"EVALUATION OF SHILAJIT WITH SPECIAL REFERENCE TO ANTIOXIDANT AND HEPATOPROTECTIVE ACTIVITY"

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Dedicated to
my
Grand Father

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Subhash Chand Yadav

ATP	Adenosine Triphosphate
ALP	Serum Alkaline Phosphatase
DIBL	Direct Bilirubin
DPPH	1,1-Diphenyl-2-Picrylhydrazyl
DTNB	5,5 Dithiobis 2- Nitro Benzoic Acid
FAs	Fulvic Acid
GA	Gallic Acid
GPT-ALP	Glutamate-Pyruvic Transaminase
HMs	Humic Acid
HPTLC	High Performance Liquid Chromatography
IR	Infra-Red
LDH	Lactate Dehydrogense
LPS	Lab Processed Shiajit
MPS	Market Processed Shilajit
MDH	Malatedehydrogenase
NT	Neotetrazolium Chloride
PDB	Protein Data Bank
RS	Raw Shilajit
SGPT	Serum glutamate Pyruvate Transaminase Levels
SGOT	Serum Glutamate Oxaloacetate Transaminase
SOT-AST	Aspartate transaminase
TLC	Thin Layer Chromatography
TBA	Thiobarbituric acid
TBIL	Total Bilirubin
UV	Ultra Violet
ρ-PDA-	ρ –phenylenediamine

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Abstract

Shilajit 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 the standardization of Shilajit from various sources and trying to establish a scientific base for its supposed anti-oxidant and hepatoprotective activity.

The Shilajit samples collected from Amritsar, Punjab. Raw Shilajit (RS), Market Processed Shilajit (MPS) and Lab Processed Shilajit (LPS) developed in the lab and Processed Shilajit (PS) purified from RS sample. Shilajit samples were evaluated by TLC/HPTLC chromatography, IR and Raman spectometry. Silymarin was used as standard. Cow milk was used instead water. Alteration in the biochemical markers of hepatic damage like AST, ALT, ALP, TBIL, DBIL and total protein were evaluated along with antioxidant parameters like SOD, MDA, GSH and catalase.

Mechanistic evaluation like DPPH free radical scavenging activity, *Insilico* study of cytochrome p450 metabolism interaction and hepatoprotective activity. Cytochrome p450metabolism activity in liver homogenate was also evaluated.

Fulvic acid (FA) was evaluated as a phytomarker in various Shilajit samples. Alcohol (3 ml/ 100 g/day p.o) increased the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphate (ALP), total bilirubin (TBIL) and direct bilirubin (DBIL). Also increase in various antioxidants levels (Glutathione and Catalase) and decrease in pro-oxidant levels (MDA).

Insilico study on cytochrome P450 activity was show high docking score of fulvic acid compare to Co-Crystalize Ligand also study on hepatoprotective activity was show high

docking score of Fulvic acid (test drug) and high hydrogen bonding, compare to Silymarin (standard).

Among the test group treated by Lab Processed Shilajit (LPS) had better anti-oxidant and hepatoprotective activity. It could be an effective and promising preventing agent against alcohol induced hepatotoxicity.

1.1Introduction

Hepatic disease adversely affects the cells, tissues, structures, or functions of the liver. Liver has a wide range of functions, including detoxification, protein synthesis, and production of biochemical necessary for digestion and synthesis as well as breakdown of small and complex molecules, many of which are necessary for normal vital functions (Anil Kumar. *et al*., 2012).Liver is considered to be one of the most vital organs that functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. Additionally, it is also handling the metabolism and excretion of drugs and other xenobiotic from the body thereby providing protection against foreign substances by detoxifying and eliminating them. Liver cell injury is caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide, chronic alcohol consumption and microbes. Enhanced lipid peroxidation during metabolism of ethanol may result in development of hepatitis leading to cirrhosis (Mohamed Saleem, T.S. *et al.*, 2010). The incredible complexity of liver chemistry and its fundamental role in human physiology is so daunting to researchers that they visualize that perhaps simple plant remedies might have something to offer which is astonishing and incredible.

1.2 Hepatoprotective herbs

Medicinal plants have been used for centuries before the advent of orthodox medicine. Leaves, flowers, stems, roots, seeds, fruits and bark can all be constituents of herbal medicines (Afolabi *et al.*, 2007). The use of plants for medicinal purposes predates human history and forms the origin of modern medicine. Many synthetic drugs originated from plant sources.Herbal-based therapeutics for liver disorders has been in use in India for a long time and has been popularized world over by leading pharmaceuticals (Ajibade, A.J.*et al.*, 2011).Herbal medicine has been shownto have genuine utility, and about 80% of rural populations depend on it as their primary health care. The limiting factors that contribute to this eventuality are (i) lack of standardization of the herbal drugs; (ii) lack of identification of active ingredient(s)/principles(s); (iii) lack of randomized controlled clinical trials (RCTs), and (iv)lack of toxicological evaluation (Radha *et al.*, 2005). The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. Recently, World Health Organization (WHO) defined herbal drugs as

therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today. These practices incorporated ancient beliefs and were passed on from one generation to another by oral tradition and/or guarded literature. Therefore, these plant drugs deserve detailed studies in the light of modern science (Varsha Kashaw*et al*., 2011).The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization and randomized placebo controlled clinical trials to support clinical efficacy (Mohamed Saleem, T.S.*et al.*, 2010).A large number of plants and formulations have been claimed to have hepatoprotective activity.

