ISSN: 2322 - 0902 (P)
ISSN: 2322 - 0910 (O)

International Journal of Ayurveda and Pharma Research

Research Article

ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITY OF *SHILAJIT* (*ASPHALTUM PUNJABINUM*) AGAINST ALCOHOL INDUCED LIVER INJURY IN WISTAR RATS

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ABSTRACT

To investigate the antioxidant, hepatoprotective activity and evaluation of effects on blood factors of *Asphaltum punjabinum* demonstrable in-vivo and in-vitro by the inhibition of alcohol induced Wistar rat. In-vitro antioxidant activity of *Asphaltum punjabinum* was evaluated by various assays, including reducing power, lipid peroxidation, DPPH. Hepatoprotective activity as judged by the blood factors and serum enzymes levels viz. Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), Alkaline Phosphatase (ALP), Total Bilirubin (TBIL) and Direct Bilirubin (DBIL) as well as oxidant enzyme viz. Malon Dialdehyde (MDA) were prevented, while antioxidant enzymes viz. Super Oxide Dismutase (SOD), reduced glutathione (GSH) and catalase were elevated in liver tissues. Further histopathological examination of liver sections was carried out to support the induction of hepatotoxicity and hepatoprotective efficacy. The results showed potent activities on reducing power, lipid peroxide, DPPH, Superoxide anion. The histopathological observations supported the biochemical evidences of hepato protection. Elevated level of SOD and decreased level of MDA further strengthen the hepatoprotective observations. In the study, demonstrating the *Asphaltum punjabinum* has potent antioxidant and hepatoprotective activity against alcohol induced hepatic damage in experimental animals.

KEYWORDS: *Shilajit*, *Asphaltum punjabinum,* Ethyl Alcohol, Anti-oxidant, Hepatoprotective.

INTRODUCTION

The liver demonstrates a major role in metabolism of xenobiotics by regulating the synthesis, secretion and metabolism of xenobiotics. Various physiochemical functions of the body including oxidation, reduction, hydroxylation, hydrolysis, conjugation, sulfation, acetylation etc, are well balanced by the liver alone. Injury to liver and damage to the hepatic parenchyma are always proved to be associated with distortion of different metabolic functions of liver[1,2]. Etiologically various infectious agents including viruses and different hepatotoxic chemicals along with environmental pollutants are thought to be responsible for different type of liver damage and hepatic injury. Recent research in free radical biology also suggested the patho physiological role of free radicals and oxidative stress in liver damage and injury. Revealing the mechanism of actions of potent hepatotoxin such as Alcohol and Paracetamol etc, it also indicated the role of oxidative stress and free radicals in the pathophysiology of hepatic injury.[3] The free radicals normally generated during the normal body metabolic pathways and also they can be acquired from the environment also. Free radicals contain unpaired electrons. Oxidative stress plays an important role in many diseases including liver diseases. The production of oxidative stress can be controlled by the antioxidant systems in living organisms. The oxygen radicals, such as superoxide radical (O^{2-}), hydroxyl radical ($^{\bullet}OH$) and non free radical species, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^{\bullet}O_2$), are generated in many redox processes of normal physiochemical path ways.[4,5] Antioxidant defence system comprising different enzymes such as superoxide dismutase, catalase and glutathione peroxidise etc, trap and destroy these free radicals. Vitamin deficiency together with overproduction of free radicals and a reduced level of above mentioned enzymes is

considered as the main culprit for producing oxidative stress[5].

Research on oxidants and antioxidants over the past few years has shown a link between most diseases like cardiovascular diseases, cancer, osteoporosis, degenerative diseases etc and production of reactive oxygen species (ROS) along with oxidative stress[3,6,7]. Free radicals mainly act by attacking the unsaturated fatty acids in the bio membranes which causes membrane lipid peroxidation (a hallmark sign of hepatotoxicity), decrease in membrane fluidity and reduction of enzyme and receptor activity and damage to membrane protein which finally triggers the cell inactivation and death.[4,5] Therefore, antioxidants can be used to reverse the harmful and pathological action of free radicals. These antioxidants generally restore the normal physiological system by scavenging the free radicals. The antioxidants in use are either derived naturally from plants or synthetically. Due to carcinogenic probability, synthetic antioxidants are not the preferred type of anti oxidants.[5] Current research in the field of free radical biology therefore accentuates the use of antioxidants from natural origin and in view of this more and more antioxidants of natural origin are being investigated.

