{sample} is the absorbance of the reaction mixture with the tested sample. EC_{50} values were determined to be the concentration at which DPPH radical is scavenged by 50%.

Iron-induced lipid peroxidation in liver homogenate— The antioxidant activity of the plant extract was evaluated by quantifying the ability of different concentrations of plant extract to suppress iron (Fe^{2+}) induced lipid peroxidation in rat liver homogenates[9-11]. Liver homogenates were prepared from male Wistar rats scheduled to be sacrificed and the liver was dissected. The dissected livers were washed with 0.15 *M* saline and homogenated in ice cold 0.1 *M* phosphate buffer (*p*H=7.4). The resultant homogenate was filtered and protein concentration of the homogenate was determined as per Henry *et al*[12]. The final protein concentration was adjusted to 10 mg protein/mL. Lipid peroxidation of liver homogenate was determined by estimation of MDA-BA adduct according to the method of Yoshiyuki *et al*[10]. A mixture containing 0.5 mL liver homogenate, 0.1 mL Tris–HCl buffer (*p*H 7.2), 0.05 mL of 0.1mM ascorbic acid, 0.05 mL 4 mM $FeCl_2$ and 0.05 mL of various concentrations of crude drug extracts or standard antioxidant, was incubated for 1 h at 37 °C. After incubation, 9 mL distilled water and 2 mL 0.6 % TBA were added to 0.5 mL of the incubation solution and shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling, 5 mL n-BuOH was added and shaken vigorously again. The n-BuOH layer was separated by centrifugation at 4000 rpm for 10 min and MDA production was measured at 532 nm.

CCl_4 induced hepatotoxicity in rats—The method for induction of hepatotoxicity was described elsewhere[13-15]. The rats were divided into following 8 groups of 6 animals each: Gr. A: normal control without any treatment, injected olive oil (0.5 mL/kg, sc) for three alternate days i.e. 2nd, 4th and 6th; Gr. B: negative control, received CMC (0.5% w/v); Gr. C: standard i.e. silymarin (50 mg/kg); Gr. D: CE (300 mg/kg); Gr. E: MF (300 mg/kg); Gr. F: EF (150 mg/kg); Gr. G: BF (300 mg/kg); and Gr. H: PAF (300 mg/kg). Gr. B, C, D, E, F and G were injected with 1:1 (v:v) mixture of CCl_4 and olive oil, administered subcutaneously at a dose 0.7 mL/kg body weight on day 2nd, 4th and 6th. CE, MF, BF, BF and PAF were administered orally between 10:00-11:00 hrs for 7 days. After 48 h of last CCl_4

injection, blood was withdrawn from ratino bulbar venous plexus under light anesthesia (pentobarbitone sodium at a dose 40 mg/kg of body weight of animals, ip) with the help of a glass capillary. Immediately, after blood withdrawal from animals, animals were sacrificed by decapitation for liver collection. Serum separated from the blood was used to analyze various marker enzymes like SGPT, SGOT, ALP as well as bilirubin levels using commercially available kits. 10 % liver homogenates were made in ice cold phosphate buffer saline (*p*H 7.4) solution using motor driven Teflon pestle. Liver homogenates were used for the estimation of protein, superoxide dismutase (SOD), MDA, catalase and GSH activity. A portion of liver was washed in phosphate buffer saline. Sections (4 μm thick) were taken and stained with hematoxylin-eosin (H & E) using standard technique for histopathological assessment.

Statistical analysis—Statistical evaluation of the data was done by one way ANOVA followed by Tukey's multiple comparison test using graphpad prism 5 software package. The values have been expressed as mean ± SE.

Results and Discussion

Phytochemical analysis—The roots were found to be very rich in saponins and polysaccharides (Table 1). Saponins were present in CE, MF and BF, while sub-fraction EF was rich in flavonoids. CE and PAF were rich in sugars and polysaccharides.

DPPH radical scavenging activity—The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants[16-20]. In the present study, measured decrease in absorbance was read to calculate the % scavenging of free radical in presence of different concentrations of extracts and standard. In order to quantify the antioxidant activity, the EC_{50}, which is the concentration of sample required to decrease the absorbance of specific free radical (DPPH) at specific λ_{max} by 50%, was calculated. The lower the EC_{50} value, greater the free radical-scavenging activity of the extract. The percentage of DPPH remaining in presence of the CE and its fractions at different concentrations are shown in Table 2.