Hepatotoxicity of primary drugs is a major problem. Mostly drug-induced hepatotoxicity has been thought to be self-limiting event but drug withdrawal is fraught with reactivation of focus as well as risk of developing multi drug resistance. This has led to the idea of providing some hepatoprotective remedies to minimize the hepatotoxicity related dropouts, particularly, with the use of some herbal hepatoprotective (Sharma, Y. K. *et al.,* 2003). Nearly 160 phytoconstituents from 101 plants have been claimedto possess liver protecting activity.In spite of the tremendous advances made, no significant and safe hepatoprotective agents are available in modern therapeutics. Therefore, due importance has been given globally to develop plant-based hepatoprotective drugs effective against a variety of liver disorders.A number of plants have shown hepatoprotective property. Developing therapeutically effective formulation from natural products may reduce the risk of toxicity when the drug is used clinically and can give the benefit of synergetic effect of many medicinal plants (Rakesh *et al*., 2011). Studies reveal that the poly herbal formulations showed better hepatoprotective activity than a single herb. So, if any single herbal formulation shows better hepatoprotective activity then polyherbal formulation that would give the new insights in research area.

1.3 Shilajit

Shilajit is described as a sticky, brown to blackish (Fig.1.1) physiologically active organic matter exuded from steep rocks in mountainous regions of the world (Garedew *et al.,* 2004) especially in Central Asia (Himalaya, Pamir and Altai) and of unclear age (Kwon *et al.,* 2004). In other words, Shilajit is a tarry, solid or elastic natural product

(Rakhmatullaeva *et al.,* 2005) typically in the form of shapeless pieces with non-uniformly porous or smooth surface having a characteristic balsamic odour (Frolova *et al.,* 1996).

The organic exudate may vary in colour from blackish to brown and is found at high altitudes between 1000 and 5000m on the walls of caves embedded in rocks or as rock exudates with specific weather conditions concerning summer and winter temperatures, duration of sunshine and amount of precipitation (Ali *et al.,* 2004). Shilajit is commonly found in the Himalayas, from Arunachal Pradesh in the East to Kashmir in the West. It is also found in other countries, such as Afghanistan (Hindukush), CIS (Tien Shan, Ural), Tsao-Shing (Ghosal, 1990) Australia (Agarwal*et al.,* 2007) Mongolia, China, Bhutan, Nepal, Pakistan (Bowman*et al.,* 2000) Tajakistan (Khalikov *et al.,* 2003) and Tibet-Himalayan belt (Kwonet *et al.,* 2004). It is also available in Japan, Algeria (Garedew *et al.,* 2004) and Saudi Arabia known as momia imported from Yemen or India (Al-Himaidi *et al.,* 2003).

Shilajit in the capacity of rasayana prevents ailments and enhances the quality of life, the two major attributes of Indian Ayurvedic and Siddha medicine (Ghosal *et al.,* 1991). Even though shilajit is described in traditional literature, it is so far mostly unknown in the West. For nearly more than 3000 years, shilajit plays a vital role with soaring economic value in the folk medicine of the former Soviet Union and also in traditional Indian medicine and Tibetan pharmacology. It is also used as growth accelerator even for plants. Currently, shilajit is prohibited to be exported from the Soviet Union because it is being considered as a ‘treasure of the country’ (Garedew *et al.,* 2004). Amongst the numerous active principles of shilajit, fulvic acid and humic substances are important. In Tajikistan, it is part of the routine diet of the general population to use shilajit.Many bioactive dietary supplements or food additives contain shilajit which have been patented are manufactured in Tajikistan. Shilajit is used in the form of an aqueous extract for therapeutic applications such as, immuno stimulants and anabolic food additives (Schepetkin*et al.,* 2003). Shilajit, is prescribed for varied disorders of different aetiology in Russia, notably, a few of them are genitourinary diseases, diabetes, angina, jaundice, digestive disorders, nervous diseases, chronic bronchitis, anaemia, menorrhagia and osteoporosis
(Schepetkin *et al.,* 2002).

