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Assessment of *Bixaorellana* L. shells extract for Hepatoprotective activity against acute ethanol-induced liver toxicity in wistarrats

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ABSTRACT

Background and Purpose: Medicinal herbs and traditional medicines have been used in our country since pre-Hispanic times and are significantly effective against a large variety of liver disorders. *Bixaorellana* is one of the herbal drugs used traditionally for fever, buccal tumors, antiseptic, antibacterial, and different types of hepatic ailments. To investigate the hepatoprotective activity of *Bixaorellana* L. (BO) shell extract against acute ethanol-induced hepatotoxicity in rats.

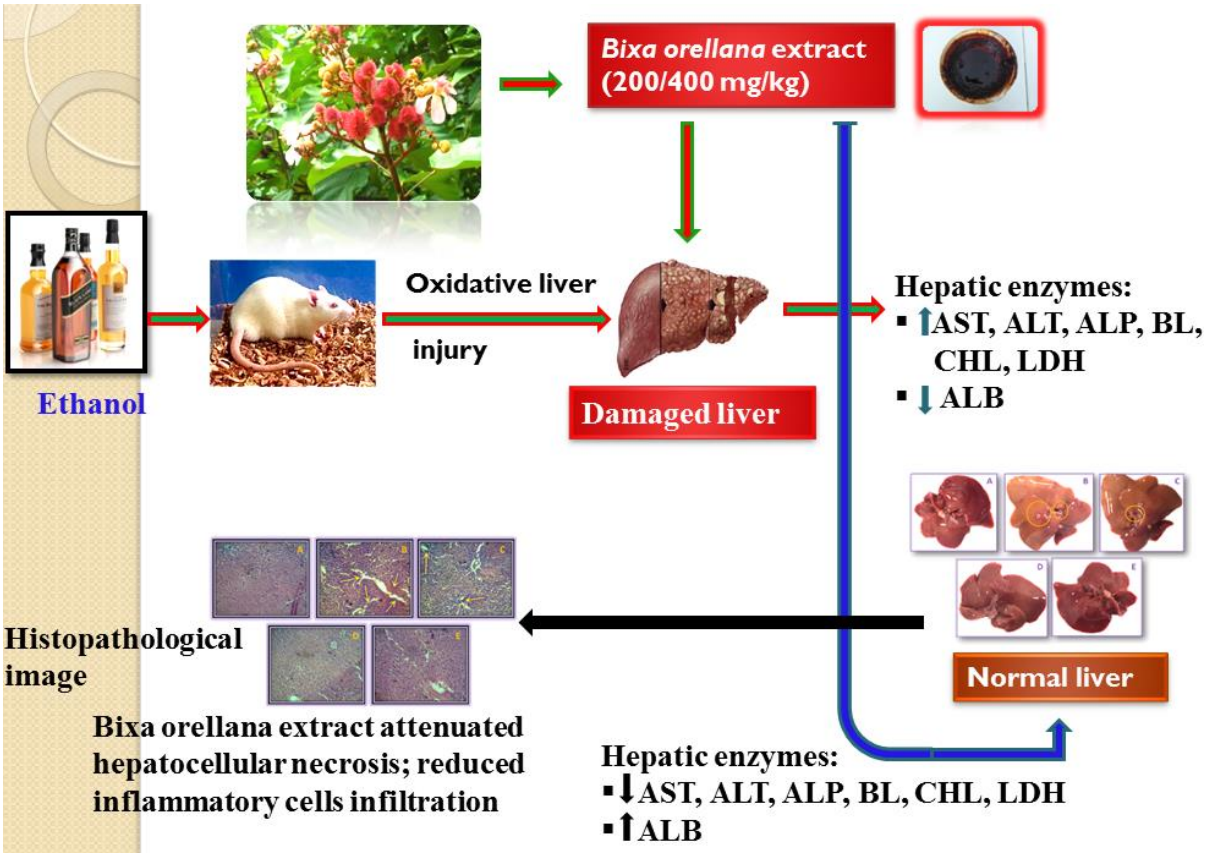
Experimental approach:The characterization of BO shells extract was performed using standard biochemical analysis. 50% ethanolic extract of *Bixaorellana* (BOE 200 and 400 mg/kg body weight) was administered daily for 8 days in experimental animals for the assessment of hepatoprotective activity. To develop hepatotoxicity, animals were orally administered with alcohol (40%) 12 ml/kg at 2 h after the doses of BOE every day for eight consecutive days except the rats of normal group. The hepatoprotective activity was assessed using various biochemical parameters like aspartate aminotransferase, alanine aminotransferase, alkaline phosphatise, Bilirubin, albumin, Cholesterol and lactate dehydrogenase.

Findings / Results: Theresults demonstrated that the treatment with *Bixaorellana*shells extract significantly (P<0.05-P<0.001) and dose-dependently prevented alcohol-induced increase in serum levels of hepatic enzymes such as AST, ALT, ALP, BIL, ALB, CHL and LDH. Histopathological study further attenuated the hepatocellular necrosis and led to reduction in inflammatory cells infiltration.

Conclusion and implications: The present study concludes and suggests that *Bixaorellana* shells extract significantly protected the liver from alcohol induced toxicity. It also showed that the extract contains numerous antioxidant compounds with hepatoprotective effect.

Keywords: Antioxidant; *Bixaorellana*; ethanol; hepatotoxicity; hepatocellular necrosis; inflammation.

Graphical Abstract



INTRODUCTION

Alcoholism is a major contributor to personal fatalities and disabilities[1]. In terms of alcohol consumption, India is in the top. After China, it is the second-largest consumer of spirits. More than 663 million liters of alcohol are used in India each year, an increase of 11% from 2017. Over 45% of all alcohol sold in India is distributed among the five southern states of Andhra Pradesh, Telangana, Tamil Nadu, Karnataka, and Kerala. Of course, taxes on alcohol sales account for more than 10% of their revenue. Alcohol use among Indian men is 33%. Over 14 percent of all Indians were of legal drinking age between the ages of 10 and 75.In comparison to the global average of 16 percent, the World Health Organization (WHO) believes that 11 percent of Indians are binge eaters. This study makes an effort to analyze the alcohol consumption trend in India. The study makes use of information from the 2019 National Survey on the Amount of Substance Use in India. Alcohol use accounts for over 5.1 percent of all infections globally and accounts for nearly 3.3 million annual fatalities (WHO, 2018)[2]. Long-term excess alcohol exposure leads to alcoholic liver disease (ALD)[3] - a global health problem without effective therapeutic approach[4] and is one of the leading causes of liver cancer, cirrhosis, fibrosis, acute and chronic liver failure which can therefore lead to significant morbidity and mortality[5]. ALD is increasingly considered as a complex and multifaceted pathological process, involving oxidative stress, inflammation and excessive fatty acid synthesis[3]. However, the reports might differ in the data, but the current consensus states that about 100-120 g of ethanolis ingested by alcoholic hepatitis patients every day since last 10 to 20 years. This quantity is measured to be standard with the drink of pure alcohol containing 14g, which is considered equivalent to 1.5 ounces (44.36 mL), 5 ounces (147.87 mL) of wine, 12 ounces (354.88 mL) of beer and a “shot” of 80-proof liquor[6].

