EVALUATION OF *IN VITRO* ANTIOXIDANT AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF *BAUHINIA VARIEGATA* ROOT ETHANOLIC EXTRACT AGAINST CCL_4 INDUCED LIVER INJURY IN RATS

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ABSTRACT

Plant derived herbal formulations and remedies provide an effective means for the treatment of various types of disease that are dogmatic and incurable in other types of systems of medicines, but it is essential to explore and establish the scientific basis for therapeutic action of herbal plant medicines. *Bauhinia variegata* root ethanolic extract was studied for the hepatoprotective activity against CCl_4 induced liver injury in rats. For estimation of hepatoprotective role of *B. variegata*, total bilirubin count, serum enzymes level and finally histopathological study of liver were performed. This extract's DPPH radical scavenging potential was also studied. The ethanolic extract of *B. variegata* root administered orally to animal showed significant depletion in CCL_4 induced increased level of SGPT, SGOT, ALP and total bilirubin concentration. Significantly ($p<0.05$), hepatotoxicity is reversed by treatment with Liv 52 syrup also. For initiation of biochemical analysis, the histopathological studies are provided supportive evidence. This extract also showed better activity in quenching DPPH radical. The ethanolic extract of *B. variegata* root shows antioxidant property by preventing the formation of trichloromethyl peroxy radicals, and thus reduce tissue damage, which is examined and confirmed by the histopathological studies. Therefore, the hepatoprotective activity of ethanolic extract of *B. variegata* root may be due to its antioxidant potential. Previously studies have reported that plants containing flavonoids possess antioxidant properties. The antioxidant and hepatoprotective properties of the test plant may be attributed to the presence flavonoids. *B. variegata* root ethanolic extract is shown to have hepatoprotective and antioxidant action.

Keywords: *Bauhinia variegata*, hepatotoxicity, antioxidant, CCL_4 induced hepatopathy, histopathology, flavonoids

INTRODUCTION

Metabolism, secretion and storage are the essential vital functions of the liver. Any injury to the liver can result in many disorders, ranging from transient elevation in liver enzymes to life threatening liver cirrhosis and hepatic failure. The common causative agents of liver injuries are toxic chemicals (e.g. CCl_4, aflatoxin etc.), therapeutic drugs (e.g., antibiotics, anti-tubercular drugs etc), alcohol and microbial agents e.g. hepatitis virus, leptospira and malarial parasites[1]. Reactive oxidant species (ROS) are responsible for the pathologies of various diseases. Several pieces of evidence suggest that ROS are necessary for normal metabolism but can be detrimental to health as well including outcome of various diseases like metabolic disease (diabetes mellitus), neurodegenerative disorders, immunosuppression and several other of diseases[2].

Free radicals lead to cellular necrosis, which is implicated in some membrane pathophysiological conditions, including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotics[3]. Liver diseases

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remain a serious health problem. Oxidative stress causing cellular damage through mechanism of covalent binding and lipid peroxidation with subsequent tissue injury is well established. Antioxidant agents of natural origin have attracted special interest because they can protect the human body from free radicals[4]. Numerous medicinal plants and their formulations are used for liver disorders in ethnomedical practices as well as in traditional systems of medicine in India[5]. Various plants species have been utilized as traditional medicines because the plant derived herbal medicine formulations have potential to cure the symptoms of a number of diseases. But it is necessary to establish the hidden potential of traditional medicines on scientific basis for the therapeutic action of traditional plant medicines, as these may serve as the major source for the development of more effective drugs.

B. variegata Linn (Caesalpiniaceae) tree, commonly known as Kachnar, is found throughout India. It is a small tree with an openly branched irregular crown, and slightly rough, brownish bark. Flowers are large, white or pink in color and orchid shaped.