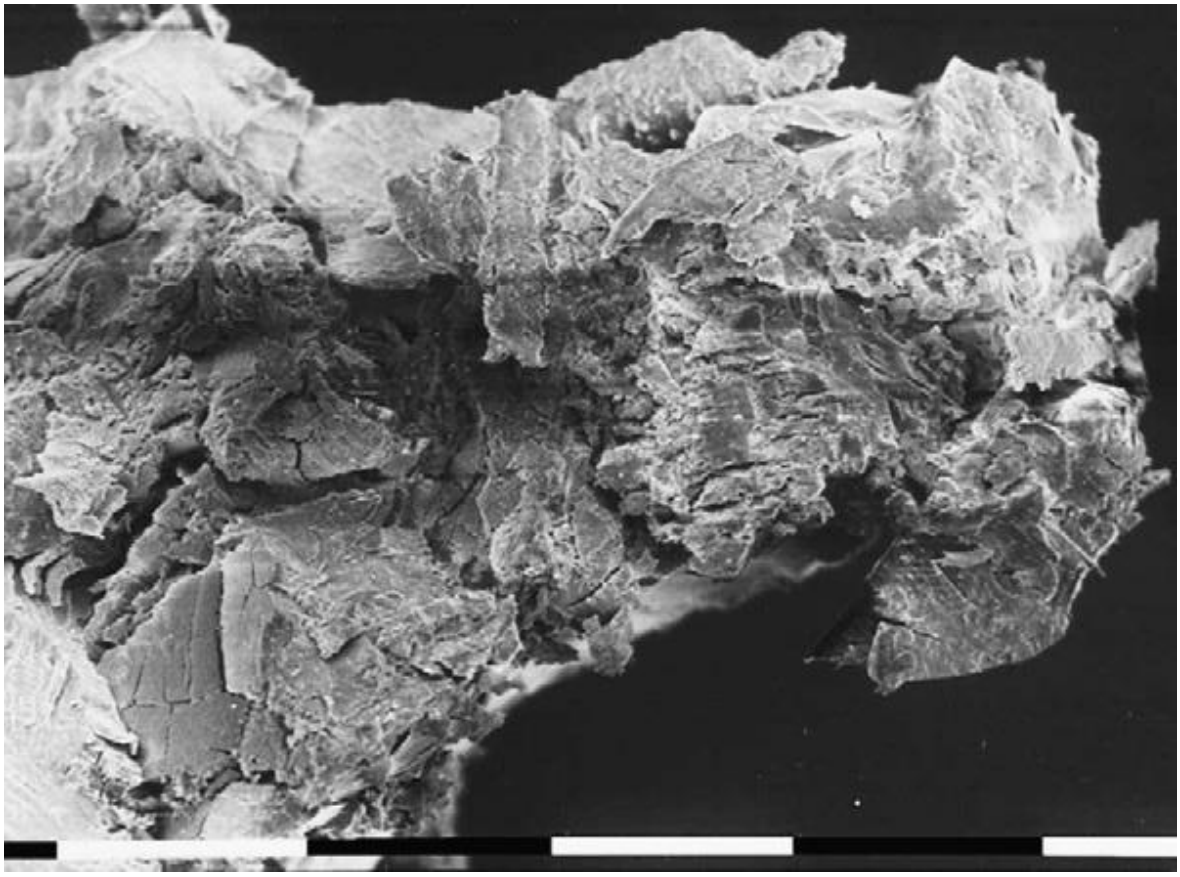

Fig. 1.1SEM photographs of a piece of raw shilajit. White and black bars correspond to 0.1mm.
Source: Garedew et al. (2004).

1.4 *Insilico* study

Molecular modelling encompasses all theoretical methods and computational techniques used to model or mimic the behaviour of molecules. The techniques are used in the fields of computational chemistry, drug design, computational biology and materials science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies(KitchenD.B., *et al.*, 2004). Molecular modelling methods are now routinely used to investigate the structure, dynamics, surface properties and thermodynamics of inorganic, biological and polymeric systems. In this field docking is a well-established computational technique which predicts the interaction energy between two molecules. This technique mainly incorporates algorithms like molecular dynamics, Monte Carlo stimulation, and fragment based search methods etc. These studies are used to determine the interaction of two molecules and to find the best orientation of ligand which would form a complex with overall minimum energy. The small molecule, known as ligand usually fits within protein's cavity which is predicted by the search algorithm. (WeiB.Q., *et al*, 2004).

Aim: Evaluation of Shilajit with special reference to antioxidant and hepatoprotective activity

Objectives:

- To study various available shilajit samples in market with raw sample by comparing various physio-chemical parameters.
- To develop TLC and HPTLC fingerprint profile of shilajit and compare various samples with raw sample of shilajit.
- To explore solubility profile of shilajit with milk and its possible interactions.
- Comparison of various spectroscopic parameters like FTIR and Raman Spectrometry of Shilajit Samples.
- To perform in-vivo hepatoprotective activity on alcohol induced liver model by evaluation of various parameters like SGPT, SGOT, ALP, DBIL, TBIL and Total Protein.
- To do in-silico study of fulvic acid with cytochrome (CYP2C9 and CYP3A4) and hepatoprotective activity (Pregenone X and NFkB receptor).
- To perform *In-Vitro* and *In-Vivo* antioxidant activity of Shilajit and comparison like MDA, SOD, GSH and Catalase. .
- To perform role of cytochrome P450 in Metabolic interaction of Shilajit drugs.

4.1 Raw materials and standard drugs

Shilajit samples were collected from Amritsar, Punjab in the month of August, 2012.

1. Raw Shilajit (RS)
2. Market Processed Shilajit (MPS)
3. Laboratory Processed Shilajit (LPS)
4. Processed Shilajit (PS)

Laboratory Processed Shilajit (LPS) are prepared from Market Processed Shilajit (MPS),Raw Shilajit was purified and used for physical and chemical characterization.

4.2 Chemicals

All the solvents were procured from CDH chemicals and enzymatic kits were acquired from Labcare diagnostic Pvt. Ltd.

4.3 Purification and Isolation of Shilajit

Shilajit (10 g), collected from Amritsar, Punjab was triturated with water and the water insoluble materials were removed by filtration. The aqueous solution was evaporated under reduced pressure (at 37 ^{o}C), to give a brown viscous residue (7.6 g). 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 (2.1 g). From the supernatant acidic solution, Fulvic acids (FAs) and the freshly released low molecular weight organic compounds were isolated (Ghosal *et al.*, 1988a)

4.4 Characterization Shilajit

4.4.1 Physical Characterization of Shilajit

a) Ash Value

1) Determination total ash:

About 2 to 3 g accurately weighed drug was incinerated, in a tarred platinum or silica dish at a temperature not exceeding 450^{o} until free from carbon, cool and weigh. Incinerate the residue and filter paper, add the filtrate, evaporate to dryness, and ignite at a temperature

not exceeding 450º.The percentage of ash with reference to the air-dried drug was calculated.

2) Determination of acid insoluble ash

The obtained ash was boiled for 5 minutes with 25 ml of dilute hydrochloric acid; collect the insoluble matter in a Gooch crucible or on an ashless filter paper, wash with hot water and ignite to constant weight. The percentage of acid-insoluble ash with reference to the air dried drug was calculated.

b) Phenolic estimation

The concentration of phenolics in purified Shilajit was determined using spectrophotometric method (Singleton *et al.,* 1999). Methanolic solution of the Shilajit in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45 ^oC for 45 min. The absorbance was determined using spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

4.4.2 Chromatography of Various Shilajit Samples

a) High Performance Liquid Chromatography (HPTLC/TLC)

- Camag Linomat 5
- Semiautomatic application, band application by spray on technique (2 - 500µl)
- Camag twin trough glass chamber (10 x 10 and 20 x 10)
- Camag TLC scanner 3
- Scanning speed upto 100mm/s, Spectral range 190 – 800nm
- CamagReprostar 3 with digital camera
- Camag UV cabinet with dual wavelength UV lamp

- Dual wavelength 254 / 366nm
- Stationary Phase: Silica gel G60 F_{254} coated on aluminium sheet.
- Hamilton 100µl HPTLC syringe.

The analysis was performed with HPTLC (Camag, Switzerland). The sample extracts were applied with the linomat III applicator on the HPTLC silica gel 60 F_{254} plates (E. Merck, Germany). The plates were developed with a twin-trough developing chamber. After development, the plates were scanned with a Camag TLC scanner 3, and the data were processed with WINCATS software.