In the initial stage of ALD, long-term heavy alcohol exposure leads to fatty liver (hepatic steatosis), characterized by triglyceride (TG) accumulation in hepatocytes, which has been widely assumed to be a benign and reversible condition, on continued alcohol consumption hepatic steatosis progresses to the advanced stages of ALD, such as steatohepatitis, fibrosis, cirrhosis, and even hepatocellular carcinoma, particularly in the presence of co-factors including hepatitis virus infection, smoking and diabetes[7]. Fatty liver, an early response to alcohol consumption, develops in most (more than 90%) heavy drinkers, with early-mild steatosis in zone 3 (perivenular) hepatocytes; it can also affect zone 2 and even zone 1 (periportal) hepatocytes when liver injury is more severe[3].

Around 16.0% of drinkers in the world who are 15 or older engage in heavy episodic drinking. The biggest impact is seen in Europe, where liver-related mortality from alcohol contributes to 7% and 12% of mortality and 5% of disability adjusted life years (DALY), respectively1.However, according to the WHO, the global alcohol consumption rate in 2016 was 6.4 liters of pure alcohol per person aged 15 or older. There were appropriate regional variations. Disability-adjusted life years (DALYs) were 2.3% for females and 8.9% for males in 2016, and alcohol usage contributed to 2.2% of female deaths and 6.8% of male deaths[1].

Bixaorellana L., commonly known as Annatto (also called as Chinese dye tree or the lipstick tree), belongs to the family Bixaceae and is native to tropical and subtropical regions of the world[10], but now cultivated in many tropical countries of the world including India. *Bixaorellana* is an evergreen shrub or small tree, 2-8m high bark light to dark brown, tough, smooth, sometimes. Flowers in terminal branched panicles, 8-50 flowered, covered with reddish brown scales; petals 4-7, obovate, 2-3 x 1-2 cm, pinkish, whitish. Fruit a spherical or broadly elongated ovoid capsule, 2-4 x 2-3.5 cm, flattened, green, greenish-brown or red when mature; shellss numerous, with bright orange-red fleshy coats[11]. Natural colorants

obtained from the shellss of achiote plant (annatto) have been used since pre-Hispanic times [12].Its leaves have antiseptic, antibacterial, and antiemetic effects, whereas the shellss were used for fever and buccal tumors, jaundice. Many pharmacologic studies have been conducted on its potential as a source of medicine[13]. *B. orellana* was shown activity against protozoan, helminths and had platelet anti-aggregant Activity[14]. Annatto tocotrienols (AnT3), which contain approximately 90% d-tocotrienol (d- T3)[15]

The fruit shell of Bixaorellana L. was used to isolate naringenin-7-O-glucoside (Prunin (Pru).[16]. Naringenin 7-O-beta-D-glucoside is a flavanone 7-O--D-glucoside that has had a -D-glucopyranosyl moiety replaced for naringenin at position 7 via a glycosidic linkage. It has been asserted that it contains antilipemic and hypoglycemic properties[17]. In the study, it was shown that naringenin (NA) and naringenin-7-O-glucoside (NAG) downregulated the diameter of 3T3-L1 cells while upregulating intracellular lipid accumulation and adiponectin release during adipocyte development. Because NA and NAG's intracellular lipid accumulation in connection with adipogenesis was inhibited by the PPAR antagonist BADGE and the PI3K/Akt inhibitors wortmannin and LY29004, it was assumed that NA and NAG exhibited the aforementioned activities through PPAR activation as well as phosphorylation of the PI3K/Akt pathway[18]

*Bixaorellana*is a safe hepatoprotective agent against various hepatotoxins (carbon tetrachloride and acetaminophen) in albino rats and act similar to that of silymarin as hepatoprotective and is more effective inducer of biochemical enzymes indicating the hepatoprotective property[17, 18]. Hence, an attempt has been made in this study to investigate the hepatoprotective activity of shells extract of *Bixaorellana* against acute ethanol-induced liver injury in Wistar rats.

MATERIALS AND METHODS

Chemicals and reagents

All the analytical grade chemicals and reagents used were of analytical grade, and procured from Sigma chemicals Co., USA and Qualigens fine chemicals, Mumbai, India. Organic solvents such as formic acid, n-hexane, petroleum ether, chloroform, ethyl acetate, and methanol (high-pressure liquid chromatography grade, Merck) were used. The drug used as standard was Silymarin (Sigma Chemicals, USA (BCBF6608V) Made by china). All the enzymatic kits used were obtained from Span Diagnostics Ltd. Surat (76LS200-60).

Plant collection and authentication

The ripped fruits of *Bixaorellana* Linn. (Bixaceae) were collected from local market of Lucknow, in the month of January 2014. The plant material was authenticated by Mr. Muhmmad Arif (Assistant Professor) and Dr. Arshan Hussain (Associate Professor & Head) Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Integral University, Lucknow- 226 022, A voucher specimen of *Bixaorellana* Linn. (IU/PHAR/HRB/14/02) was deposited in the institute for further reference.

Extract preparation for animal study

The freshly collected fruits of *Bixaorellana* Linn.were washed with distilled water to remove dirt and soil and shade dried in a ventilated place at room temperature. Dried shellssand shells were separated and shells were cut into small pieces and reduced to coarse powder by mechanical grinder and further extraction was carried out with 50 % hydroalcoholic by cold percolation method to avoid damage due to heat. The extract was filtered and concentrated under reduced pressure below 40±1°C using rotevavaccum rotary evaporator (Model no-UDOIAB-2391 Medica instrument) to dryness to get a constant weight. The % yield was

found to be 20.25 % w/w. The extract was stored in −20 °C freezer and used for Pharmacological investigation.

