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IN-VITRO ANTIOXIDANT AND *IN-VIVO* HEPATOPROTECTIVE ACTIVITY OF *GANODERMA LUCIDIUM* STRAINS DARL-4 & MS-1 AGAINST PARACETAMOL INDUCED HEPATOTOXICITY

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ABSTRACT: The purpose of current research work is to evaluate the *in-vitro* antioxidant and *in-vivo* hepatoprotective activity of *Ganoderma lucidum* strains DARL-4 and MS-1. Antioxidant activity of different extracts was evaluated by using DPPH and ABTS method. Hepatoprotective activity of hydroalcoholic extract of *G. lucidum* strain DARL-4 and MS-1 were evaluated by paracetamol-induced liver damage in rats. The hydroalcoholic extract of DARL-4 and MS-1 were orally administered to the animals at a dose of 300 mg/kg once daily for 14 days. On the 14th day the paracetamol (2 g/kg) was also orally administered. The degree of protection was measured by estimating the serum biochemical parameters. The result showed that the hydroalcoholic extract of DARL-4 and MS-1 showed potent antioxidant activity than the ethanolic, methanolic and aqueous extract. The serum biochemical parameters which were elevated due to paracetamol toxicity were significantly reduced. The histopathological studies also supported that extracts markedly reduced toxicity of paracetamol. The hydroalcoholic extract of MS-1 showed a more potent hepatoprotective effect than that of DARL-4. The present study revealed that *in-vitro* cultivated *G. lucidum* strains DARL-4 and MS-1 have significant free radical scavenging potential and liver protection property.

INTRODUCTION: Free radicals are highly reactive molecules that damage lipids, proteins, and DNA, which may result in major complications such as carcinogenesis, mutagenesis, aging and atherosclerosis [1], cataracts, liver diseases, renal failure, cardiovascular disease, diabetes mellitus and brain dysfunction [2, 3, 4].

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Antioxidants are substances that directly scavenge ROS or indirectly up-regulate antioxidant defense system [5]. Liver cells have the antioxidant defence system which posses antioxidants like glutathione, vitamin E, and ascorbic acid and antioxidant enzymes like catalase, superoxide dismutase act against oxidative stress, which protect from the destruction of cell components and cell death [6].

The liver has been known as a central place to detoxify and metabolize different drugs and xenobiotics [7]. The excessive or inaccurate use of drugs leads to liver dysfunction [8]. Hepatotoxicity is defined as an injury or damage to the liver that is associated with an impaired liver functions caused

by continuously and wide exposure to hepatotoxin, xenobiotics, excess alcohol, and chemotherapeutic agents [9]. It can eventually result in liver diseases such as hepatitis, cirrhosis, alcoholic liver disease. Today, liver diseases are one of the most fatal diseases in the world. Oxidative stress has been involved in the pathogenesis of various liver diseases such as alcoholic liver disease, chronic hepatitis C and non-alcoholic fatty liver disease [10].

Ganoderma lucidum commonly known as Reishi, is a lignicolous high-value medicinal mushroom belonging to family Ganodermataceae. *G. lucidum* is the oldest medicinal mushroom, which is used for more than 2,000 years in Traditional Chinese Medicine (TCM). *G. lucidum* used in the treatment of several types of ailments including bronchitis, hepatitis, lupus erythematus, hypertension, asthma, hypercholesterolemia, CV diseases, migraine, nephritis, gastritis, dysmenorrhoea, arthritis and cancer [11]. The fruiting body, mycelia, and spores of *G. lucidum* consist different 400 bioactive constituents including triterpenoids, proteins, polysaccharides, sterols, fatty acids, steroids, and trace elements. Ganoderma contains triterpenoids as main bioactive constituents that could serve as a potent hepatoprotective agent in the food supplement [12]. Wild *G. lucidum* is less abundant in nature so its *in-vitro* cultivation was developed for its easy availability for pharmaceutical development. DARL-4 is an indigenous strain and MS-1 is an exotic Malayasian strain which was *in-vitro* cultivated under sterile condition. The present study was conducted to evaluate the *in-vitro* antioxidant and *in-vivo* hepatoprotective activity of *Ganoderma lucidum* strains DARL-4 & MS-1.