Test Sample: Methanolic solution of samples MPS, RS, LPS and PS.

Standard Sample: Methanolic solution of Fulvic acid and 4-OH Coumarin.

Solvent system:- Methanol: Chloroform: n-Butanol: Acetic acid: Distilled water (7:9:3:1:0.5)

4.4.3 Spectrometry of Shilajit

a) IR (Infra-Red Spectrometry)

For FT-IR analysis, 15 mg of dried FA, MPS, LPS and RS sample were mixed with 100 mg KBr and compressed into a pellet on an IR hydraulic press. These pellets were made immediately prior to recording of the spectrum. The infrared spectrum was recorded on an FTS 40 (Bio-Rad, USA) FTIR instrument (wavelength 4000–450 cm-1).

b) Raman Spectrometry

Raman spectroscopyis a spectroscopic technique used to observe vibrational, rotational, and other low-frequency modes in a system. It relies on inelastic scattering, or Raman scattering, of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the vibrational modes in the system. Infrared spectroscopy yields similar, but complementary, information. Typically, a sample is illuminated with a laser beam. Light from the illuminated spot is collected with a lens and sent through monochromatic. Wavelengths close to the laser line due to elastic Rayleigh scattering are filtered out while the rest of the collected light is dispersed onto a detector.Raman spectra analysis of dried FA, MPS, RS and LPS.

4.5 Anti-oxidant and Hepatoprotective Activity

4.5.1 Alcohol Induced Hepatotoxicity

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 present 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.

Chemicals

All the enzymatic kits were acquired from (Lab care Diagnostics (India) Pvt.Ltd). Standard drug Silymarin was procured from medical store.

Treatment protocol

Rats (n=24) were randomized into following groups:-

Wistar rats, weighing (250- 300 g) were divided into 6 groups consisting of 4 animals in each group.

Group 1: Normal group: Animal of this group receive cow milk p.o. for 30 days.

Group 2: Control group: Animal of this group received vehicle, cow milk instead of water and alcohol 3ml/ 100g/day p.o. for 30 days.

Group 3: Standard group: Animal of this group receivevehicle, cow milk instead of water, Silymarin 50 mg/ Kg/day p.o. with cow milk and alcohol 3 ml/ 100g/day p.o. for 30 days.

Group 4: Test group: Animal of this group receive vehicle, cow milk instead of water,Raw Shilajit (RS) with cow milk (300 mg/Kg/day) and alcohol 3 ml/100 g/day p.o. for 30 days.

Group 5: Test group: Animal of this group receive vehicle, cow milk instead of water, Market Processed Shilajit (MPS) with cow milk (300 mg/Kg/day) and alcohol 3 ml/ 100 g/day p.o. for 30days.

Group 6: Test group: Animal of this group receive vehicle, cow milk instead of water, Lab Processed Shilajit (LPS) with cow milk (300 mg/Kg/day) and alcohol 3 ml/100 g/day p.o. for 30 days.

Parameters assessed

Serum: Serum glutamate Pyruvate Transaminase Levels (SGPT), Serum Glutamate Oxaloacetate Transaminase SGOT), Serum Alkaline Phosphatase (ALP), Direct Bilirubin (DBIL), Total Bilirubin (TBIL) and Total Protein.

Collection of serum

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 4^0 C until used.

Quantitative determination of activity of SGOT, SGPT, ALP, TBIL and DBIL in serum was done using enzymatic kit (Lab care Diagnostics (India) Pvt. Ltd)

A. **Biochemical Estimations**

a) Estimation of Serum Glutamate Pyruvate Transaminase Levels (SGPT)

In vitro quantitative determination of activity of SGPT in serum was done using enzymatic kit (Lab care Diagnostics (India) Pvt.Ltd)

Principle

Glutamate-pyruvic Transaminase (GPT-ALP) catalyses the reaction between alpha ketoglutaric acid and alanine giving L-glutamic acid and pyruvic acid. Pyruvic acid in presence of lactate dehydrogense (LDH) reacts with NADPH giving lactate acid and NAD. The rate of NADPH consumption is determined photo metrically and is directly proportional to the GPT activity in the sample.

Procedure: Pipette into test tube

Sample	20 µl
Reagent	1000 µl

Mixed well and allowed to stand for 1min at 37^0C. Measure absorbance decrease per min during 3 min (pA/min)

Calculation:

pA/min. x 1746 = U/l ALT

b) **Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT**)

In vitro quantitative determination of activity of SGOT in serum was done using enzymatic kit (Lab care Diagnostics (India) Pvt.Ltd)

Principle:

Aspartate transaminase (SOT-AST) catalyses the reaction between alpha ketoglutaric acid and L-aspartate giving glutamate and oxaloacetate. Oxaloacetate, in the presence of malatedehydrogenase (MDH) reacts with NADPH giving malate and NAD. The rate of NADPH decrease is determined photometrically and is directly proportional to the GOT activity in the sample.

Procedure: Pipette into test tube

Sample	100 µl
Reagent	1000 µl

Mix well and let stand for 1min at 37^0C. Measure absorbance decrease per min during 3 min (pA/min)

Calculation:

pA/min. x 1746 = U/l AST

c) **Estimation of serum Alkaline Phosphatase level (ALP)**

In vitro quantitative determination of activity of ALP in serum was done using enzymatic kit(Lab care Diagnoostics (India) Pvt.Ltd)

Principle:

p-NitrophenylPhosphateis converted to p-nitrophenol and phosphate by alkaline phosphatase. The increase of absorption at 405 nm is proportional to the alkaline Phospahtase concentration in the sample.