Preparation of TLC Sample

Coarsely powdered sample of fruit shells 10 g was extracted with 300 ml methanol in a soxhlet extractor until discolouration of the sample. The extract was filtered and concentrated to dryness under reduced pressure below 40 ± 1°C using a rotary vacuum evaporator (Model no- UDOIAB-2391 Medica instrument). Percentage (%) yield was found to be 13.69 % w/w. A preparative TLC plate was cut into small pieces and activated at 110°C for 30 minutes. Single spot of the sample was applied on the plate using the capillary. Further the plate was developed in a chamber having desired solvent systems for bixin and amino acids as mentioned below in **Table 1**. The ratios of the distances travelled by the samples compared to the distances travelled by solvents were identified on the TLC plates as colored spots. Each plate was then placed inside a closed container with iodine vapour which helped to specifically identify each and every spot more clearly for better visualization.

Animal experiments

Adult female Wistar rats weighing 160 ± 20 g procured from National Laboratory Animal Center, Central Drug Research Institute (CDRI), Lucknow were used for the study. The animals were housed separately in polypropylene cages for acclimitization at a temperature of (23 ± 2 ºC) and 50–60% relative humidity, with a 12 hr light/dark cycle one week before and during the commencement of the experiment. Animal were kept on standard pellet diet (Dayal animal feed, Unnao, India) and drinking water *ad libitum* throughout the housing period. All experimental procedures involving animals were conducted in accordance with the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The study protocols were approved by the Institutional Animal Ethics Committee (IAEC) of Integral University, Faculty of Pharmacy, Lucknow, India (Reg. no. 1213/GO/ac/08/CPCSEA).

Experimental Protocol

Totally 25 experimental animals (female wistar rats) randomly divided into five groups consisting of five rats (n=5) per group were used in this study as follows:

Group I: Control received 0.3% CMC (vehicle) orally for 8 days throughout the study.

Group II: Female wistar rats received 12 ml/kg bwt of 40% ethanol which served as acute ethanol-intoxicated control.

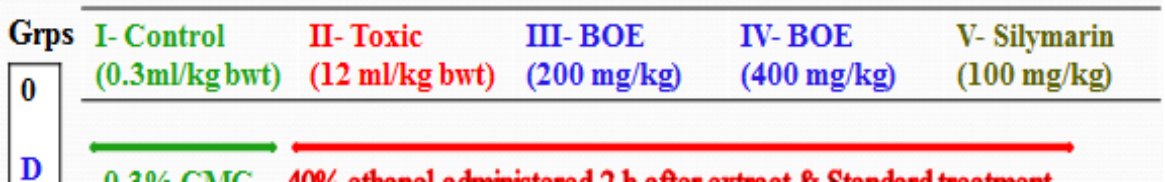
Group III: Rats were treated with*Bixaorellana*shell extract (BOE) dose 200 mg/kg bwt before the dose of ethanol orally for 8 consecutive days

Group IV: Rats received *Bixaorellana*fruit shell extract (400 mg/kg bwt) as in group III 2 h before the ethanol dose (12 ml/kg bwt)

Group V: Rats received standard drug Silymarin (100 mg/kg bwt) as in group III followed by the dose of ethanol for 8 continuous days[16].

Serum collection and organ isolation

At the end of the treatment i.e., six hours after the administration of last single dose of 40% ethanol, rats were anaesthetized and blood was collected by retro-orbital plexus followed by heart puncture as per the regulations of Good Laboratory Practices. After withdrawal of blood the animals were sacrificed. The serum was separated by centrifugation at 3000 rpm at 4°C for 20 minutes and stored at −80°C for the analysis of various biochemical parameters. The isolated liver tissue was washed twice with ice cold saline, blotted, dried and then weighed. The relative liver weight was calculated as the percentage ratio of liver weight to



the body weight. A small portion of the tissue was fixed in formalin for histopathalogical examination.

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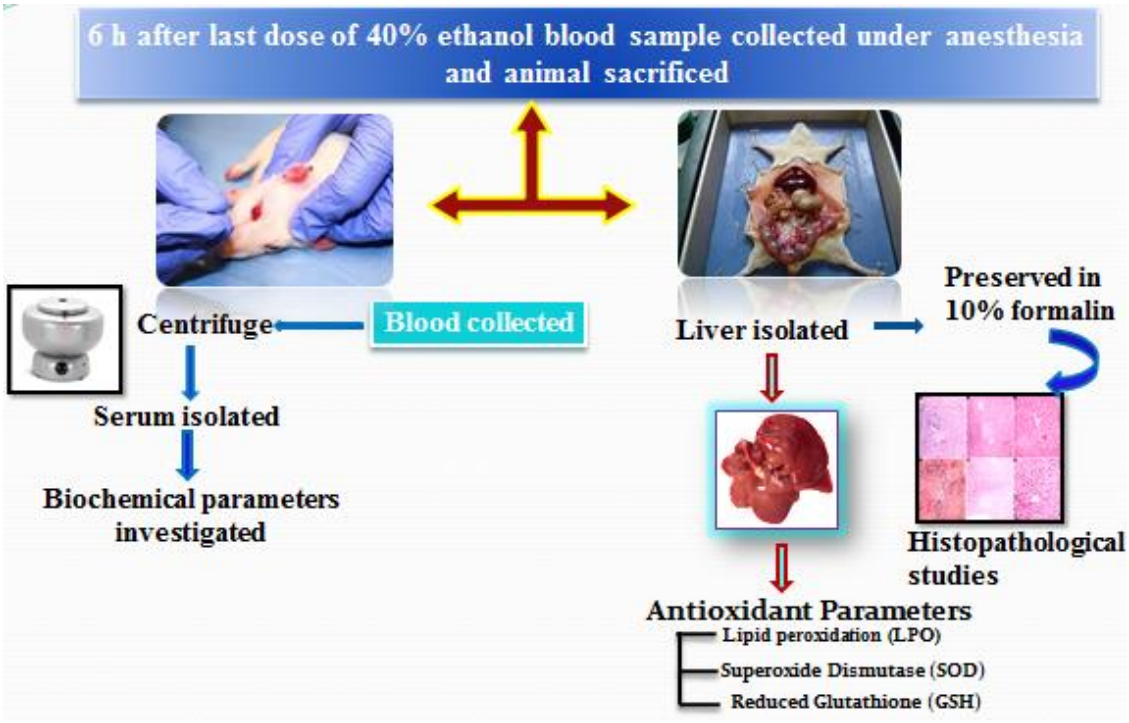

Assessments of Liver Function Test (L.F.T)

Serum enzymes like, Serum alanine transaminase (ALT), Alkaline Phosphate (ALP), Aspartate Transaminase (AST), Bilurubin, Total Cholesterol and Albumin (ALB) were determined by using standard kits from Span diagonistic ltd, Surat, India. Serum lactate dehydrogenase (LDH) was estimated by using standard kits from Accurex biomedical Pvt. ltd, Mumbai, India. All estimations were carried out using UV spectrophotometer (Shimadzu, India) as per standard kit methods.