MATERIALS AND METHODS:

Preparation of Plant Extract: *In-vitro* cultivated sample of *Ganoderma lucidum* strains DARL-4 and MS-1 were collected from the polyhouse of DIBER, DRDO. The strains were authenticated by the Mycology department, DIBER, DRDO. Samples were then air-dried and grinded into powdered form.

The powdered sample of DARL-4 and MS-1 were extracted with hydroalcohol (50:50), methanol, ethanol and aqueous by the cold maceration process. All the extracts were filtered with the help of the Whatman no. 1 filter paper. After repeated extraction three times, the filtered extract was combined and finally evaporated to dryness. The extracts were finally lyophilized and stored in a desiccator.

Chemicals: DPPH and ABTS were procured from Sigma. Paracetamol was obtained from Cipla. Standard drug Silymarin was obtained from Admac. All reagents used were of analytical grade. Standard kits for SGOT, SGPT, ALP, bilirubin, total protein, albumin, and globulin were obtained from Siemens Ltd., India.

Animals: Male Wistar rats (180-250 gm) were used for the study. The animals were maintained at a temperature 23-25 °C with a 12 h light-dark cycle and at humidity 45-50%. They were provided feed (Ashirwad Industries, Mohali) and water during the study. Ethical clearance for performing experiments on animals was obtained from Institutional Animal Ethics Committee (1306/C/09/CPCSEA/ IAEC/12 dt 13/2/15) prior to the initiation of the experiment and the care of the laboratory animals was taken as per the CPCSEA regulations.

Antioxidant Activity:

DPPH Radical-Scavenging Activity: The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of free radical-scavenging activity of the different extract. Different aliquots of each extract (hydroalcoholic, methanolic, ethanolic and aqueous) were taken in test tubes and volume was made up to 1 ml with methanol. Then 2 ml methanolic solution of DPPH (0.1 mM) was added in each test tubes. After 30 min at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid was used as standard [13]. Percentage inhibition was calculated as

$$\% \text{ radical scavenging activity} = Abs_{control} - Abs_{sample} / Abs_{control} \times 100$$

Where,

Abs control is the absorbance of DPPH radical with methanol;

Abs sample is the absorbance of DPPH radical with sample extract/standard.

ABTS Radical-Scavenging Activity: Free radical scavenging ability of *G. lucidum* was also determined by the of 2,2'-azinobis [3-

ethylbenzthiazoline] -6-sulfonate (ABTS) assay. 7.0 mM ABTS solution in methanol and 2.45 mM potassium persulfate solution in water were prepared as separate stock solutions in equal quantities and allowed them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1.0 ml of ABTS solution and appropriate volume of methanol to obtain the absorbance 0.702 ± 0.001 units at 734 nm. Different aliquots of each extract (hydroalcoholic, methanolic, ethanolic and aqueous) were taken and volume was made up to 1 ml with methanol. Then 1 ml ABTS solution was added, and absorbance was taken at 734 nm after 10 min reaction time.

The ABTS scavenging capacity of the extracts was compared with ascorbic acid. Ascorbic acid was used as the standard [13].

% radical scavenging activity = $Abs_{control} - Abs_{sample} / Abs_{control} \times 100$

Where,
Abs control is the absorbance of ABTS radical with methanol.
Abs sample is the absorbance of ABTS radical with sample extract/standard.

Animal Study:

Acute Toxicity Study: The hydroalcoholic extract of DARL-4 and MS-1 were administered at different dosages 30, 125, 500 and 2000 mg/kg body weight orally to different groups of rats for acute toxicity. Acute toxicity study was carried out according to the OECD Guidelines and animals were observed for 14 days for any toxic symptoms.