The fruit is a pod with ten to fifteen seeds[6]. Both roots and bark are astringent, acrid, constipating and anthelmintic. They are useful in diarrhea, dysentery, cough, leprosy and diabetes. Dried buds are useful in diarrhea, worms, piles and dysentery. Bark is tonic to the liver. The plant is used in malaria and as an antidote to snake poison[7]. Plant is also having promising antitumour, cytotoxic, antimicrobial and anti-inflammatory activities[8]. Phytochemical analysis of the root bark of *B. variegata* Linn yielded a new flavanone, (2*S*)-5,7-dimethoxy-3,4-methylenedioxyflavanone and a new dihydrodibenzoxepin,5,6-dihydro-1,7-dihydroxy-3,4-dimethoxy-2-methyldibenz [b,f] oxepin, together with three known flavonoids[9]. The literature screened in the process of the proposed work indicates that the selected plant contains classes of chemical constituents which have shown antioxidant activity. Literature survey revealed that *B. variegata* root ethanolic extract has no scientific claims for hepatoprotective and antioxidant activity. Phytochemical as well as pharmacological estimation of this plant may provide useful information and evidence for further studies and also provide material for better management for preventing the production of oxidative stress.

MATERIALS AND METHODS

Animals

Healthy adult male Wistar albino rats weighing between 170-200 g were used for the hepatoprotective studies, whereas Wistar albino rats of either sex were used for determination of acute toxicity study. The animals were housed in groups of 5 per cage with free access to commercial rat pallet diet (Lipton India Ltd., Mumbai, India) and water *ad libitum*. The animal room was maintained at 25 °C ± 2 °C with timed lighting on from 6 am to 6 pm and relative air humidity of 30 % to 60 % . The Institutional Animal Ethics Committee (CPCSEA/1/15/2007) approved the study.

Chemicals

All chemicals and solvents used were of analytical grade from Merck Ltd., Mumbai, India and Sigma Aldrich Co., USA. Liv 52 syrup was obtained from Himalaya Drug Company, India.

Collection of plant material

The roots of *B. variegata* Linn was collected from local areas of Kolhapur (Maharashtra) and Belgaum (Karnataka) India. The specimen was authentificated from Dr. S.R. Yadav, Prof., Dept. of Botany, Shivaji University, Kolhapur (Maharashtra) India. The voucher specimen (KLEU/Pharm/ 07/15) was retained in the Herbarium of Department of Pharmacognosy, K.L.E. University, College of Pharmacy, Belgaum (Karnataka) India.

Preparation of plant extract

The collected plant material was washed thoroughly in water, chopped, shade dried at room temperature, reduced to a coarse powder in a mechanical grinder and passed through a 40 # sieve for desired particle size. The powder obtained was subjected for the extraction, with 95 % ethanol in a Soxhlet apparatus. The extract was concentrated under reduced pressure and dried. The yield of *B. variegata* root ethenolic extract was 4.3 % (w/w). The obtained extract was stored in a refrigerator at 2-8 °C until usage.

Preliminary phytochemical investigations

Preliminary phytochemical investigation revealed the presence of tannins, alkaloids (bark), sesquiterpenes and monoterpenes in volatile oils (leaves)[10], flavanone and flavonoids (root)[9] and phenanthraquinones (stem)[11] in the *B. variegata* plant.

Experimental design

Screening of *B. variegata* root ethanolic extract for hepatoprotective and antioxidant action was done in rats.

Acute toxicity study

Determination of LD_{50} for extracts was done as per OECD guidelines for fixing the dose for biological

evaluation. The animals were fasted overnight prior to the experiment and maintained under standard conditions. The LD_{50} of the extract as per OECD guidelines 2001 falls under 5 mg, 50 mg, 300 mg and 2000 mg kg^{-1} bw with no signs of acute toxicity at respective doses. The biological evaluation of extract is carried out at 1/10 doses of LD_{50}[12].

Hepatoprotective activity

Hepatoprotective activity was carried out by using albino rats. The animals were divided into four groups of six rats in each. 1% gum acacia suspension was given to groups I and II as a vehicle for 10 days by oral route. Liv 52 was administered as a standard drug to group III at a dose of 1mL kg^{-1} by oral route up to 10 days and IV^{th} group received *B. variegata* ethanolic extract 200 mg kg^{-1} by oral route up to 10 days.