Histopathological assessment

For histologic studies, the liver tissues were fixed with 10% phosphate buffered neutral formalin, dehydrated in graded (50-100%) alcohol and embedded in paraffin. Thin sections (5 M) were cut stained with routine hematoxylin and eosin stain for photo microscopic assessment. The examination was qualitative, with the purpose of determining histopathological lesions in liver tissue.

Statistical analyses

The data were represented as mean ± standard error of mean (S.E.M.) for five rats. Student t-test was followed by individual comparison by Newman- Keuls test using GraphPrism Pad software (Version 6.05, GraphPad Software, Inc. USA) for the determination of level of significance. The value of probability less than 5% ($P < 0.05$) was considered statically significant.

RESULTS

Standardization of plant extract by Thin Layer Chromatography

A number of spots on the TLC plates of the plant extract identified in **figure 1** clearly indicate the presence of various chemical compounds. Retention factors (R_f) was calculated. Different ratios of 1) EtOAc-MeCOEt-HCO,H-H,O; (2) n-BuOH-HOAc-H,O;(3) CHCl,-EtOAc-HCO,H; Methanol: water: acetic acid (50/44/6, v/v/v)(Prunin, Naringin,

andNaringenin) gave many R_f values for the plant extract of *Bixaorellana*as mentioned in **Table 1.**

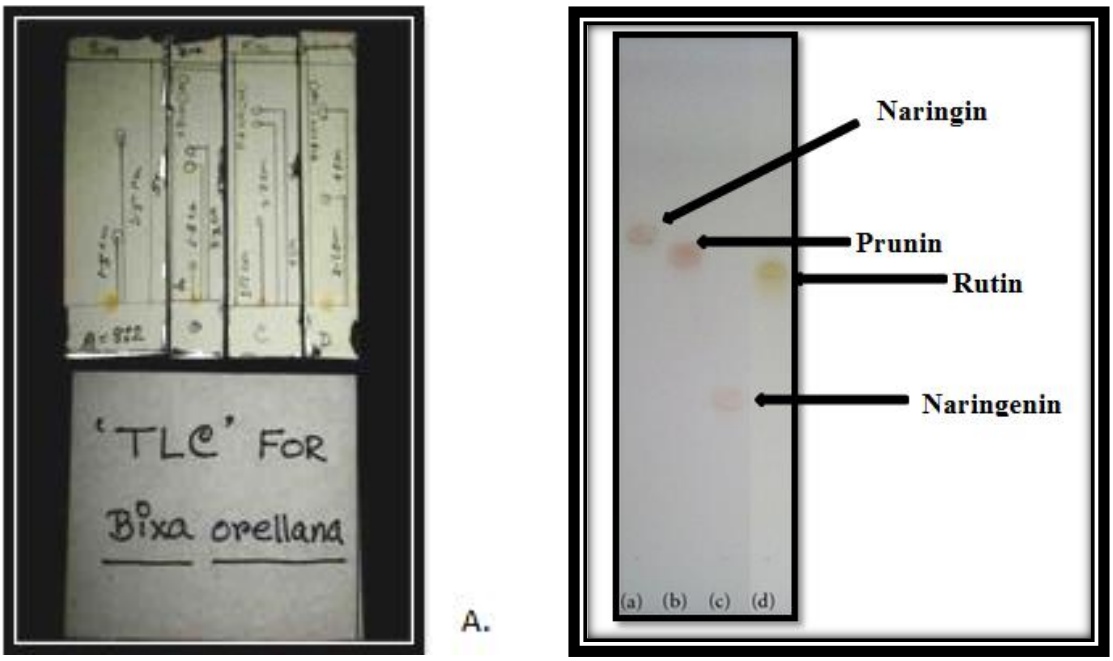

FIG 1: TLC PLATE FOR IDENTIFICATION of: (A) BIXIN (B) AMINO ACIDS (LEUCINE, GLYCINE and PROLINE) FROM *BIXA ORELLANA* EXTRACT.

Table 1: VARIOUS R_f VALUES CALCULATED FOR DIFFERENT SOLVENTS RATIOS FOR *B. ORELLANA* EXTRACT.

Extract of *Bixaorellana* for identification	Solvent system	Ratio	R_f values
Prunin **Naringin** **Naringenin**	TLC was performed on sihca gel fluorescent plates. Plates were developed in the following ascending solvent systems at room temp. (1) EtOAc-MeCOEt-HCO,H-H,O; (2) n-BuOH-HOAc-H,O; (3) CHCl,-EtOAc-HCO,H;	1) (5 : 3 : 1: 1) 2) (4: 1.5) 3) (5: 13:2)	0.5; 0.75; 1.0;
Prunin; Naringin; Naringenin	Methanol: water: acetic acid (50/44/6, v/v/v)	5:4:1	0.58; 0.62; 0.30

Animal experiment

Administration of ethanolic BOE extract in rats without introducing ethanol was performed to determine the BOE effect alone if it can cause hepatic damage. The effect of 50% Bixaorellana ethanolic extract on liver weight, body weight and serum marker enzymes was found to attenuate the toxic effect of 40% ethanol on rats, thereby contributing to its antihepatotoxic potential. The effect of 50% ethanolic extract of Bixaorellana on body weight, liver weight and relative liver weight in control and 40% ethanol - induced hepatotoxicity in rats is summarised in Table 2.

Table 2: EFFECT OF *BIXA ORELLANA*SHELLS EXTRACT ON BODY WEIGHT, LIVER WEIGHT AND RELATIVE LIVER WEIGHT OF CONTROL AND ETHANOL INDUCED HEPATOTOXICITY IN RATS.

Groups	Treatment	Initial body weight (g)	Final body weight (g)	Liver weight (g)	Relative liver wt. (liver weight/100g b.w.)
I	Control	184.2 ± 6.9	201.2 ± 11.3	6.15 ± 0.42	3.05 ± 0.27
II	40% ethanol 12ml/kg	180.5 ± 8.4	165.7 ± 10.4$^{\#}$	9.02 ± 0.64$^{\#}$	5.44 ± 0.52$^{\#\#}$
III	*B. orellana*200 mg/kg	179.4 ± 9.3	191.5 ± 12.4*	7.39 ± 0.58*	3.85 ± 0.43**
IV	*B. orellana* 400 mg/kg	186.6 ± 8.5	203.7 ± 13.5*	6.21 ± 0.42**	2.69 ± 0.25***
V	Silymarin	183.2 ± 6.7	202.8 ± 14.7**	6.11 ± 0.57**	3.01 ± 0.32***

Values are expressed as mean ± SEM of 5 rats in each group. P values :$^{\#}$<0.01, $^{\#\#}$<0.001 compared with respective control group I. P values :*<0.05, **<0.01 and *** <0.001 compared with group II (Ethanol).