Procedure: Pipette into test tube

Sample	20 µl
Reagent	1000 µl

Mix well and let stand for 1min at 37^0C. Measure absorbance increase every 30 sec for 2 min determine (pA/min)

Calculation:

pA/min. x 2720 = U/l ALP

d) Estimation of Direct Bilirubin (TBIL)

In vitro quantitative determination of activity of TBIL in serum was done using enzymatic kit (Lab care Diagnoostics (India) Pvt.Ltd)

Principle:

The method uses dyphylline to dissociate unconjugated bilirubin from albumin. Unconjugated bilirubin, conjugated bilirubin, and albumin-linked bilirubin (delta) subsequently react with the diazonium salt 4-(N-carboxymethylsulfonyl) benzenediazoniumhexafluorophosphate to produce azobilirubinchromophores that have similar molar absorptivities and absorbance maxima around 520 nm.

Procedure: Pipette into test tube

Sample	100 µl
Reagent	1000 µl

Mix well and let stand for 1min at 37^0C. Measure absorbance increase every 30 sec for 2 min determine (pA/min)

e) Estimation of Total Bilirubin (DBIL)

In vitro quantitative determination of activity of DBIL in serum was done using enzymatic kit (Lab care Diagnoostics (India) Pvt.Ltd)

Principle:

Total bilirubin (TBIL) is measured by the diazo derivatives of unconjugated Bu, conjugated, and covalently protein-bound "delta" bilirubin. Conjugated and unconjugated bilirubins are measured simultaneously using a dual-wavelength spectral measurement.

Procedure: Pipette into test tube

Sample	100 µl
Reagent	1000 µl

Mix well and let stand for 1min at 37^0C. Measure absorbance increase every 30 sec for 2 min determine (pA/min)

4.5.2 Anti-Oxidant Activity

A. Preparation of the Liver Tissue for Enzyme Assay

Liver, kept in cold conditions (precooled in inverted petridish on ice) was removed. It was cross chopped with surgical scalpel into fine slices and was chilled in the cold 0.25 M sucrose, quickly blotted on a filter paper. The tissue was minced and homogenized in 10 mMTris-HCl buffer, pH 7.4 (10%w/v) with 25 strokes of tight teflon pestle of glass homogenizer at a speed of 2500 rpm. The clear supernatant was used for other enzymes assays.

a) Superoxide dismutase (SOD)

SOD was estimated by the method of Mishra and Fridovich, 1972.

Principle:

Rate of auto oxidation of epinephrine & the sensitivity of this auto oxidation to inhibition by SOD were augmented as pH was raised from 7.8-10.2, O_2, generated by xanthine oxidase reaction, caused by the oxidation of epinephrine to adrenochrome and the yield of adrenochrome produced per O_2 introduced. The auto oxidation of epinephrine proceeds by least two distinct pathways only one of which is free radical chain reaction involving O_2 and hence inhabitable by SOD.

Reagent:

1. Carbonate buffer (0.05 M pH 10.2):16.8 gm of $NaCO_3$ was dissolved in 500 ml of distil water & the final volume was made up to 1000 ml with distill water.
2. EDTA 0.49 M: 1.82 gm of EDTA was dissolved in 1000 ml of distill water.
3. Epinephrine (3 mM): 0.99 gm of epinephrine bitartarate was dissolved in 1000 ml of distill water.
4. SOD standard: Dissolve 1 mg (1000 units /mg) of SOD from bovine liver in 100 ml of carbonate buffer.

Procedure:

All the reagents required were kept in cold condition to maintain the reaction condition.

Blank	Test
0.1 ml DW	0.1 ml sample
0.1 ml EDTA	0.1 ml EDTA
0.5 ml carbonate buffer	0.5 ml carbonate buffer
1 ml of epinephrine	1 ml of epinephrine
Absorbance at 480 nm for 3 min at 30 sec interval	

The reaction was initiated by the addition of epinephrine and the change in optical density / min. was measured at 480 nm, readings were taken for 3 min with 30 second interval.

Calculation: SOD= (0.025 – Y) ÷ (Y × 50) × 100

Y = Final reading – Initial reading, Units = Units/ mg of protein.

b) Catalase:

Catalase was estimated by the method of Aebi*et al.*, 1987.

Principle: In the ultra-violet range H_2O_2 shows a continuous increase in absorption with decreasing wavelength. The décor position of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in the absorbance per unit time is a measure of the catalase activity.

Reagent:

1. Phosphate buffer (50 m mol/L pH 7)

(i) 6.81 gm of KH_2PO_4 dissolved in distill water and make up volume to1000 ml with distillwater.

(ii) 8.9 gm of Na_2HPO_4 dissolved in distill water and make up volume to 1000 ml with distill water.

2. Hydrogen peroxide (30 n mol/L)

Procedure:

Blank	Test
2910 µL of phosphate buffer pH 7	2910 µL of phosphate buffer pH 7
50 µL of Distilled water	5 µL of Homogenate.
40 µL of hydrogen peroxide solution	40 µL of hydrogen peroxide solution

Add H_2O_2 just before taking OD at 240 nm; readings were taken for 3 min. with 15 second interval.

Calculation: Log A_1/A_2 × 229.7 (factor) A_1= initial Absorbance, A_2= final Absorbance.

Unit = units / mg of protein.

c) Reduced glutathione (GSH):

Reduced of glutathione (GSH) was estimated by the method of Moran *et. al.*, 1979.

Principle: Glutathione present in RBC consist of sulfhydryl groups. 5,5 dithiobis 2- nitro benzoic acid (DTNB), A disulphide compound, gets readily attacked by these sulfhydryl groups and forms a yellow coloured anion which measured calorimetrically at 412 nm.

Reagents:

1. Trichloroacetic acid (10%):10 gm of TCA was dissolved in 100 ml of distill water.
2. Dithiobis nitro benzoic acid (DTNB):40 gm of DTNB was dissolved in1% Sodium citrate solution.
3. Phosphate buffer (0.2 M, pH 8.0):1.36 gm of KH2PO4 was dissolved in 100 ml of distill water and dissolve in 0.8 gmNaOH in 100 ml distill water.
4. Reduced of glutathione standard 10 gm of GSH standard was dissolved in 100 ml of distill water (100ìg/ml).