Paracetamol Induced Hepatotoxicity: This study was undertaken to evaluate the hepatoprotective effect of hydroalcoholic extract of *in-vitro* cultivated DARL-4 and MS-1 in paracetamol-induced hepatotoxicity. The rats were divided into Group I (normal control), Group II (Toxin control), Group III (DARL-4), Group IV (MS-1) and Group V(Std Silymarin). Each group contains five rats. Group I (Control) served as normal and received the vehicle alone orally (Sterile distilled water) for 14 days. Group II (Toxin control) animals received paracetamol (2 g/kg orally) on the 14th day. Group III and IV were treated with hydroalcoholic extract of DARL-4 and MS-1 at a dose of 300 mg/kg body weight orally per day respectively for 14 days and on the 14th day paracetamol (2 g/kg orally) was given 1 h after the treatment of the extract. Group V (Standard) was treated with standard drug silymarin (25 mg/kg orally) for 14 days and on the 14th day paracetamol (2 g/kg orally) was given 1 h after the treatment of the drug. After 48 h of dose of paracetamol the animals were weighed and anaesthetized with ketamine i.m.

The blood was withdrawn by retro-orbital puncture, collected and allowed to stand for 30 min at 37 °C. Then the serum was separated from blood with the help of centrifugation for further estimation of serum biochemical parameter. The animals were sacrificed under mild ether anaesthesia. Livers from all animals were removed, washed with ice cold saline and weighed.

Biochemical Parameters Analysis: The biochemical parameters including serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase **(**ALP), serum glutamic oxalacetic transaminase (SGOT), total bilirubin (TB), total protein, albumin and globulin were analyzed using RA-50 Bayer chemistry analyzer.

Histopathological Studies: The liver tissue was collected & transferred in 10% buffered formalin solution for fixation. The formalin-fixed liver samples were stained for photomicroscopic observation. The histopathology examination was done from Department of Veterinary Pathology, G.B Pant University of Agriculture and Technology, Pantnagar.

Statistical Analysis: In an animal study, the data were expressed as mean ± SD. For statistical analysis data were subjected to analysis of variance (ANOVA) by using Graph pad instat. Values are considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION:

DPPH Scavenging Assay: The results indicate that hydroalcoholic extract of DARL-4 and MS-1 exhibited higher potency than the other extracts. All the data were significant statistically, and means differed significantly with each other. The antioxidant effect of DARL-4 and MS-1 against DPPH were observed in the following sequence: hydroalcoholic > methanolic > aqueous > ethanolic as shown in **Fig. 1** & **2**. The data were shown in **Table 1**.

ABTS Scavenging Assay: In ABTS assay, IC_{50} values for hydroalcoholic extract in MS-1 and DARL-4 were observed to be 0.96 ± 0.013 and 0.99 ± 0.014 mg/ml, while methanolic extract exhibited 1.07 ± 0.032 in MS-1 and 1.10 ± 0.021 mg/ml in DARL-4 respectively. In ethanolic extract, IC_{50} values showed 1.18 ± 0.030 in MS-1 and 1.25 ± 0.027 mg/ml in DARL-4, similarly aqueous extract exhibited the same at 1.13 ± 0.027 and 1.15 ± 0.019 mg/ml respectively. The antioxidant effect of DARL-4 and MS-1 against ABTS was found in the following sequence: hydroalcoholic > methanolic > aqueous > ethanolic, as shown in **Fig. 3** and **4**. The data were shown in **Table 2**.