Alcohol is second of the most common hepato toxin used for experimental induction of liver injury in animal studies. Impoverishment of modern system of medicine in terms of a reliable liver protective drug switched on the exploration of traditional systems of medicine including Ayurveda, Siddha, Unani etc, for a probable answer to hepatotoxicity. Numerous medicinal plants are being researched for an effective hepatoprotective remedy. A number of medicinal preparations in the Indian System of Medicine (Ayurveda) have been used as effective hepatoprotective. In view of this several medicinal preparations and a number of medicinal plants mentioned in Ayurveda for treatment of liver disorders are being investigated *Asphaltum punjabinum* is a multi-component natural occurring mineral substance used in Ayurveda and Siddha systems of medicine which originated in India. Its source can be traced to the mountainous regions, where the hilly tribes first identified its beneficial use. It has been proposed and used indigenously for the treatment of various ailments ranging from genitourinary to immunomodulatory. It is mainly found as exudates in the mountainous regions in India, Russia and other selected parts of the world. It is a multi-component agent among which Fulvic acid and humic acid form the major part. Though it has been traditionally used from ages, little scientific basis for its standardization and therapeutic activities exist. Proper standardization of *Shilajit* forms a prerequisite owing to the great geographic and chemical diversity in the source. Taking this into consideration the present study has been carried out undertaking to establish a scientific base for its supposed anti-oxidant and hepatoprotective activity.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used were of analytical grade from CDH chemicals and enzymatic kits were acquired from labcare diagnostic Pvt. Ltd. 1,1-diphenyl-2-picrylhydrazyl (DPPH), TrisHCl, thio barbituric acid (TBA), CCl4, glutathione (GSH), ascorbic acid Biochemical kits for determining serum-glutamate pyruvate trans aminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), bilirubin content and silymarin were purchased from Sigma Aldrich (St. Louis, USA).

Samples

Test samples were collected from Amritsar, Punjab in the month of August, 2012. Raw *Shilajit* (RS), Market Processed *Shilajit* (MPS), Laboratory Processed *Shilajit* (LPS) and Processed *Shilajit* (PS). Laboratory Processed *Shilajit*, are prepared from Market Processed *Shilajit*.

Purification of *Asphaltum punjabinum*

Shilajit (10g) was triturated with water and the water insoluble materials were removed by filtration. The aqueous solution was evaporated under reduced pressure (at 37°C), to give a brown viscous residue (7.6g). The residue was exhaustively extracted successively with hot n-hexane, EtOAc and MeOH. The solution was filtered to remove the insoluble humins (HMs) were collected by centrifugation and dried in vacuum (2.1g).[8-9]

Animals

Wistar albino rats (250-300g) were maintained in the animal house of Institute of Pharmacy, Nirma University, Ahmedabad, for experimental purpose. Then all the animals were acclimatized for seven days under standard husbandry conditions, i.e. room temperature of 25 ± 10°C; relative humidity 45-55% and a 12:12h light/ dark cycle. The animals had free access to standard rat pellet, with water supplied ad libitum under strict hygienic conditions. Animals were habituated to laboratory conditions for 48 hours prior to experimental protocol.

All experiments and protocols described in study were approved by the Institutional Animal Ethics Committee (IAEC) of Institute of Pharmacy, Nirma University, Ahmedabad and with permission

from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. Protocol number is IP/PCOG/ MPH/12-1/011.

Preparation of test drug solution

Silymarin 50mg/ Kg/day p.o. with cow milk, Raw Shilajit (RS) with cow milk (300mg/Kg/day), Market Processed Shilajit (MPS) with cow milk (300mg/Kg/day) and Lab Processed Shilajit (LPS) with cow milk (300mg/Kg/day).

DPPH radical scavenging activity

The antioxidant activity of the test samples, based on the scavenging activity of the stable 1, 1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined[10]. 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%.