The CE, MF and EF showed a concentration-dependent antiradical activity by inhibiting DPPH radical. Among all above fractions EF showed maximum activity at EC_{50} 153.3 μg/mL.

The method is based on the reduction of methanolic DPPH solution in presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. Cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g. hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g. p-phenylene diamine, p-aminophenol), reduce and decolourise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability[21]. It appears that the EF possesses maximum hydrogen donating capabilities and acts as an antioxidant at lower concentration (i.e. EC_{50} = 153.3 μg/mL). The scavenging effect increased with increasing concentration of the extract. However, scavenging activity of ascorbic acid, a known antioxidant, used as positive control, was relatively more

Table 1— Phytochemical screening of different extracts of roots of *A. racemosus*

Sr. No.	TEST	CE	MF	EF	BF	PAF
1	Alkaloids	-	-	-	-	-
2	Flavonoids	+	++	+++	-	-
3	Saponins	++	+++	+	++++	-
4	Tannins	-	-	-	-	-
5	Sterols	-	+	-	+	-
6	Carbohydrates	++	-	-	+	++++

+ Less, **++** Moderate**, +++** High **, ++++** Highest, - Negative
CE: Crude Extract; MF: Methanolic Fraction; EF: Ethyl acetate Fraction; BF: n-Butyl alcohol Fraction; PAF: Purified Aqueous Fraction

Table 2— Effect of different extracts of roots of *A. racemosus* on DPPH scavenging activity

Sr. No.	Extract	Concentration (mg/3 mL)	Protection %	EC_{50}
1	CE	2.50	20.02±1.25	8.514 mg/3mL (2838 µg/mL)
		5.00	32.41±1.32	
		7.50	45.07±1.21	
		10.00	57.35±2.12	
2	MF	2.50	40.75±1.22	4.1 mg/3mL (1366.6 µg/mL)
		5.00	57.15±2.11	
		7.50	78.78±2.15	
		10.00	87.03±2.35	
3	EF	0.25	25.99±0.24	0.460 mg/3mL (153.33 µg/mL)
		0.30	33.12±1.24	
		0.40	42.58±1.55	
		0.50	56.68±1.33	
4	BF	2.50	12.70±0.55	Insignificant
		5.00	17.70±0.65	
		7.50	27.57±0.41	
		10.00	32.44±0.88	
5	PAF	2.50	12.60±0.14	Insignificant
		5.00	16.93±0.32	
		7.50	26.81±0.26	
		10.00	29.94±1.14	
6	Ascorbic acid	0.010	38.68±2.14	0.0141 mg/3mL (4.7 µg/mL)
		0.015	56.94±2.15	
		0.020	72.39±2.31	
		0.025	88.07±1.24	

pronounced than that of EF. The effectiveness of the extracts were calculated by comparing CE and its active fractions, the free radical-scavenging activities decreased in the order of maximum inhibition to minimum likewise EF, MF, CE, BF, and PAF respectively. The free radical scavenging activity was found good with CE and MF; while maximum with concentration-dependent manner in EF indicating that *A. racemosus* possesses potent free radical-scavenging activity due to constituents present in EF. The free radical-scavenging activity of the CE and MF were less than those of EF, while BF and PAF exhibited negligible activity. CE and its fractions were compared with that of a standard antioxidant, ascorbic acid.

Iron-induced lipid peroxidation in liver homogenates—Incubation of the liver homogenate for 1 h with increasing concentrations of the different extracts of roots of *A. racemosus* (CE, MF, EF, BF and PAF) was evaluated to prevent lipid peroxidation caused by ferrous sulphate ($FeSO_4$) in liver homogenates. CE, MF and EF showed good protection for lipid peroxidation in rat liver homogenates. The addition of 25 mM $FeSO_4$ to liver homogenates for 1 h caused significant increase in the extent of lipid peroxidation compared to the normal samples. When different concentrations of different extracts were added to the Fe^{2+}containing homogenates, there was significant concentration dependent inhibition in the extent of lipid peroxidation specifically with CE, MF and EF. Highest protection was found with EF (Fig. 1).
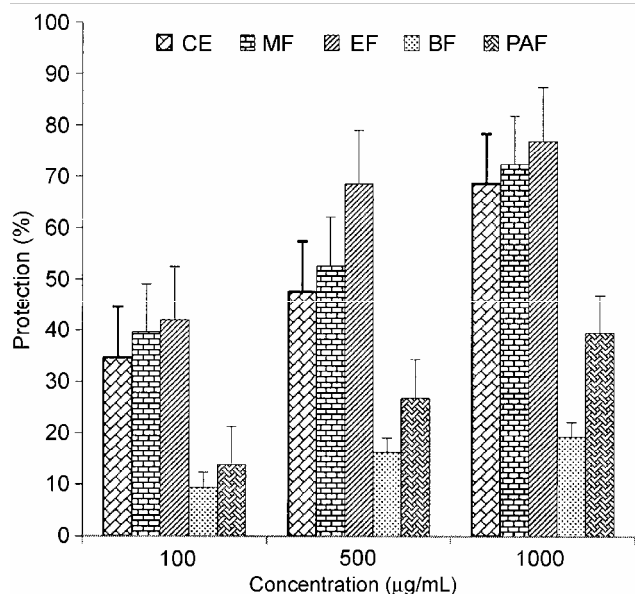