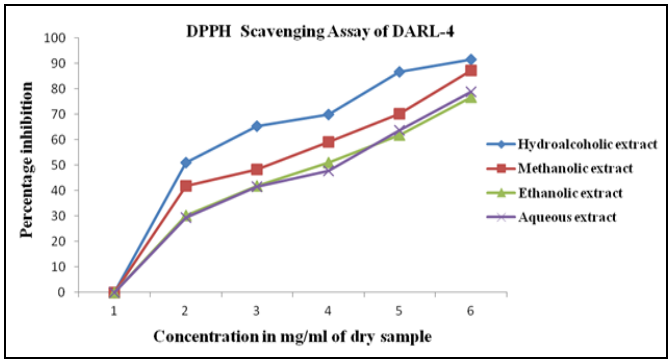

FIG. 1: DPPH SCAVENGING ASSAY OF DARL-4

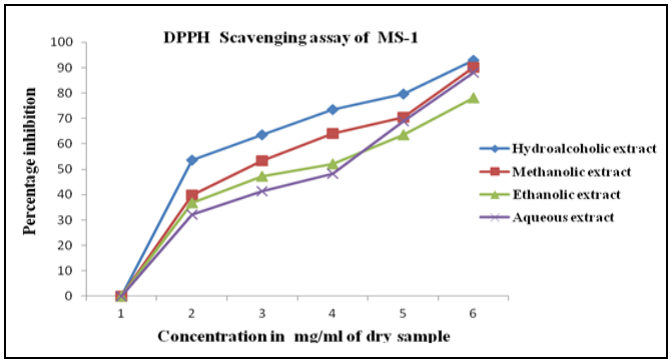

FIG. 2: DPPH SCAVENGING ASSAY OF MS-1

TABLE 1: IC_{50} VALUES OF DARL-4 AND MS-1 IN mg/ml AGAINST *IN-VITRO* DPPH RADICAL SYSTEM

S. no.	Extracts	DARL-4	MS-1
1	Hydroalcoholic extract	0.737 ± 0.01 mg/ml	0.733 ± 0.01 mg/ml
2	Methanolic extract	0.97 ± 0.01 mg/ml	0.92 ± 0.03 mg/ml
3	Ethanolic extract	1.18 ± 0.02 mg/ml	1.11 ± 0.03 mg/ml
4	Aqueous extract	1.17 ± 0.04 mg/ml	1.08 ± 0.02 mg/ml