Alcohol induced hepatotoxicity in Wistar rats

Wistar rats, weighing (250- 300g) were divided into 6 groups consisting of 4 animals in each group. Gr-1: Normal group, animal of this group receive cow milk p.o. for 30 days. Gr-2: Control group, animal of this group received vehicle, cow milk instead of water and alcohol 3ml/ 100g/day p.o. for 30 days. Gr-3: Standard group, animal of this group receive vehicle, cow milk instead of water, Silymarin 50mg/ Kg/day p.o. with cow milk and alcohol 3ml/ 100g/day p.o. for 30 days. Gr-4: Test group, animal of this group receive vehicle, cow milk instead of water, Raw *Shilajit* (RS) with cow milk (300mg/Kg/day) and alcohol 3ml/100 g/day p.o. for 30 days. Gr-5: Test group, animal of this group receive vehicle, cow milk instead of water, Market Processed *Shilajit* (MPS) with cow milk (300mg/Kg/day) and alcohol 3 ml/ 100g/day p.o. for 30days. Gr-6: Test group, animal of this group receive vehicle, cow milk instead of water, Lab Processed *Shilajit* (LPS) with cow milk (300mg/Kg/day) and alcohol 3ml/100 g/day p.o. for 30 days.

The blood samples were withdrawn from retro-orbital plexus under light ether anaesthesia without any anticoagulant and allowed to clot for 10 min at room temperature. It was centrifuged at 2500 rpm for 20 min. The serum was kept at 40°C until used.

Quantitative determination of activity of SGOT, SGPT, ALP, TBIL and DBIL in serum was done using enzymatic kit.

10% liver homogenates were made in ice cold phosphate buffer saline (pH 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 ìm thick) were taken and stained with hematoxylin-eosin (H&E) using standard technique for histopathological assessment.

Iron-induced lipid peroxidation in liver homogenate

The antioxidant activity of the *Asphalatum punjabinum* was evaluated by quantifying the ability of different solution of samples to suppress iron (Fe2+) induced lipid peroxidation in rat liver homogenates[10-11]. Liver homogenates were prepared from male Wistar rats scheduled to be sacrified 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[12]. The final protein concentration was adjusted to 10mg 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 0.5mL liver homogenate, 0.1mL Tris–HCl buffer (pH 7.2), 0.05mL of 0.1mM ascorbic acid, 0.05mL 4 mM FeCl2 and 0.05mL of various concentrations of crude drug extracts or standard antioxidant, was incubated for 1 h at 37°C. After incubation, 9mL distilled water and 2mL 0.6% TBA were added to 0.5mL of the incubation solution and shaken vigorously. The mixture was heated for 30 min in a boiling water bath. After cooling, 5mL 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.

Statistical analysis

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

RESULTS AND DISCUSSION

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[13-14]. In the present study, measured decrease in absorbance was read to calculate the percentage scavenging of free radical in presence of different samples of Shilajit and standard. In order to

quantify the antioxidant activity, the EC50, which is the concentration of sample required to decrease the absorbance of specific free radical (DPPH) at specific lmax by 50%, was calculated. The lower the EC50 value, the greater the free radical-scavenging activity of the samples. The *Shilajit* samples were found to scavenge the free radical generated from methanolic solution of DPPH. The percentage protection were found to be 7.98, 20.34, 28.67, 48.23, 61.83 and 66.56 and it is shown in table 1. The DPPH activity of RS, MPS and LPS were found to be 37.06, 41.06 and 48.23% respectively. From the table it's showed that the sample LPS have more % inhibition (48.23±0.85).

The method is based on the reduction of methanolic DPPH solution in presence of hydrogen donating anti oxidant, due to the formation of the non-radical form DPPH-H by the reaction. The samples was able to reduce the stable radical DPPH to the yellow-coloured diphenyl picryl hydrazine. Cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g. hydroquinone, pyrogallol, gallic acid), and aromaticamines (e.g. p-phenylene diamine, p-aminophenol), reduce and decolourise 1, 1-diphenyl-2-picrylhydrazyl bytheir hydrogen donating ability[15]. It appears that the LPS possesses maximum hydrogen donating capabilities andacts as an antioxidant at lower concentration (i.e. EC50= 48.23±0.85 µg/mL).