Procedure:

Blank	Test
1 ml of D.W.	1 ml of Homogenate
1 ml of TCA (10%)	1 ml of TCA (10%)
Cool for 10 min and centrifuged at 2000 rpm take 0.5 ml of supernatant	
0.5 ml of above supernatent	0.5 ml of above supernatent
2 ml sodium hydrogen phosphate	2 ml sodium hydrogen phosphate
0.25 ml DTNB	0.25 ml DTNB

Mixed well and allowed to stand for at room temperature, readings were taken against blank at 412 nm using spectrophotometer.

Calculation: Y = 0.0002X + 0.0049, X = Conc. of reduced of glutathione

Y = Abs of test sample. Units: µg of GSH / mg of protein.

d) Lipid peroxidation:

Malondialdehyde formation (MDA) was estimated by the method of Ohkawa et al., 1979.

Principle:

The method estimates Malondialdehyde(MDA), a product of lipid peroxidation process. One molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) under mildly acidic conditions to form a pink coloured chromogen, whose intensity was measured calorimetrically at 535 nm.

Reagents:

1. Thiobarbituric acid (1% in Tris hydrochloride, pH 7):1 gm of thiobarbituric acid was dissolved in 100 ml of Tris hydrochloride buffer.

2. Trichloroacetic acid (10%):10 gm of trichloroacetic acid was dissolved in distilled water.

3. SLS (8%): 8 gm of SLS in 100 ml of water.

Procedure:

Blank	**Test**
0.2 ml of D.W.	0.2 ml of Homogenate
0.2 ml of SDS	0.2 ml of SDS
1.5 ml acetic acid in HCl	1.5 ml acetic acid in HCl
1.5 ml TBA	1.5 ml TBA
0.6 ml DW	0.6 ml DW
Heated for 45 min in water bath at 95^{0} C and cool	
2ml mixture + 2 ml TCA	2ml mixture + 2 ml TCA
Centrifuge on 1000 rpm for 5 min	
Pink color measure at 532 nm	

$A = a * b * c$

A = abs.

a = mol. Extinction coefficient ($1.56 * 10^{5}$ cm^{-1})

b = Path length (1 cm^{2})

c = conc. of sample

Units: nm of MDA / gm of tissue.

4.6Histopathological Evaluation

Histopathological evaluation was carried out at prime path solutions.

4.7 Mechanistic Evaluation

4.7.1 DPPH radical scavenging activity

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was measured by spectrophotometric method (Qureshi NN, et al, 2009). 2 ml of methanolic solution of the extracts of various concentrations (0-500 μg/ml) were mixed with 1 ml of ethanolic solution of DPPH (1.5 mg/10ml). A mixture of 1 ml of ethanol and 1 ml of ethanolic solution of DPPH served as control. After mixing, all the solutions were incubated in dark for 20 min and then absorbance was measured at 517 nm. The experiments were performed in triplicate and EC50 was measured.

4.7.2*Insilico* Study

Molecular modelling encompasses all theoretical methods and computational techniques used to model or mimic the behaviour of molecules. The techniques are used in the fields of computational chemistry, drug design, computational biology and materials science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies(KitchenD.B., *et al.,* 2004). Molecular modelling methods are now routinely used to investigate the structure, dynamics, surface properties and thermodynamics of inorganic, biological and polymeric systems. In this field docking is a well-established computational technique which predicts the interaction energy between two molecules. This technique mainly incorporates algorithms like molecular dynamics, Monte Carlo stimulation, and fragment based search methods etc. These studies are used to determine the interaction of two molecules and to find the best orientation of ligand which would form a complex with overall minimum energy. The small molecule, known as ligand usually fits within protein's cavity which is predicted by the search algorithm. (WeiB.Q.,*et al,* 2004).

To study the molecular mechanism, active constituent fullvic acid was selected. ChemDraw Ultra 8.0 software was used to design the ligand followed by 3D optimization. The Sybyl Mol2 format files of these ligands were converted into Protein

data bank (PDB) format. The potent target receptors involved in hepatoprotection namely NFkB and Pregnane X receptor were elicited from literature survey (Watkins R.E., *et al*, 2001) and their respective PDB files ID: 1VKX [21] and ID: 1ILG (Watkins R E, Wisely G B, Moore L B et al. Science 2001; 292: 2329-2333) were retrived from Protein data bank. Using Tripose force field and MMFF94 charges, solvent deletion and hydrogens were added into the receptors files for the preparation of receptor in docking simulation. Docking analysis was done using SYBYL-X 1.3 with formation of 10 conformers of the ligand. Results were analysed to study the interactions, binding energy, hydrogen bond interactions and the binding distance between the hydrogen bond donors and acceptors for the best conformers.(Saliou C., *et al*, 1998).

Scoring functions in SYBYL-X 1.3 Surflex-Dock uses an empirically derived scoring function that is based on the binding affinities of protein-ligand complexes and on their X-ray structures.TheSurflex-Dock scoring function is a weighted sum of non-linear functions involving van der Waal surface distances between the appropriate pairs of exposed protein and ligand atoms.Surflex-Dock scores are expressed in $-\log_{10}(K_d)$ units to represent binding affinities. The Scoring functions include hydrophobic, polar, repulsive, entropic, solvation and crash value.

A. *Insilico* Study (Hepatoprotective Activity)

To study the molecular mechanism, active constituent fulvic acid was selected. ChemDraw Ultra 8.0 software was used to design the ligand followed by 3D optimization. The SYBYL Mol2 format files of these ligands were converted into Protein data bank (PDB) format. The potent target receptors involved in hepatoprotection namely NFkB and Pregnane X receptor were elicited from literature survey and their respective PDB files ID: 1VKX and ID: 1ILG (Watkins R E, Wisely G B, Moore L B et al. Science 2001; 292: 2329-2333) were retrived from Protein data bank. Using Tripose force field and MMFF94 charges, solvent deletion and hydrogen's were added into the receptors files for the preparation of receptor in docking simulation. Docking analysis was done using SYBYL-X 1.3 with formation of 10 conformers of the ligand. Results were analysed to study the interactions, binding energy, hydrogen bond interactions and the binding distance between the hydrogen bond donors and acceptors for the best conformers.