I. **BIOCHEMICAL LIVER ENZYMES:** The effect of 50% ethanolic extract of Bixaorellana on liver injury in control and ethanol -induced hepatotoxicity in rats is shown in figure 2, figure 3, and figure 4. Various biochemical enzymes levels were affected by the effect of 50% ethanolic Bixaorellana extract including serum aspartate transaminase (AST), alanine transaminase (ALT), Alkaline Phosphate (ALP), Lactate Dehydrogenase (LDH), Cholesterol (CHL), Bilirubin, and Albumin.

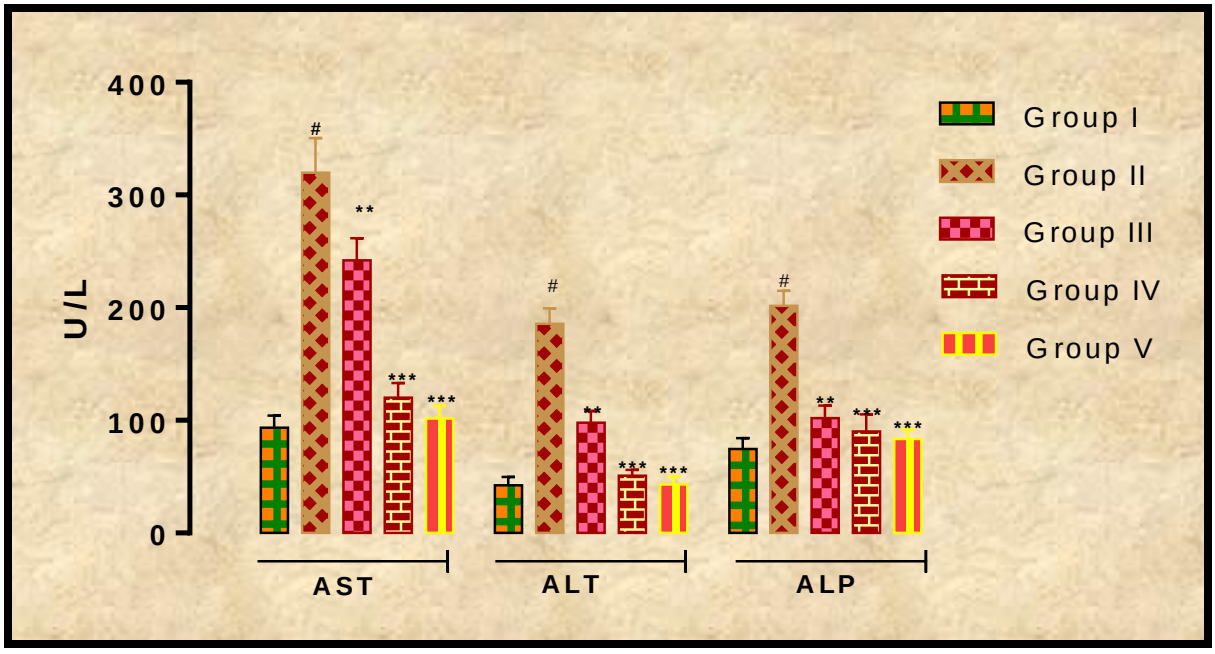

FIG 2: Effect of *Bixaorellana*shell extract on the activities of Aspartate Transaminase (U/L), Alanine Transaminase (U/L) and Alkaline Phosphatase (U/L) against ethanol induced hepatotoxicity in rats. Values are expressed as Mean ± S.E.M. of 6 rats in each group. P values: #<0.001 compared with respective control group I. P values: *<0.05, **<0.01, ***<0.001 compared with group II (Ethanol).

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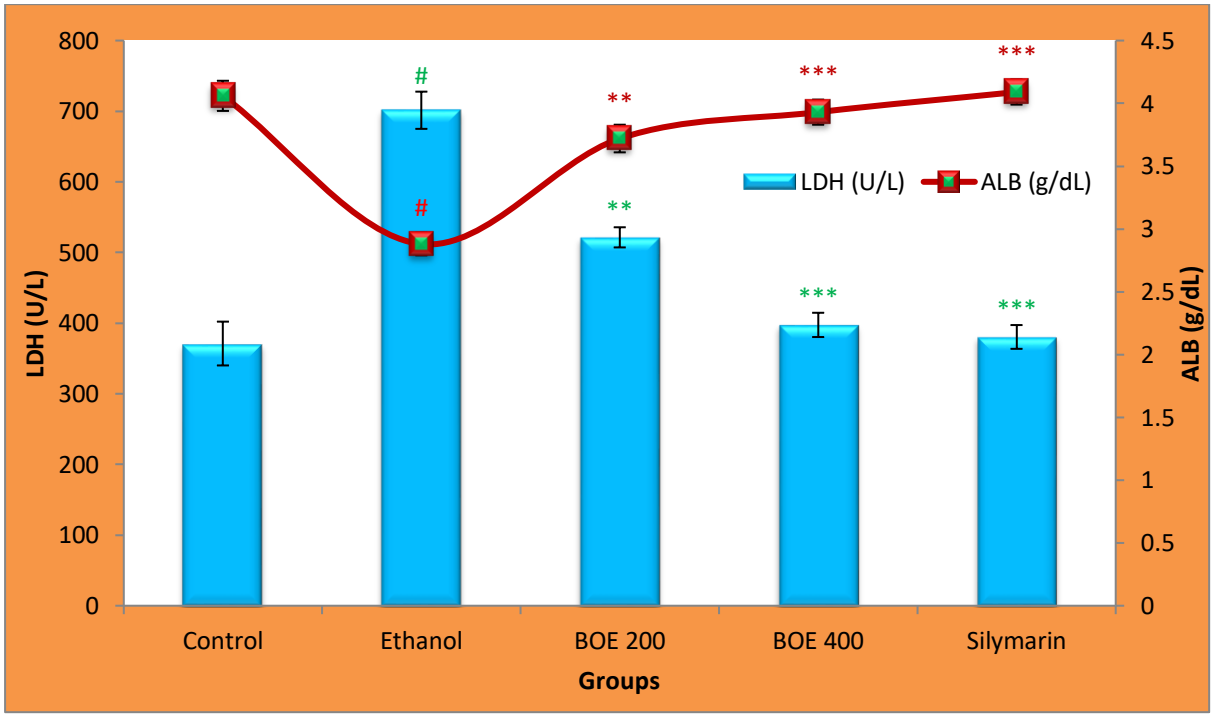

FIG 3: Effect of *Bixaorellana*shells extract on Lactate Dehydrogenase (LDH) and Albumin (ALB) against ethanol induced hepatotoxicity in rats. Values are expressed as Mean ± S.E.M. of 6 rats in each group. P values:#<0.001 compared with respective control group I; *<0.05, **<0.01, ***<0.001 compared with group II (Ethanol).