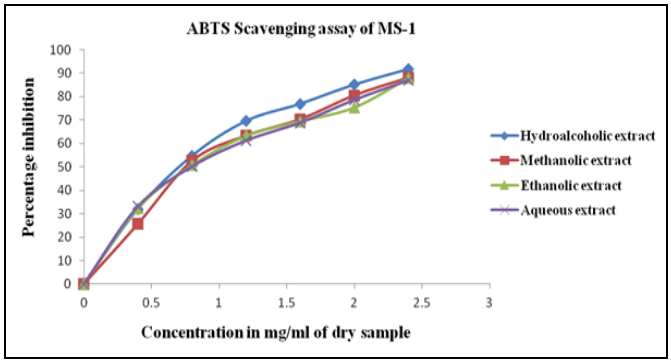

FIG. 3: ABTS SCAVENGING ASSAY OF MS-1

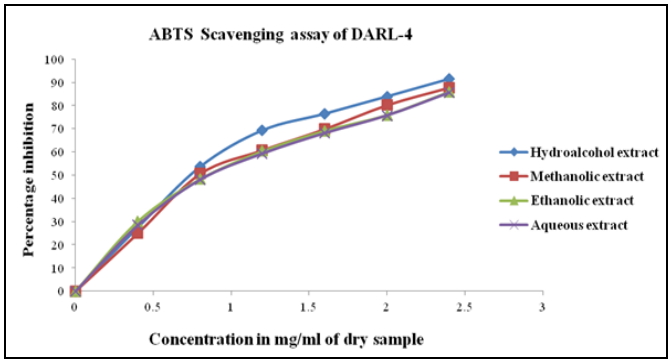

FIG. 4: ABTS SCAVENGING ASSAY OF DARL-4

TABLE 2: IC_{50} VALUES OF DARL-4 AND MS-1 IN mg/ml AGAINST *IN-VITRO* ABTS RADICAL SYSTEM

S. no.	Extracts	DARL-4	MS-1
1	Hydroalcoholic extract	0.99 ± 0.014 mg/ml	0.96 ± 0.013 mg/ml
2	Methanolic extract	1.10 ± 0.021 mg/ml	1.07 ± 0.032 mg/ml
3	Ethanolic extract	1.25 ± 0.027 mg/ml	1.18 ± 0.030 mg/ml
4	Aqueous extract	1.15 ± 0.019 mg/ml	1.13 ± 0.027 mg/ml

TABLE 3: IC_{50} VALUE OF STANDARD ANTIOXIDANT IN µg/ml AGAINST *IN-VITRO* DPPH AND ABTS RADICAL SYSTEM

Standard	DPPH	ABTS
Ascorbic acid	8.52 ± 0.014 µg/ml	6.3 ± 0.034 µg/ml

Acute Toxicity (LD_{50}) Studies: Acute toxicity studies were carried out according to OECD guidelines. No mortality was observed at 30, 125, 500 and 2000 mg/kg body weight. Therefore 300 mg/kg dose was taken as an effective dose for *in-vivo* study.

***In-vivo* Hepatoprotective Activity:**

Body Weight: The body weight in the control and pcm induced rats were shown in **Fig. 5**. The body weight was reduced in the PCM treated rats as

compared to control rats. Treatment with hydroalcoholic extract of DARL-4, MS-1 (300 mg/kg orally) and silymarin (25 mg/kg orally) to pcm induced rats caused an increase in the body weight during the experimental period. The hydroalcoholic extract of MS-1 showed more increase in body weight in rats than DARL-4.

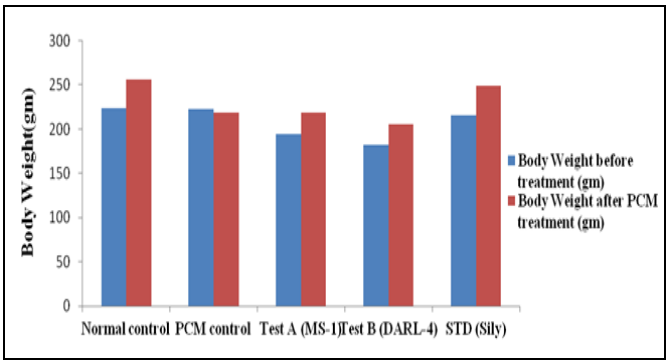

FIG. 5: EFFECTS OF HYDROALCOHOLIC EXTRACT OF DARL-4 AND MS-1 ON THE BODY WEIGHT (BEFORE AND AFTER TREATMENT) IN PCM INDUCED HEPATOTOXICITY IN RATS

Feed Intake: The effect produced by the administration of hydroalcoholic extract of DARL-4 and MS-1 on feed intake of rats were shown in **Fig. 6**.

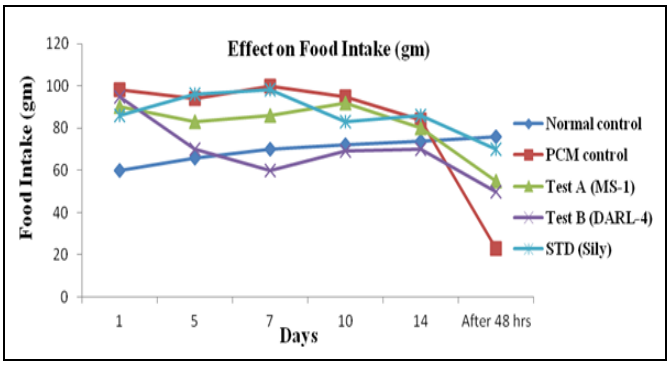

FIG. 6: CHANGES IN FEED INTAKE (g) OF RATS DURING EXPERIMENTAL PERIOD

After paracetamol induction, the feed intake in rats was significantly reduced in Group II (toxin control), Group III (DARL-4), Group IV (MS-1) and Group V (Silymarin) in comparison with Group I (normal control). After PCM induction the feed intake was reduced more in DARL-4 treated rats as compared to MS-1 treated rats.