B. *Insilico* Study (Human Cytochrome P450 Enzymes Activity)

Based on the inhibition results, potent protein targets were assessed for further inhibition mechanisms by molecular docking analysis. The software SYBYL X 1.2 was used to perform molecular docking of HM-1 to human CYP450 isoforms with the crystal structures from the Protein Data Bank (Trott and Olson *et al.*,2010). The docking parameters were set to the default values. The grid boxes were 20 ˚A × 20 ˚A × 20A˚, encompassing their active site cavities. The binding modes of HM-1 to CYPs with lowest binding free energy were chosen for further optimum docking conformation, while probe substrates in the metabolism position closer to the heme iron were identified. Molecular docking studies for CYP2C9 and CYP3A4 were further performed to confirm the inhibition modes for each CYP isoform.

4.7.3 Cytochrome P450 Metabolism Interaction Activity (Liver Homogenate)

Fresh liver tissue homogenates of Wistar rats were mainly used as a sourceof enzyme for the test of methods, and applying these methods colorimetric estimation of the enzyme activities.

Procedure:

Incubation medium for cytochrome c-cytochrome oxidasesystem contained generally 0.2 ml. of 0.2M ρ -phenylenediamine (ρ-PDA),0.2 ml. of 0.2% neotetrazolium chloride (NT) (Fig. 1) (these were mixed immediately before incubation), 0.2 ml. of tissue homogenate containing20 or 40 mg of liver tissue in 0.1 M phosphate buffer (pH 7.6). For cytochromec oxidase 0.2ml.of 10-4 M cytochrome c was added. The substratecontrol, i. e. endogenous dehydrogenase, 0.2 ml. of distilled waterwas used in place of *p-PDA* solution, and for enzyme control, 0.2 ml of0.lM phosphate buffer was used in place of tissue homogenates. The incubation was conducted at 37°C during 30 minutes. The reactionwas stopped by the addition of 0.4 ml. of IN sulphuric acid. Whenthe reaction was stopped by adding sulphuric acid, the yellowish brown ordark brown oxidized product of ρ-PDAbecame insoluble in ether-acetone,while the reaction product of NT, diformazan, could be easily extractedwith ether-acetone (1:1). The extracts were collected and the volume wasread. The absorption spectrum of the diformazan in ether-acetone extract(Fig. 2) was written with the Beckman DK style of

autorecordingphotoelectricspectrophotometer and the optical density was determined at thewave length of the maximum absorption, 520 *m/l*, with the photo-electricspectrophotometer.

Estimation of Cytochrome Oxidase: The values of the optical densities were multiplied by the volumesof the extracts in ml. divided by 5, and thus the optical densities of thediformazan in 5 ml. of solvent were obtained. The results were expressedin the optical densities. If the amounts of neotetrazolium chloride reducedor oxygen consumption was required, the former was obtained from thestandard curve of neotetrazolium chloride reduced and the later fromthe conversion of μM of the formazan to µM of oxygen in proportion of **(**1:1).

4.8Statistical significance

All the values are expressed as mean ± S.E.M. statistics was applied using GraphPad Prism software version 5.0. Statistical significance between normal control and induced control group was tested using student's t- test and ANOVA test. Differences were considered to be statistically significant at $P < 0.001$.

According to the World Health Organization (WHO), traditional medicine (TM) incorporates health practices of plant; mineral and animal based medicines, applied singularly or in combination to treat and prevent illnesses/maintain well-being (World Health Organization, 2000).

Shilajit is widely considered as a herbomineral drug which is mainly indegeneous to India, Tibet and Russia. There are several schools of thought regarding the origin of shilajit. It was originally thought as a plant fossil, a substance of mixed plant and animal origin (Ghosal, 1990; Ali et al., 2004). Shilajit is described as a sticky, brown to blackish physiologically active organic matter exuded from steep rocks in mountainous regions of the world (Garedew et al., 2004) especially in Central Asia (Himalaya, Pamir and Altai) and of unclear age. Many researchers claim that shilajit exudates from a layer of rocks of mountains with plant secondary metabolites. Shilajit is commonly found in the Himalayas from Arunachal Pradesh in the East to Kashmir in the West. (Wilson et al.,2011)

Shilajit is used in the form of an aqueous extract for therapeutic applications such as, immune stimulants and anabolic food additives (Schepetkin et al., 2003). Shilajit, is prescribed for varied disorders of different aetiology in Russia, notably, a few of them are genitourinary diseases, diabetes, angina, jaundice, digestive disorders, nervous diseases, chronic bronchitis, anaemia, menorrhagia and osteoporosis Ample experimental and epidemiological studies support the involvement of oxidative stress in the pathogenesis and progression of several chronic diseases. Amongst the numerous active principles of shilajit, fulvic acid and humic substances are important. Proper characterization of Shilajit with emphasis on its active constituents has not yet fully explored. Hence, this study addresses that issue with its possible therapeutic implications in alcohol induced liver damage. (Wilson et al., 2011)
Shilajit well known drug but herb mineral drug but it’s not evaluated so TLC, HPTLC, Ash value and phenolic estimation included.

It is now known that oxygen, indispensable for maintaining life, sometimes becomes toxic and results in the generation of most aggressive agents such as Reactive oxygen species (ROS). The high reactivity of ROS may trigger a host of disorders in body

resulting in tissue damage and necrosis in many instances. The alcohol induced model of liver injury was adopted in this study as it is the most frequent cause of liver damage in humans lately. Alcohol induces hepatic microsomal enzyme systems and vice versa by antioxidants which mop up the free radicals. (Ahsan et al., 2009)

The invitro free radical scavenging activity of Shilajit in its various forms raw, market processed and the lab processed was assessed by the DPPH assay with ascorbic acid as standard. The lab processed shilajit showed a better activity compared to the raw and market processed shilajit. Its activity was almost similar to that os ascorbic acid. This preliminary study proved that Shilajit has an antioxidant activity. Further invivo activity was also carried out.