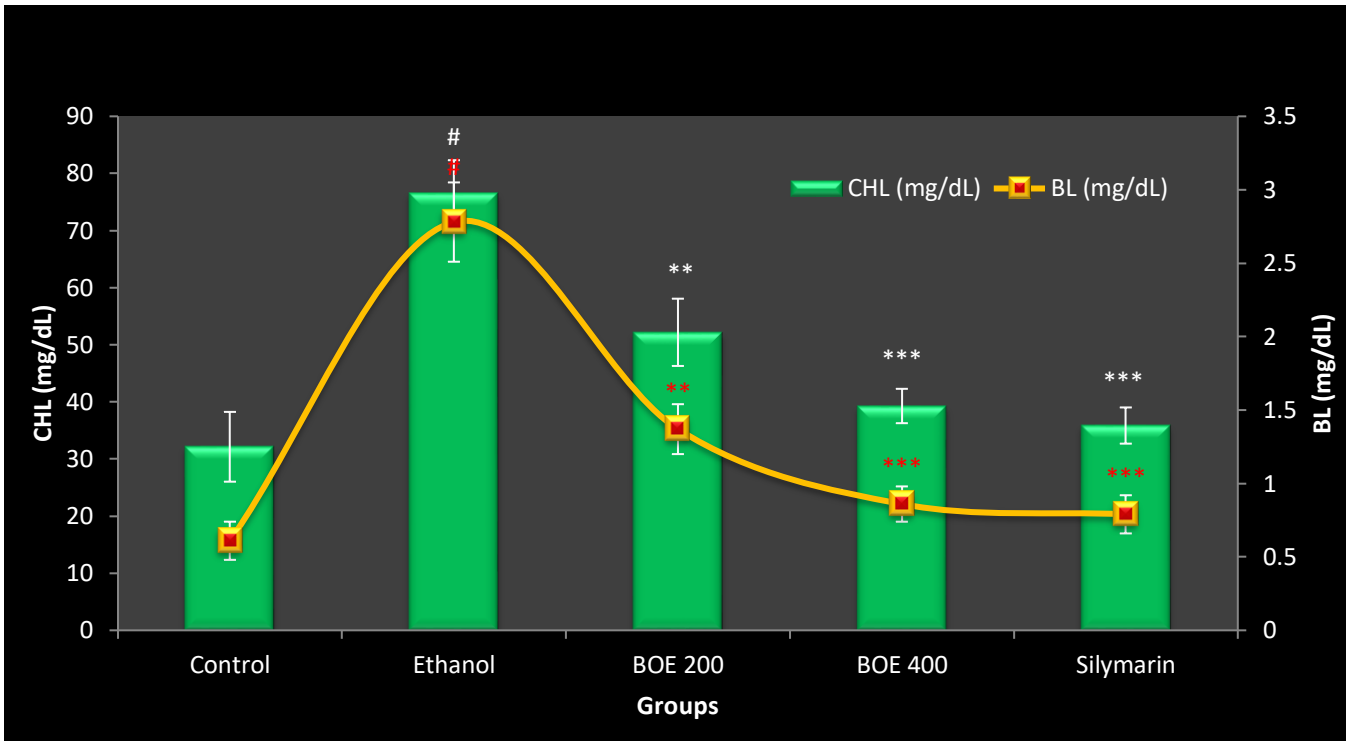

FIG. 4: Effect of *Bixaorellana*shells extract on the activity of Cholesterol (CHL) and Bilirubin (BLB) in the serum control and ethanol induced hepatotoxicity in rats. Values are expressed as Mean ± S.E.M. of 6 rats in each group. P values: $^{\#}$<0.001 compared with respective control group I; *<0.05, **<0.01, ***<0.001 compared with group II (Ethanol).

II. Antioxidant Liver enzymes

The LPO levels in liver homogenate were found to be significantly increased in the ethanol treated Group II rats. The LPO value of control Group I was 0.39 ± 0.06 U/mg protein which increased to 0.58 ± 0.08 U/mg protein ($P < 0.001$) [Table 2]. Administration of BOE showed a significant reduction in LPO at dose of 400 mg/kg as 0.38 ± 0.06 showing the significant value of $P < 0.001$. Ethanol treatment (Group II) decreased the levels of hepatic GSH from 0.98 ± 0.08 to 0.29 ± 0.05 ($P < 0.001$) when compared to Group I animals [Table 2]. However, BOE at 200 and 400 mg/kg significantly increased the levels of GSH from 0.65 ± 0.04 to 0.97 ± 0.07 ($P < 0.05$ to $P < 0.001$), when compared to Group II rats. Silymarin at 100 mg/kg significantly reduced the elevated LPO level (0.36 ± 0.05 U/mg protein, $P < 0.001$) but increased the levels of GSH, (0.99 ± 0.05 ($P < 0.001$), compared to ethanol-treated Group II rats.