Table 1: Effect of different samples of *Asphaltum punjabinum* on DPPH scavenging activity

DPPH Conc. (Ug/ml)	% Protection			
	Ascorbic Acid	MPS	RS	LPS
2	12.76±0.35	5.30±0.97	3.48±0.78	7.98±0.12
4	28.81±1.42	17.70±0.69	18.14±0.75	20.34±0.63
8	33.18±1.56	28.67±0.23	24.18±0.96	27.43±0.56
12	51.66±4.78	41.06±0.25	37.08±1.23	48.23±0.85
16	69.45±0.75	57.67±0.29	49.91±0.29	61.83±0.75
20	71.01±0.8	61.11±0.85	53.43±0.74	66.56±0.95

Alcohol induced hepatotoxicity in rats

Alcohol induced hepatic injury which was indicated by the great increased in SGPT, SGOT, ALP, total and direct bilirubin levels in disease control animals shown table 2, when compared to normal group of animals and these increase in levels were significantly decreased in all above serum enzymes by pre-treatment with STD, MPS, RS and LPS when compared to disease control group [with cow milk (300 mg/Kg/day) and alcohol 3 ml/100 g/day p.o.].

Table 2: Effect of different samples of *Asphaltum punjabinum* of on serum marker enzymes in alcohol induced hepatotoxicity [Values are mean ± SE from 4 animals in each group]

Groups/ Parameters	Normal Control (NC)	Disease Control (DC)	Silymarin Treated (STD)	Market processed Shilajit (MPS)	Raw Shilajit (RS)	Laboratory processed Shilajit (LPS)
SGOT(IU/L)	207.48 ± 2.04	323.02 ± 0.37#	233.96 ± 0.72*	219.45± 0.99*	220.43 ± 0.56*	172.78± 1.29*@
SGPT(IU/L)	37.16 ± 2.05	101.46 ± 0.22#	37.70 ± 0.49*	45.21 ± 0.47*	48.27 ± 0.47*	39.52 ± 1.18*
ALP(KA/dl)	8.84 ± 0.49	80.74 ± 1.72#	26.91 ± 0.84*#	41.23 ± 0.51*#	39.46 ± 0.73*#	14.14 ± 2.28*
Total Bilirubin (IU/L)	0.23 ± 0.63#	1.94 ± 0.07*	0.41 ± 0.39*	0.24 ± 0.55*	0.34 ± 0.44*	0.22±0.22
Direct Bilirubin (IU/L)	0.07 ± 0.89	0.60 ± 0.53#	0.17 ± 0.72*	0.18 ± 0.97*	0.19 ± 0.59*	0.125 ± 0.66*
Total Protein (mg/ml)	40.63 ± 0.62	16.205 ± 0.07#	35.525 ± 0.38*	31.1975± 0.44*#	31.7175 ± 0.55*#	38.74 ± 0.22*
Sugar (mg/dl)	77.23 ± 0.62	80.06 ± 0.16	77.65 ± 0.25	74.03 ± 0.65	75.0 ± 0.10	72.16 ± 0.12

Urea (mg/dl)	25.06 ± 0.29	34.06 ± 0.72	29.06 ± 0.12	24.02 ± 0.52	24.52 ± 0.60	23.01 ± 0.16
Creatinine (mg/ml)	1.02 ± 0.70	1.95 ± 0.50	1.01 ± 0.75	1.0 ± 0.95	1.01 ± 0.62	0.90 ± 0.36
Uric Acid (mg/dl)	4.62 ± 0.25	6.09 ± 0.69	4.60 ± 0.36	3.69 ± 0.26	3.70 ± 0.30	3.50 ± 0.16
Albumin (g/dl)	3.96 ± 0.20	3.02 ± 0.21	3.96 ± 0.62	4.95 ± 0.36	4.09 ± 0.25	4.99 ± 0.34
Cholesterol (mg/dl)	145.90 ± 0.36	160.09 ± 0.26	145.25 ± 0.36	125.06 ± 0.58	125.09 ± 0.36	123.06 ± 0.25
HDL (mg/dl)	41.50 ± 0.21	36.63 ± 0.23	42.62 ± 0.26	44.63 ± 0.39	44.96 ± 0.58	45.65 ± 0.69
LDL (mg/dl)	78.44 ± 0.36	80.26 ± 0.85	78.25 ± 0.85	75.06 ± 0.28	75.96 ± 0.42	74.02 ± 0.02
VLDL (mg/dl)	22.96 ± 0.25	28.52 ± 0.23	22.60 ± 0.13	18.02 ± .01	18.95 ± .09	17.02 ± 63