Fig. 1—Effect of different extracts of roots of *A. racemosus* in iron induced lipid peroxidation in liver homogenate

CCl₄ induced hepatotoxicity in rats—CCl_4 caused hepatic injury which was indicated by the great increased in SGPT, SGOT, ALP, total and direct bilirubin levels in negative control animals when compared to normal group of animals and these increase in levels were significantly decreased in all above serum enzymes by pretreatment with CE, MF and EF when compared to negative control group. While with sub-fractions BF and PAF (300 mg/kg, po) results were insignificant (Table 3).

Above conclusion was also further confirmed by measuring the effect of the different extracts of roots of *A. racemosus* on lipid peroxidation, in terms of MDA production, SOD, GSH level and catalase activity in liver homogenates of rats of all groups. The negative control group of animals showed significant increase of MDA production and decrease in SOD, GSH and catalase contents in the liver homogenate. The pretreatment of EF (150 mg/kg, po, 7 days), showed maximum activity amongst all active groups (CE, MF) by significant decrease of MDA production with increase in SOD, GSH and catalase content. Results were also compared with that of standard drug silymarin (50 mg/kg/7days, po) (Table 4). This fact was also confirmed by histopathological studies. The all above results were indicating that EF (150 mg/kg, po) and MF (300 mg/kg, po) were the most effective fractions of roots of *A. racemosus.*

CCl_4 is the potent hepatotoxins producing centrilobular hepatic necrosis, which causes liver injury. 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. CCl_4 has the toxic intermediate trichloromethyl radical (CCl_3) producing maximum damage to liver[22,23].

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[24,25]. Although several isoforms of cytochrome P450 may metabolize CCl_4, attention has been focused largely on the cytochrome P450 2E1 (CYP2E1) isoform, which is ethanol inducible[26,27]. Alterations in the activity of CYP2E1 affect the susceptibility to hepatic injury from CCl_4[28-30].

The preventive actions of different extracts in liver damage induced by CCl_4 have widely used as an

Table 3— Effect of different extracts of roots of *A. racemosus* on serum marker enzymes in CCl$_4$ induced hepatotoxicity

[Values are mean ± SE from 6 animals in each group]

Groups	SGOT (IU/L)	SGPT (IU/L)	ALP (KA/dl)	Total Bilirubin (IU/L)	Direct Bilirubin (IU/L)
Normal control	30.35±1.32	32.25±1.23	5.3±0.60	0.36±0.09	0.05±0.01
Negative control	177.25±3.45[*]	130.25±4.53[*]	94.5±1.50[*]	1.857±0.04[*]	0.48±0.05[*]
Standard	55.2±1.20[#]	43.5±1.34[#]	33±1.20[#]	0.2625±0.05[#]	0.12±0.02[#]
CE	77.25±2.54[#]	58.5±2.80[#]	57±2.5[#]	0.5954±0.15[#]	0.13±0.03[#]
MF	70.35±2.63[#]	53.5±2.15[#]	44±1.50[#]	0.4135±0.16[#]	0.23±0.01[#]
EF	62.5±1.3[#]	39.5±1.20[#]	36.3±1.60[#]	0.5355±0.21[#]	0.14±0.02[#]
BF	109.75±3.86	105.5±3.45	68.5±1.80	0.5853±0.23[#]	0.35±0.02[#]
PAF	151±4.23	126.5±3.32	79.8±2.10	0.6953±0.31[#]	0.53±0.03

P value: <0.01; significantly different from [*]normal group, [#]control group
All the data was evaluated by one way ANOVA followed by Tukey's multiple comparison test. Differences were considered to be statistically significant if *P*<0.05.