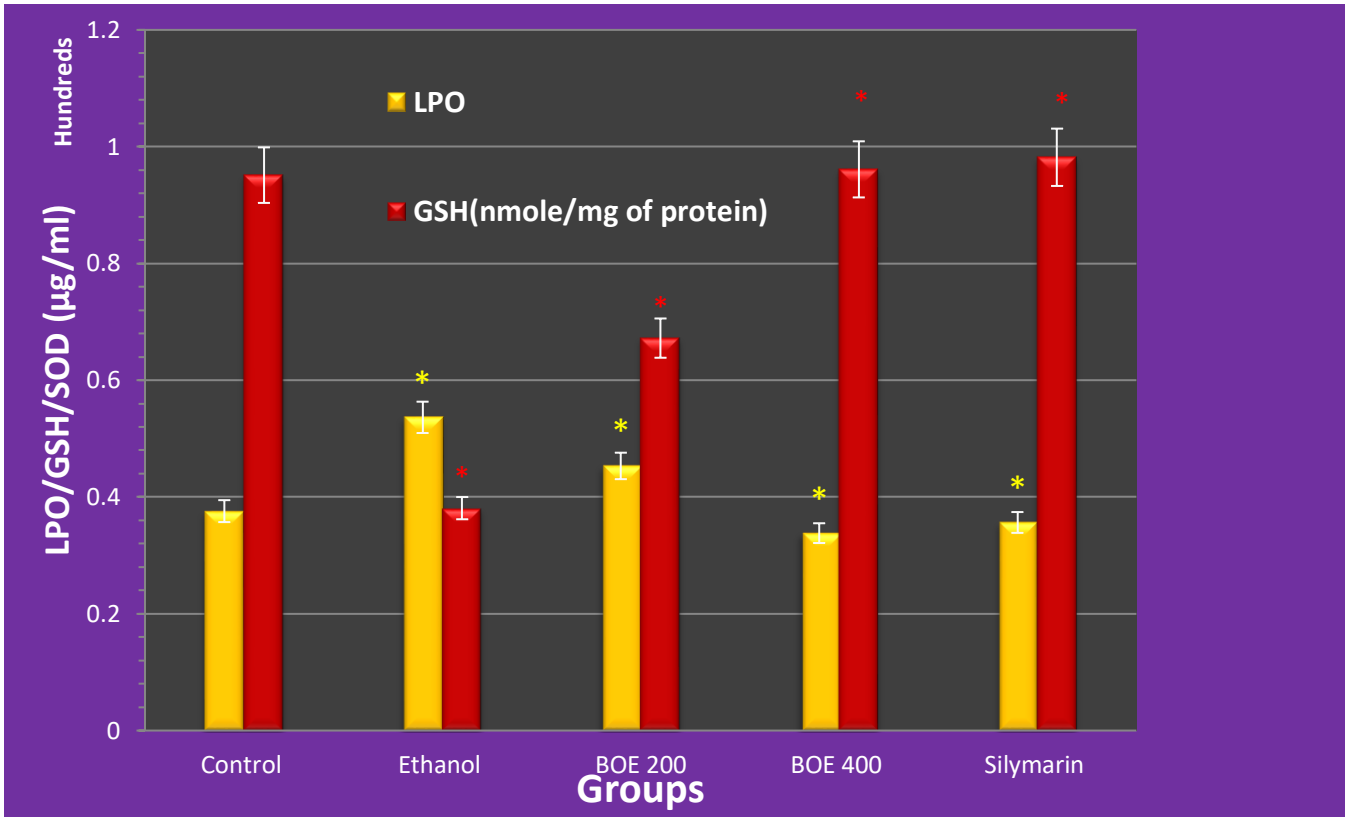

FIG: Effect of*Bixaorellana*shells extract on Lipid Peroxidase (LPO), Reduced Glutathione (GSH), Superoxide Dismutase (SOD) against ethanol induced hepatotoxicity in rats. Values are expressed as mean ± S.E.M. of 6 rats in each group. P values:*<0.05 compared with control group and group II (Ethanol)

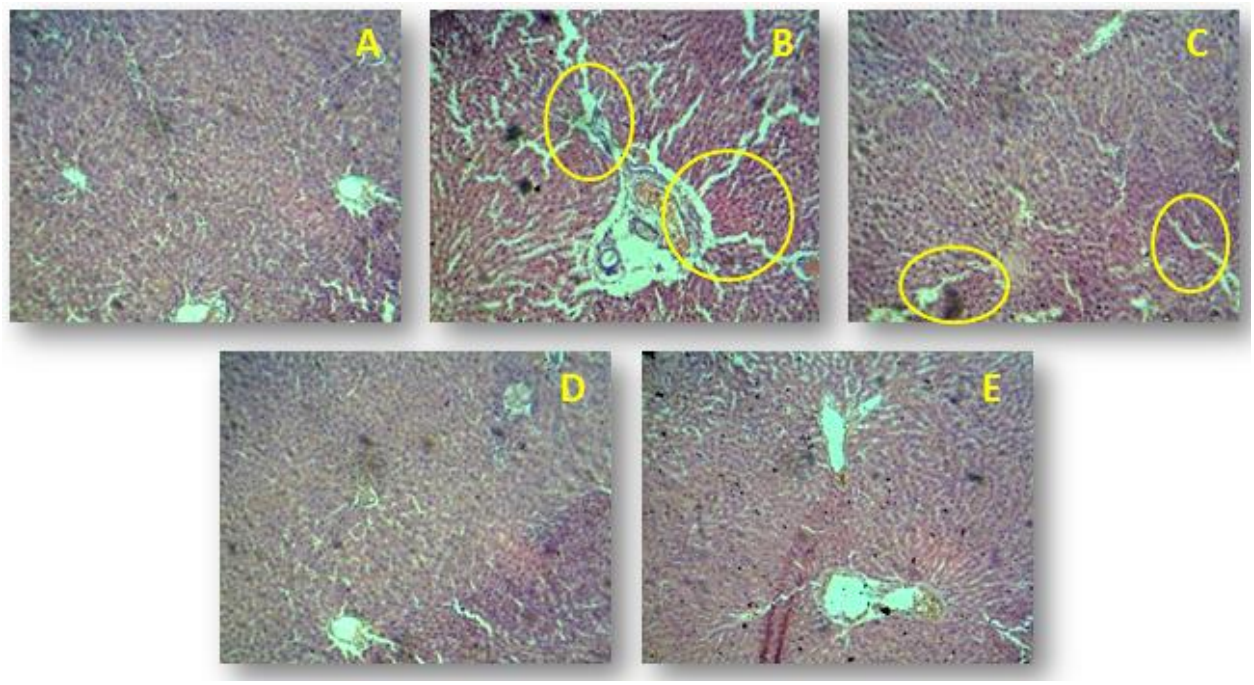

FIG 5: HISTOPATHOLOGY OF LIVER TISSUES (Hematoxylin AND Eosin). (A) Liver section of normal control (B) Liver section of ethanol treated rats group II (C) Liver section of rats treated ethanol and 200 mg/kg of BOE group III (D) Liver section of rats treated ethanol and 400 mg/kg OF BOE group IV (E) Liver section of rats treated ethanol and 100 mg/kg of Silymarin group V.