Above conclusion was also further confirmed by measuring the effect of the different samples of *Shilajit* on lipid peroxidation, in terms of MDA production, SOD, GSH level and catalase activity in liver homogenates of rats of all groups shown table 3. The disease control group of animals showed significant increase of MDA production and decrease in SOD, GSH and catalase contents in the liver homogenate. The pre-treatment of LPS [with cow milk (300mg/Kg/day) and alcohol 3ml/100 g/day p.o for 30 days] showed maximum activity amongst all active groups (MPS, RS) by significant decrease of MDA production with increase in SOD, GSH and catalase content. Results were also compared with that of standard drug Silymarin (50mg/ Kg/day p.o. with cow milk and alcohol 3ml/100g/day p.o. for 30 days) (Table 4). This fact was also confirmed by histopathological studies.

Alcoholic liver disease (ALD) it was found that cytokine and chemokine levels in blood accompanied the fluctuating levels of blood EtOH, indicating that they are directly influenced by absolute EtOH concentration. During the early phases of ALD in this model, a strong initial Th1 response was observed as revealed by increased levels of cytokine as well as transcription factor mRNAs, followed by a down regulation, whereas response was decreased by EtOH over the entire treatment period of four weeks. We found that supplementation with the antioxidant NAC to ethanol treated animals decreases severity of liver damage and somewhat decreases initial inflammatory response mediated by TNFα. NAC also diminished the ethanol-induced formation of protein adducts of lipid peroxidation products like MDA and HNE. Also, the formation of antibodies against neo-antigens formed by MDA, HNE and HER protein adducts was lowered.

Table 3- Effect of different samples of *Asphaltum punjabinum* on oxidant/antioxidant enzymes in liver homogenate in alcohol induced hepatotoxicity [Values are mean ± SE from 6 animals in each group]

Groups/ Parameters	Normal Control (NC)	Disease Control (DC)	Silymarin Treated (STD)	Market processed Shilajit (MPS)	Raw Shilajit (RS)	Laboratory processed Shilajit (LPS)
Total Protein (mg/ml)	40.63 ± 0.62	16.205 ± 0.07$^{\#}$	35.525 ± 0.38*	31.1975± 0.44$^{*\#}$	31.7175 ± 0.55$^{*\#}$	38.74 ± 0.22*
MDA (nmoles/mg protein)	10.66±1.295	131.3±2.733$^{\#}$	25.65±1.361$^{*\#}$	35.90±0.5008$^{*\#@}$	32.14±1.216$^{*\#}$	30.81±0.7798$^{*\#}$
SOD (U/min/mg of protein)	9.647 ± 1.644	3.718±0.6476$^{\#}$	8.473±1.043*	6.440±0.6430	7.444±0.7955*	8.036±0.8244*
Catalase (nmoles of H_2O_2	319.0±19.53	124.7±5.405$^{\#}$	265.7±3.884$^{\#*}$	197.6±2.403$^{*\#@}$	232.6±3.948$^{*\#}$	232.6±3.948$^{*\#}$

utilized/min/mg/protein						
Reduced Glutathione ng of GSH/mg protein)	29.84±1.065	13.22±1.308[#]	25.79±1.353[*]	19.10±1.026[*#@]	218.1±5.995[*#@]	23.85±1.412[*#]

The histological evidence of alcohol produced by an experimental liver damage resembles that of with viral hepatitis. In the present investigation, alcoholtreated 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. Histological examination of the liver of animals of disease control group, which were exposed to alcohol showed necrotic lesions and extensive vacuolisation of cytoplasm when compared with normal group (Fig. 1).