Liver Weight: The liver wt in the toxin control group was increased significantly ($p<0.01$) in comparison with control group. Pretreatment with hydro-alcoholic extracts (DARL-4, MS-1) and silymarin showed significantly reduced in liver weight as compared to PCM control rats were shown in **Fig. 7**.

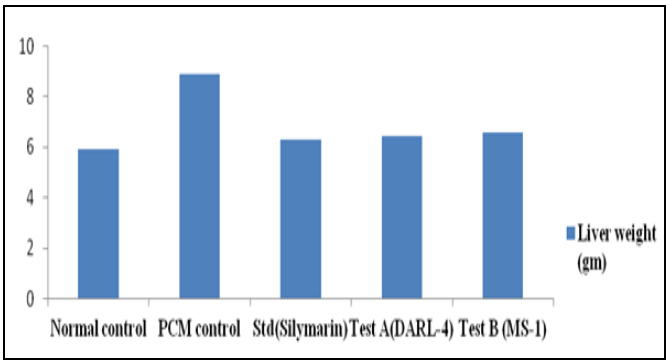

FIG. 7: EFFECT OF DARL-4 AND MS-1 ON LIVER WEIGHT

Estimation of Biochemical Parameters: The effect of hydroalcoholic extract of DARL-4 and MS-1 on different biochemical parameters in paracetamol-induced hepatotoxicity in rats was observed as shown in **Table 4** and **5**. The seum levels of SGOT, SGPT and ALP were significantly increased ($p<0.05$, $p<0.01$) in pcm treated group in comparison with control group. Total bilirubin and direct bilirubin were also increased ($p<0.01$) in PCM treated rats as compared to control rats. The level of total protein, albumin and globulin were reduced significantly ($p<0.01$) in the toxin group as compared with the normal control group.

TABLE 4: EFFECT OF DARL-4 AND MS-1 ON SGOT, SGPT, ALP, TB AND DB IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

Treatment Group	SGPT (IU/L)	SGOT (IU/L)	ALP (IU/L)	Total Bilirubin (mg/dl)	Direct Bilirubin (mg/dl)
Control	46.58 ± 1.06	74.42 ± 1.38	105.8 ± 5.51	0.37 ± 0.01	0.12 ± .005
PCM (2g/kg)	229.9 ± 4.70$^{\#\#}$	378.2 ± 7.14$^{\#}$	398.5 ± 1.89$^{\#\#}$	2.02 ± 0.05$^{\#\#}$	0.66 ± 0.01$^{\#\#}$
MS-1 (300mg/kg)	71.18 ± 1.18*	106.8 ± 2.24*	165.3 ± 1.29*	0.49 ± 0.02*	0.27 ± .007*
DARL-4 (300mg/kg)	91.86 ± 0.92*	116.7 ± 0.65*	173.3 ± 5.60*	0.53 ± 0.01*	0.30 ± .006*
Silymarin (25mg/kg)	58.18 ± 4.31*	92.94 ± 2.65*	124.4 ± 0.08*	0.47 ± 0.02*	0.19 ± .008*

Statistical significance was determined by one way ANOVA followed by Dunnett test. Values are expressed as mean ± SEM, n=5 $^{\#}p<0.05$, $^{\#\#}p<0.01$ as compared with normal control group;*$p<0.01$ as compared with toxin control group

Pre-treatment with hydroalcoholic extract of DARL-4, MS-1, and silymarin (std) significantly decreased ($p<0.01$) the level of elevated serum enzymes such as SGOT, SGPT, ALP, total bilirubin and direct bilirubin as well as increased the level of total protein, albumin, and globulin when compared with toxin control group.