Histopathological Studies

The histopathological evaluation of liver tissues of experimental groups (I-V) of rats (hematoxylin and eosin) was observed as described. (A) Liver section of normal control rat shows uniform hepatocytes with small vesicular nuclei and architecture of liver is well maintained. (B) Liver section of ethanol treated rats showed smaller hepatocytes and eosinophilic cytoplasmic with indistinct cell boundaries showing proliferation. Architecture not well maintained but vascularity has relatively increased (indicated by arrow). (C) Liver section of rats treated ethanol and 200 mg/kg of BOE indicates less inflammatory cells, mild focal necrosis with sinusoidal dilatation. (D) Liver section of rats treated ethanol and 400 mg/kg of BOE showed normal vesicular nuclei and abundant eosinophilic cytoplasm with distinct cell boundaries. Architecture well maintained with normal interstitial cells and vascularity. (E) Liver section of rats treated ethanol and 100 mg/kg of Silymarin showing normal morphology of hepatocyte.

DISCUSSION

Liver disease due to alcohol consumption is a common cause of death in adults; medicinal support to alcohol-induced liver dysfunction is indeed very meager. In order to reflect the protective effect of *Bixaorellana*, we established the alcohol-induced acute liver injury model in experiment. In the present study, the *Bixaorellana*extract (BOE) was observed to exhibit hepatoprotective effect by using 40% ethanol induced hepatotoxicity in Wistar rat.

Ethanol-induced acute liver injury demonstrated that there was a significant increase in aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Bilirubin (BIL), Cholesterol (CHL), lactate dehydrogenase (LDH) and decrease in albumin (ALB) concentrations in ethanol induced toxicity (Group II) animals. An augment in the serum levels of these marker enzymes occurred due to the enzymes leakage from liver into the circulation as a consequence of liver damage[19].The AST and ALT actions are responsive indicators of acute hepatic necrosis, and the ALP level is indicator of hepatobiliary disease[20]. Significant reduction in the levels of AST, ALT, ALP, LDH, CHL, Bilirubin and increase in the level of ALB towards the normal value was an indication of the stabilization of plasma membrane and the repair of hepatic tissue. Histological examination of the liver sections revealed lipid change of hepatocytes treated with 40% alcohol, while in the sections obtained from the rats treated with BOE and with alcohol, the lipid change of hepatocytes was alleviated.

Ethanol consumption leads to the production of excessive free radical inside the living system. This is due to the fact that ethanol is extensively metabolized by the microsomal oxidizing system to acetaldehyde and 1-hydroxyethyl radicals by cytochrome P_{450} II E1. These free radicals cause peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes. Ethanol is extensively metabolized to acetaldehyde in the liver by the enzyme alcohol dehydrogenase. Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase/oxidase, leading to the generation of reactive oxygen species (ROS)[21]. Further oxidations in alcohol metabolism are accompanied by an excessive reduction of nicotinamide adenine dinucleotide (NAD), with a shift in NADH/NAD ratio. Under normal circumstances, reduction of NAD (changing to NADH) is finely regulated by the cell Krebs cycle. These reactive species oxidize cellular biomolecules, such as proteins and DNA and initiate membrane peroxidative degradation in the adipose tissue resulting in fatty infiltration of the hepatocytes and causing impairment of gluconeogenesis and diversion of metabolism to ketogenesis and fatty acid synthesis[22].

The toxic effect of ethanol was controlled in the animals treated with ethanolic shells extract of *B. orellana* at the dose of 200 and 400 mg/kg respectively, by restoring the levels of liver function, indicating its protective activity against liver damage (**Figure 2, 3, 4**). This

protective effect could be possibly due to the reduction in the tissue damage brought by the ethanolic extract of *B. orellana.* The results were compared with the standard drug Silymarin (100 mg/kg). The increase in the levels of serum bilirubin reflects the depth of jaundice as the clear indication of cellular leakage and loss of functional integrity of cell membrane. Bilirubin is the conventional indicator of liver diseases, restoration of total bilirubin levels may be due to the inhibitory effects on cytochrome P_{450} resulting in the hindrence of the formation of hepatotoxic free radicals [21]. A marked elevation was observed in serum bilirubin level (SBL) of ethanol treated groups, whereas albumin level in the serum was markedly decreased. A reduction in synthesizing proteins was seen following intoxication of the liver with hepatotoxicants. As seen in the silymarin treated group and ethanolic extract of *B. orellana,* all studied parameters were restored to normal conditions from the abnormal ones.

The protective effect of *B. orellana*may also be attributed to its antioxidant activity as evaluated by Chisté et al., and Bell et al.,[23, 24]for acetaminophen induced- oxidative stress causing hepatic damage in rats. Therefore, from the results it is clear that *Bixaorellana*shells extract has shown dose dependent activity among which at the dose level of 400 mg/kg, p.o. shows greater activity that is comparable with the control and Silymarin treated groups (Standard).

CONCLUSION

Concluding from the above results it indicates that the ethanolic shells extract of *Bixaorellana* supplementation could antagonize the development of oxidative liver injury induced by acute ethanol exposure in Wistar rats. BOE (200 and 400 mg/kg) administration significantly attenuated the ethanol-induced liver injury. Furthermore, BOE supplementation prevented the acute ethanol-induced enhancement of hepatic enzymes. Liver histopathology images evidenced that *BOE* attenuated the hepatocellular necrosis and led to reduction in inflammatory cells infiltration, which may be attributed to its hepatoprotective effects.

The AST and ALT actions are responsive indicators of acute hepatic necrosis, and the ALP level is indicator of hepatobiliary disease. The study powerfully supports that the protective effect of *Bixaorellana* shell extract significantly attributes to the hepatoprotective activity caused by alcohol toxicity as it reverses the altered liver marker enzymes back to normal. It also showed that the antioxidant effect contributes to its antihepatotoxic activity. Significant reduction in the levels of AST, ALT, ALP, LDH, CHL, Bilirubin and increase in the level of ALB towards the normal value was an indication of the stabilization of plasma membrane and the repair of hepatic tissue.

The hepatoprotective effects of the ethanolic shells extract of *Bixaorellana* possesses antioxidant activities as proved earlier and ameliorate hepatic function of hepatic injury induced by alcohol. Clinically, application of *B. orellana* extract for liver protection against liver injury induced by ethanol in patient demands further investigation, yet proving to be the promising herbal drug to be included in the list of herbal drugs against alcohol induced hepatotoxicity.

FUTURE RESEARCH HYPOTHESIS

- Future research with *Bixaorellana* shells should focus on ethanol metabolism by the microsomal oxidizing system (cytochrome P_{450} II E1) to reduce the production of free radicals which oxidize cellular biomolecules, such as proteins and DNA, finally resulting in fatty infiltration of the hepatocytes and causing impairment of gluconeogenesis and diversion of metabolism to ketogenesis and fatty acid synthesis.
- Implications of such studies could eventually lead to novel treatment for hepatic disorders and mitochondrial damage.

CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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