Table 4— Effect of different extracts of roots of *A. racemosus* on oxidant/antioxidant enzymes in liver homogenate in CCl$_4$ induced hepatotoxicity

[Values are mean ± SE from 6 animals in each group]

Groups	Total Protein (mg/ml)	MDA (nmoles/mg protein)	SOD U/min/mg of protein	Catalase nmoles of H$_2$O$_2$ utilized/min/mg protein	Reduced Glutathione ng of GSH /mg protein
Normal Control	45±3.20	10.23±2.10	7.32±1.21	305.3±5.40	30.2±2.1
Negative control	15.8±1.20#	231.37±5.23#	2.85±0.25#	121.1±3.30#	15.3±1.3
Standard	30.5±2.50[c]	27.4±2.50[c]	6.24±1.36[c]	323.3±5.20[c]	45.7±5.2[c]
CE	33.4±2.70[c]	31±1.50[c]	4.35±1.23	251.5±4.50[c]	31.75±2.8[a]
MF	40.5±2.40[c]	35.2±2.30[c]	5.02±1.52[a]	201.5±6.3[c]	36.5±3.8[b]
EF	45.91±2.30[c]	26.33±2.55[c]	6.01±1.08[c]	383.3±6.8[c]	41±6.3[c]
BF	32.5±1.50[c]	55.2±3.45[c]	5.84±0.58[c]	191.1±4.5[c]	12.04±2.3
PAF	15.35±1.20[c]	79.53±3.85[c]	3.32±0.75	188.0±4.9[c]	18.20±1.2

P value: Significantly different from # normal group; [a]*P*<0.05, [b]*P*<0.01, [c] *P*<0.001 from control group.
All the data was evaluated by one way ANOVA followed by Tukey's multiple comparison test. Differences were considered to be statistically significant if *P*<0.05.

indicator of the liver protective activity. The histological evidence of CCl$_4$ produced by an experimental liver damage resembles that of with viral hepatitis. In the present investigation, CCl$_4$ treated rats developed significant hepatic damage, which was observed in serum through a substantial increment in the concentration of serum marker enzymes viz. SGOT, SGPT, ALP, total bilirubin and direct bilirubin levels, also confirmed by measuring various oxidant and antioxidant enzymes like MDA, SOD, catalase and reduced glutathione in liver homogenates. The above results were indicating that EF (150 mg/kg) and MF (300 mg/kg) were most effective fractions of roots of *A. racemosus* in antihepatotoxic as well as antioxidant activities revealed the same conclusion.

Histological examination of the liver of animals of control group, which were exposed to CCl$_4$ showed necrotic lesions and extensive vacuolisation of cytoplasm when compared with normal (Fig. 2 B).

Liver of animals treated with EF (150 mg/kg, po, 7 days) with CCl$_4$ were almost similar to normal in histology, size and staining properties; no vacuolisation was seen and smooth nuclei with nucleoli were clearly visible as in the normal cells (Fig. 2 F). In the liver of animals treated with CE and MF (300 mg/kg, po, 7 days), cytoplasmic vacuolization was significantly reduced (Fig. 2D and E), while in animals treated with BF and PAF the protection was insignificant in comparison to other sub fractions. (Fig. 2 G and H) Results were also well comparable with that of standard drug silymarin (50 mg/kg, po, 7 days) (Fig. 2 C).