The results indicate that pretreatment with DARL-4, MS-1, and silymarin significantly prevented the biochemical changes induced by paracetamol. MS-1 showed good hepatoprotective effect by normalizing the elevated serum enzyme levels in pcm induced liver damage in rats than that of DARL-4.

TABLE 5: EFFECT OF DARL-4 AND MS-1 ON TOTAL PROTEIN, ALBUMIN AND GLOBULIN IN PARACETAMOL INDUCED HEPATOTOXICITY IN RATS

Treatment group	Total Protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control	8.14 ± 0.05	4.67 ± 0.03	3.47 ± 0.01
PCM (2g/kg)	5.40 ± 0.10#	3.15 ± 0.01#	2.25 ± 0.08#
MS-1 (300mg/kg)	7.45 ± 0.06*	4.25 ± .022*	3.19 ± 0.04*
DARL-4 (300mg/kg)	7.01 ± 0.15*	4.16 ± 0.01*	2.84 ± 0.13*
Silymarin (25mg/kg)	7.78 ± 0.01*	4.53 ± 0.01*	3.25 ± .005*

Statistical significance was determined by one way ANOVA which followed by the Dunnett test. Values are expressed as mean ± SEM, n=5 #$p<0.01$ as compared with Group I (normal control) *$p<0.01$ as compared with Group II (toxin control).

Histopathological Examination: Group I (normal control) rats treated with distilled water showed with the normal structure of liver **Fig. 8A** while pcm treated rats showed hepatic degeneration with necrosis, vacuolization, space formation and inflammatory cell **Fig. 8B**. The hydroalcoholic extract of DARL-4, MS-1 (300mg/kg) and silymarin (25mg/kg) indicate a sign of protection by preventing hepatic necrosis, vacuolar degeneration and inflammation **Fig. 8c-e**. Animal treated with the DARL-4 and MS-1 showed improved histology of the liver as compared to that observed in animals treated with pcm (toxin group). The hydroalcoholic extract of MS-1 showed more effective hepatoprotective effect by complete regeneration of hepatic damage **Fig. 8c** as compared to DARL-4.