Photos *Shodhana* of *Shilajatu*

Raw *Shilajatu*

***Shilajatu* coarse powder**

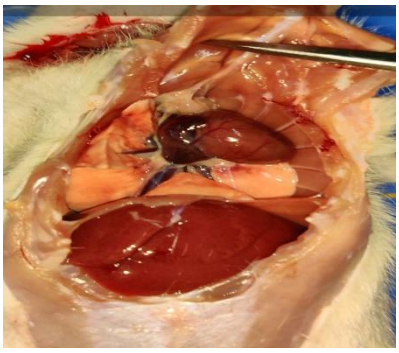
Preparation of *Triphala kashaya*

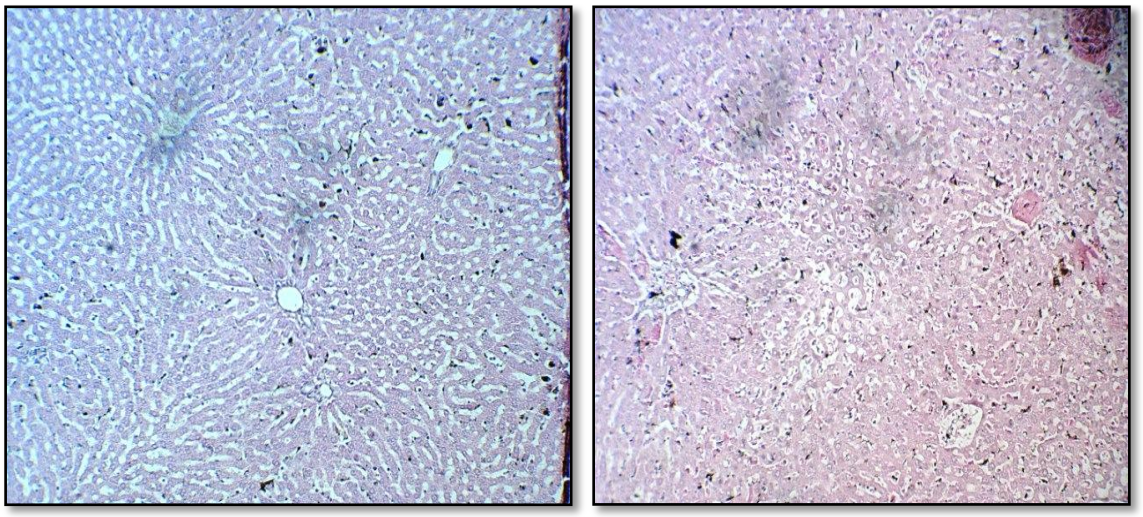
Maceration after 3 hours

Day -1

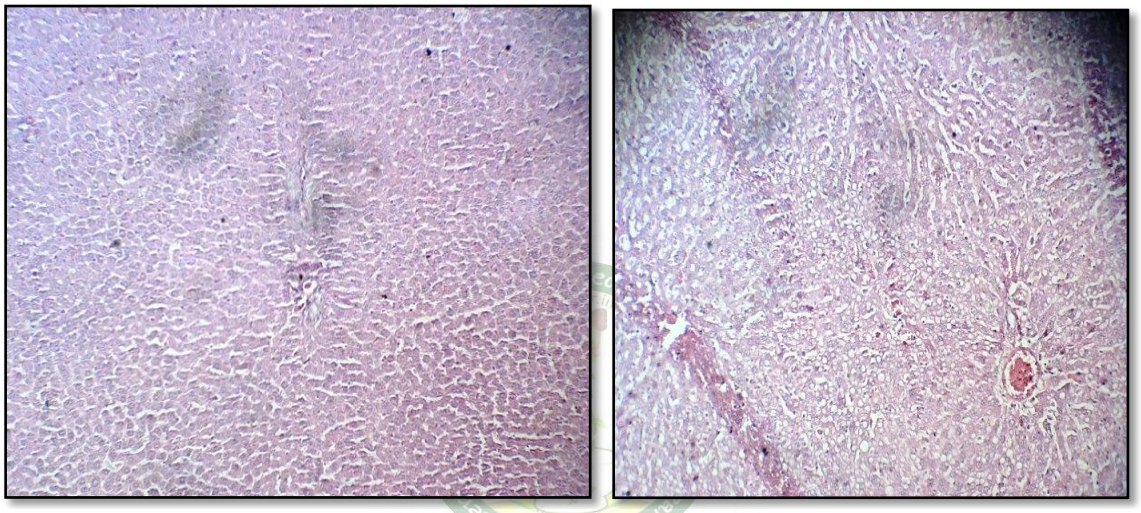
Day- 10

Photos experimental study

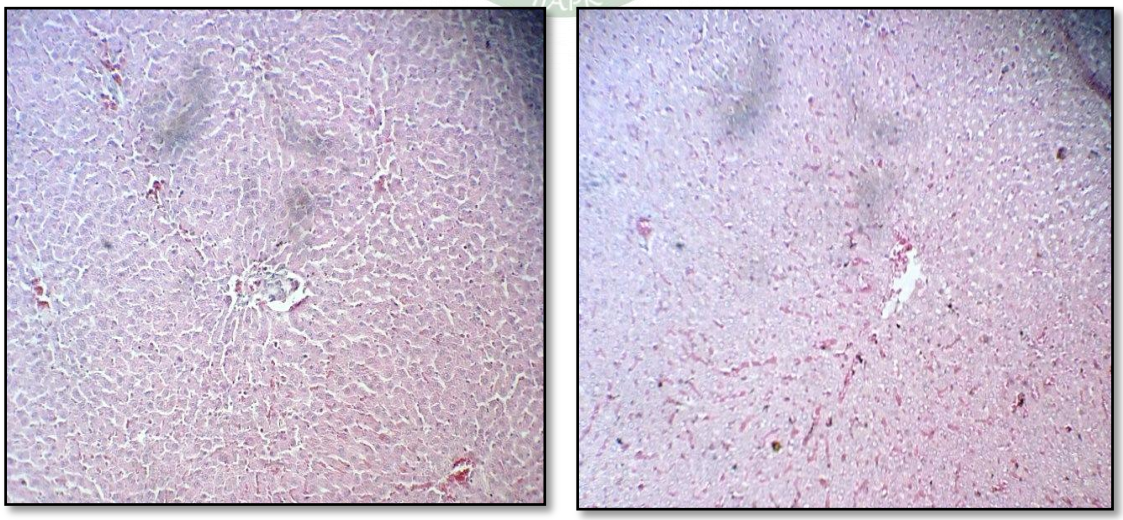




A) Normal Control (NC) B). Disease Control (DC)



C). Standard (STD) D). Market Processed Shilajit (MPS)



E) Raw Shilajit (RS) F) Lab Processed Shilajit (LPS)

Fig. 1- Effects of *Asphaltumpunjabinum* on histopathological dameges induced by alcohol in rats (H & E staining; original magnification, 40X) A) Normal control without any treatment showing normal liver architecture; B) Disease control receiving ethyl alcohol showing necrotic lesions and vacuolization of cytoplasm with 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) Marketed processed *Shilajit* treated animals showing insignificant effect compared to other sub fractions; E) Raw *Shilajit* treated animals reduction of cytoplasmic vacuolization; F) Lab processed *Shilajit*

showing regeneration of hepatocytes, normal hepatic cells and no signs of necrosis.

Liver of animals treated by LPS with cow milk (300mg/Kg/day) and alcohol 3ml/100 g/day p.o. for 30 days were almost similar to NC and STD 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 MPS and RS cytoplasmic vacuolizationwas significantly reduced (Fig. 2D and E), Results were also well comparable with that of standard drug silymarin (Fig. 2 C). 1) From the above study we can conclude that all samples of *Shilajit* MPS, RS and LPS are responsible for the hepatoprotective in which LPS are more significant as antioxidant and hepatoprotective action.

Conclusion

Asphaltum punjabinum has potent antioxidant and hepatoprotective activity against alcohol induced hepatic damage in experimental animals.

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Cite this article as:
Subhash Chand Yadav, Jeyabalan Govindasamy, Reetesh Ramnani. Antioxidant and hepatoprotective activity of Shilajit (Asphaltum punjabinum) against alcohol induced liver injury in Wistar rats. International Journal of Ayurveda and Pharma Research. 2020;8(6):1-8.
Source of support: Nil, Conflict of interest: None Declared

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