As EF is the sub fraction isolated from MF and MF is the sub fraction separated from CE, this gives clear conclusion that the free radical scavenging activity as well as *in vivo* hepatoprotective activity of the roots of *A. racemosus* is attributed due to the constituents, which are present in the fraction EF. The phytochemical screening have clearly indicated that
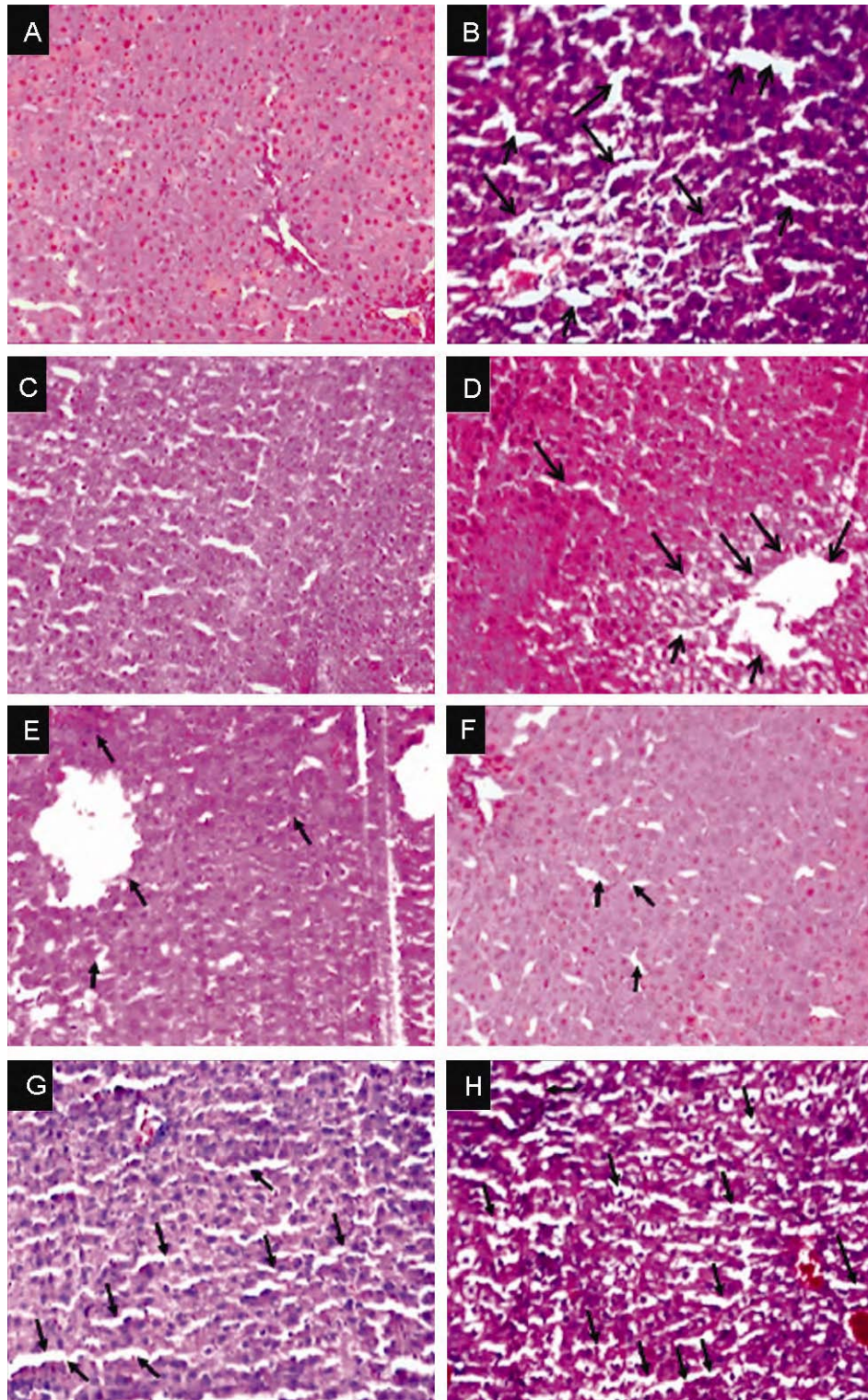
Fig. 2— Effect of roots of *A. racemosus* extracts on histopathological damages induced by CCl$_4$ in rats (H & E staining; original magnification, 40 X) (A) Normal control without any treatment showing normal liver architecture; (B) Negative control receiving CMC showing necrotic lesions and vacuolization of cytoplasm with the loss of cellular boundaries; (C) Standard i.e. silymarin treated animals showing well brought out hepatic cell with well preserved cytoplasm and cellular boundaries; (D) CE & (E) MF treated animals showing reduction of cytoplasmic vacuolization; (F) EF treated animals showing regeneration of hepatocytes, normal hepatic cells and no signs of necrosis; (G) BF & (H) PAF treated animals showing insignificant effect compared to other sub fractions

EF is rich in flavonoids (Table 1). From the above study we can conclude that flavonoids of roots of *A. racemosus* are responsible for the hepatoprotective activities which on further screening may give possibility of novel molecules for the treatment of hepatitis.

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