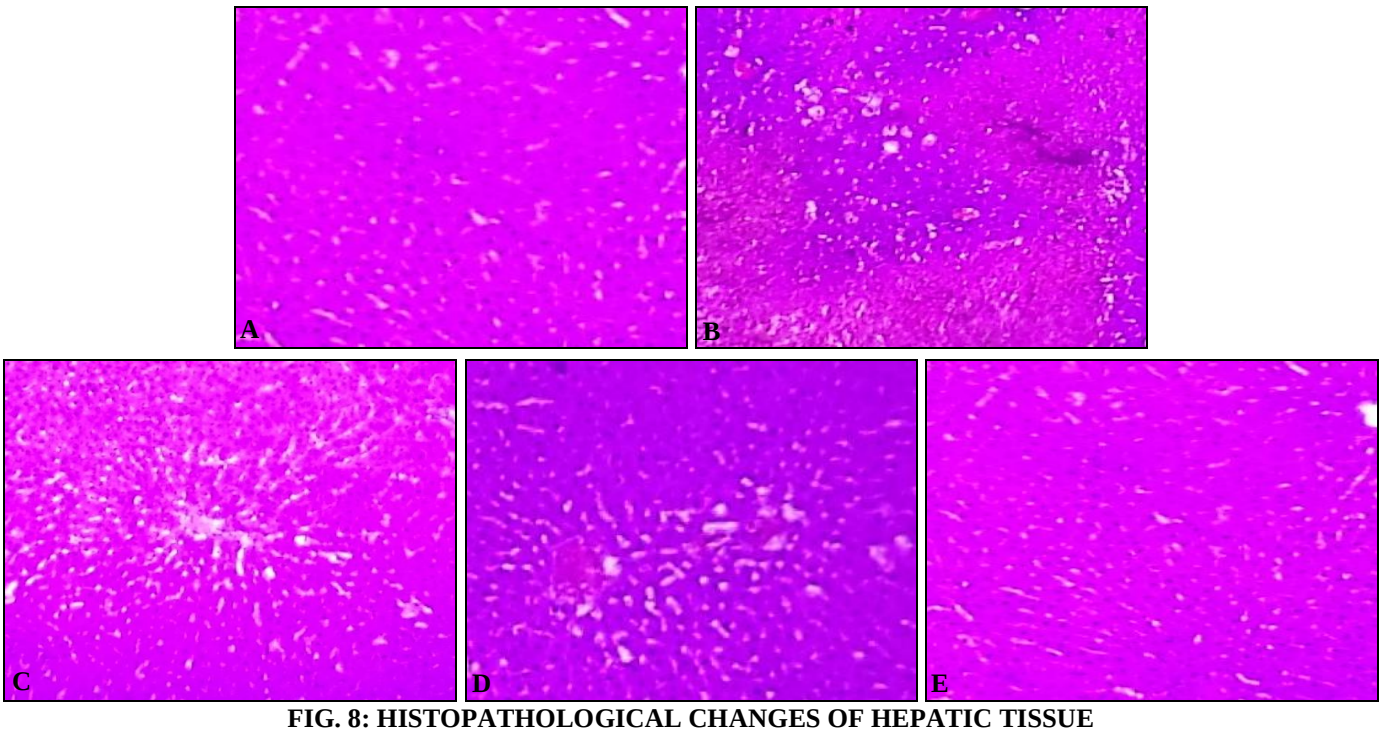

FIG. 8: HISTOPATHOLOGICAL CHANGES OF HEPATIC TISSUE

A. Control group (administered distilled water daily) showing the normal architecture of hepatic cells.
B. PCM treated group (2g/kg) showing hepatic degeneration with necrosis, vacuolar degeneration, space formation, and inflammatory cells.
C. PCM + MS-1 (300mg/kg) showing complete regeneration and hepatocyte were almost normal.
D. PCM + DARL-4 (300mg/kg) showing mild degeneration of hepatocyte, lesser inflammatory cell, and vacuolization.
E. PCM + Silymarin (25mg/kg) showing complete regeneration of hepatocytes.

CONCLUSION: In the present study, antioxidant activity of different extract of DARL-4 and MS-1 were evaluated by using DPPH and ABTS method. The hydroalcoholic extract of DARL-4 and MS-1 showed potent antioxidant activity than the ethanolic, methanolic and aqueous extract in both DPPH and ABTS method. *G. lucidum* enriched with triterpenoids and polysaccharide that influence hepatoprotective efficacy via attenuating over-production of free radicals and thereby protecting the cells from damage [14].

In the hepatoprotective study, the paracetamol toxin group increases serum biochemical parameters such as SGOT, SGPT, ALP, and BUN whereas decreases the level of total protein, albumin, and globulin. Liver weight was also increased. The pre-treatment with hydroalcoholic extract of DARL-4 and MS-1 (300mg/kg) normalizes the elevated level of SGOT, SGPT, ALP, and BUN. The effects of extracts were comparable with standard drug silymarin (25 mg/kg). The level of total protein, albumin and globulin increases in the animals pre-treated with DARL-4 and MS-1 extract. The histopathological examination indicates reduction of liver damage in rats treated with DARL-4 and MS-1. MS-1 showed more effective hepatoprotective activity as compared to DARL-4. This study showed that *in-vitro* cultivated *Ganoderma lucidum* strains DARL-4 and MS-1 have good antioxidant and hepatoprotective activity.

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CONFLICTS OF INTEREST: We declare that we have no conflicts of interest.

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