Going a step further, hepatoprotective activity was assessed. In the assessment of liver damage by hepatotoxins like alcohol, the determination of enzyme levels such as SGPT and SGOT is largely used. This is evidenced by an elevation in the serum marker enzymes namely SGPT, ALP and SGOT.Hepatic necrosis or membrane damage releases the enzymes into circulation and hence it can be measured in the serum. High level of AST indicates liver damage, such as viral hepatitis, cardiac infarction and muscle injury. AST catalyses the conversion of alanine to pyruvate and glutamate, and is released in a similar manner. Therefore, ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver. On other hand, serum levels of ALP, bilirubin and TP are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in the presence of increasing biliary pressure. The antioxidant mechanism along with the enzyme levels were carried out for the assessment of the hepatoprotective activity. (Eesha et al., 2011)

The market processed Shilajit, Lab processed and the raw Shilajit showed a significant decrease in these enzymes compared to the disease control group. Elevation of total bilirubin which results from decreased uptake and conjugation of bilirubin by the liver is caused by liver cell dysfunction, while increased levels of direct or conjugated bilirubin is due to decreased secretion from the liver or obstruction of the bile ducts. A reduction of

increase in total and conjugated bilirubin was observed in the shilajit treated groups. The activity was also compared with the standard Silymarin treated group which too showed a significant decrease.. The extract perhaps protects the liver cell from damage, thereby enhancing bilirubin uptake and conjugation by the liver and subsequent secretion into the bile ducts. Reports from the study show that Shilajit possess hepatoprotective activity.

The body has an effective mechanism to prevent and neutralize the free radical – induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as SOD, CAT, and GPX etc. When the balance between ROS production and antioxidant defences is lost, 'oxidative stress' results, which through a series of events deregulate the cellular functions leading to various pathological conditions. Alcohol induced liver damage simulates free radical induced damage. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this type of damage. In the present study, elevated level of TBARS observed in alcohol treated rats indicates excessive formation of free radicals and activation of LPO system resulting in hepatic damage. TBARS produced as by-products of LPO that occurs in hydrophobic core of bio-membranes. The significant decline in the concentration of SOD, CAT was observed in the alcohol treated group. The shilajit and the Silymarin treated groups showed an increase in the levels by the antioxidant properties. The raw shilajit and the lab processed shilajit showed a significant increase in the levels compared to the disease control, normal control and the Silymarin treated groups. This study concurs with the invitro data and proves Shilajit as a potent anti-oxidant.

GSH is a major non-protein thiol in living organisms which plays a central role in coordinating the body's antioxidant defence processes. Perturbation of GSH status of a biological system has been reported to lead serious consequences. Decline in GSH content in the liver of alcohol intoxicated rats, and its subsequent return towards near-normalcy in shilajit treated rats reveal the antioxidant effect of the extract. Explanations of the possible mechanism underlying the hepatoprotective properties of drugs include the revention of GSH depletion and destruction of free radicals. These two factors are believed to attribute to the hepatoprotective properties of the fruit pulp of L. acidissima SOD, CAT and GPX constitute a mutually supportive team of defence against ROS. SOD

is a metalloprotein and is the first enzyme involved in the antioxidant defence by lowering the steady-state level of O_2.CAT is a hemeprotein, localized in the peroxisomes or the micro peroxisomes. This enzyme catalyses the decomposition of H_2O_2 to water and oxygen and protects the cell from oxidative damage by H_2O_2 and OH—. GPX is a seleno-enzyme two third of which (in liver) is present in thecytosol and one third in the mitochondria. It catalyses the reaction of hydro peroxides and gets reduced to form glutathione di sulphide (GSSG) and the reduction product of the hydroperoxide.In our study, decline in the activities of these enzymes in alcohol administered rats revealed that LPO and oxidative stress elicited by alcohol intoxication have been nullified due to the effect of Shilajit. Since treated groups have significantly elevated the GSH, SOD, CAT contents of the liver against alcohol intoxication, it may be helpful in treating the hepatotoxicity induced by free radicals. With reference to the various groups, Lab processed Shilajit shows a greater activity compared to the marketed and the raw Shilajit.

In the histopathological studies, the first cells to be damaged are those in the centrilobular region where microsomal enzyme activity is the greatest. The initial damage produced is highly localised in the endoplasmic reticulum which results in loss of cytochrome p450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver and necrosis, a characteristic of alcohol poisoning. If the damage is severe, it leads to disturbances in the water and electrolyte balance of hepatocytes leading to an abnormal increase in liver enzymes in plasma there by impairing mitochondrial functions, followed by hepatocellular necrosis. The reverse of this phenomenon can be considered as the index of hepatoprotective activity. To validate this phenomenon, estimation of cytochrome p450 enzyme was carried out and the results concurred with the above details. The alcohol treated animals showed a marked decline in the enzyme levels and the treatment with Shilajit reversed this. Lab processed Shilajit showed a better activity among the other groups and its activity was on par with that of Silymarin treated group.

Histopathological studies of liver sections has shown that the pretreatment with Shilajit exhibited protection against alcohol induced fatty degeneration and necrosis of the liver tissue, confirming the results of biochemical studies and indicating the hepatoprotective

properties of Shilajit. Among these, the lab processed showed a greater degree of protection compared to the raw and market processed Shilajit. All the above evidence from the study puts forward the therapeutic and beneficial effects of Shilajit in hepatotoxicity.

Insilico studies for Cytochrome P-450 enzymes revealed that the Fulvic acid showed better interactions and gave more docking score when compared with the Co crystal ligands of PDB ID 1WOG and 1OG5. The studies for hepatoprotective activity also revealed that the Fulvic acid showed more hydrogen bonds with high docking score when compared with standard Silymarin on both PDB ID's 1ILG and 1VKX. The interactions and hydrogen bonds in the all cases, indicates that the Fulvic acid showed better results when compared with standards.

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