Except group I (control group), all remaining groups received carbon tetrachloride at a dose of 0.7 mL kg^{-1}, on 3, 6 and 10th day by intraperitoneal route. On 10 th day, 1 h after last dose of carbon tetrachloride, animals were sacrificed by cervical dislocation and the blood was collected from the carotid artery, serum was separated and used for estimation of biochemical parameters such as SGPT, SGOT, ALP and total bilirubin. Liver was excised, quickly fixed in 10 % formalin and then fixed in bovine solution and further histopathological study was done for observation of architectural changes[13].

In vitro antioxidant –DPPH free radical scavenging activity

The free radical scavenging activity of *B. variegata* root ethanolic extract was measured by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH)[14]. For DPPH assay, the method of Blois was adopted. The capacity of *B. variegata* root ethanolic solvent extract to scavenge the lipid-soluble DPPH radical was monitored at an absorbance of 517 nm. Ethanolic root extract (1 mL) of *B. variegata,* at different concentration was allowed to react with DPPH. Thirty minutes later, the absorbance was measured at 517 nm. The percentage inhibition of absorbance was calculated for each concentration relative to a blank absorbance using the spectrophotometer. The DPPH scavenging capacity of the extracts is compared with that of BHT (butylated hydroxytolune). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. All determinations are carried out at least three times. IC_{50} value in the tested compound is the concentration required to scavenge 50 % DPPH free radical. Percentage inhibition was calculated as DPPH radical scavenging activity.

DPPH radical Scavenging effect (%) = (Abs control – Abs sample) / (Abs control) × 100

where, Abs control is the absorbance of initial conc. of DPPH radical;

Abs sample is the absorbance of DPPH radical + sample extract / standard

Statistical analysis

Values are presented as mean ± S.E.M. Statistical difference between treatments and the controls were tested by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test using the "Stat" statistics computer program. A difference in the mean values of $P<0.05$ was considered to be statistically significant.

RESULTS

Acute toxicity study

Acute toxicity study revealed no mortality or any toxic reactions with oral administration of ethanolic extract of root of *B. variegata* even at the highest dose (2000 mg kg^{-1}). The biological evaluation of extract was carried out at 1/10 doses of LD_{50}[12].

Hepatoprotective activity study

Rats subjected to CCl_4 only, developed significant hepatocellular damage as evident from significant increase in serum activities of GPT, GOT, ALP and total bilirubin concentration as compared to normal control group, which has been used as reliable marker of hepatotoxicity. Oral administration of ethanolic extract of *B. variegata* root (200 mg kg^{-1}, p.o) exhibited significant reduction ($p<0.05$) in CCl_4-induced increase in levels of GPT, GOT, ALP and bilirubin (total) concentration. Treatment with Liv 52 syrup also reversed the hepatotoxicity significantly ($p<0.05$) (Table I).

Histopathological studies

Histopathological studies also provided supportive evidence for biochemical analysis. Histology of the liver section of normal control animals showed normal hepatic cells with well preserved cytoplasm, prominent nucleus and nucleolus and well brought out central vein (A). The liver sections of CCl_4-intoxicated rats showed massive fatty changes, necrosis, hemorrhagic foci in hepatic parenchyma, ballooning degeneration and broad infiltration of the lymphocytes and the loss of cellular boundaries (B). The histological architecture of liver sections of rats treated with ethanolic extract of *B. variegata*

Table I: Effect of *B. variegata* root ethanolic extract on the serum enzymes and total bilirubin in CCl_4-induced hepatotoxic rats after 10 days of treatment

Exp. Group (n= 6)	Treatment	SGPT (U/L)	SGOT (U/L)	ALP (U/L)	TOTAL BILIRUBIN (mg dL^{-1})
I	Normal control (1% gum acacia)	131.0±20.5*	86.00±17.1**	161.0±15.2**	0.700±0.07**
II	CCl_4 control	217.0±28.7	340.0±21.0	385.0±27.3	2.123±0.1
III	Liv 52 syrup	140.0±19.1*	182.0±5.1**	219.0±12.2**	0.800±0.1**
IV	*B. variegata* ethanolic extract (200 mg kg^{-1})	149.0±27.1*	198.5±4.4**	292.0±38.4*	0.856±0.1**
**P < 0.05 & **P < 0.01 Significant, compared to CCl_4 control*					
n= no of animals in each group					

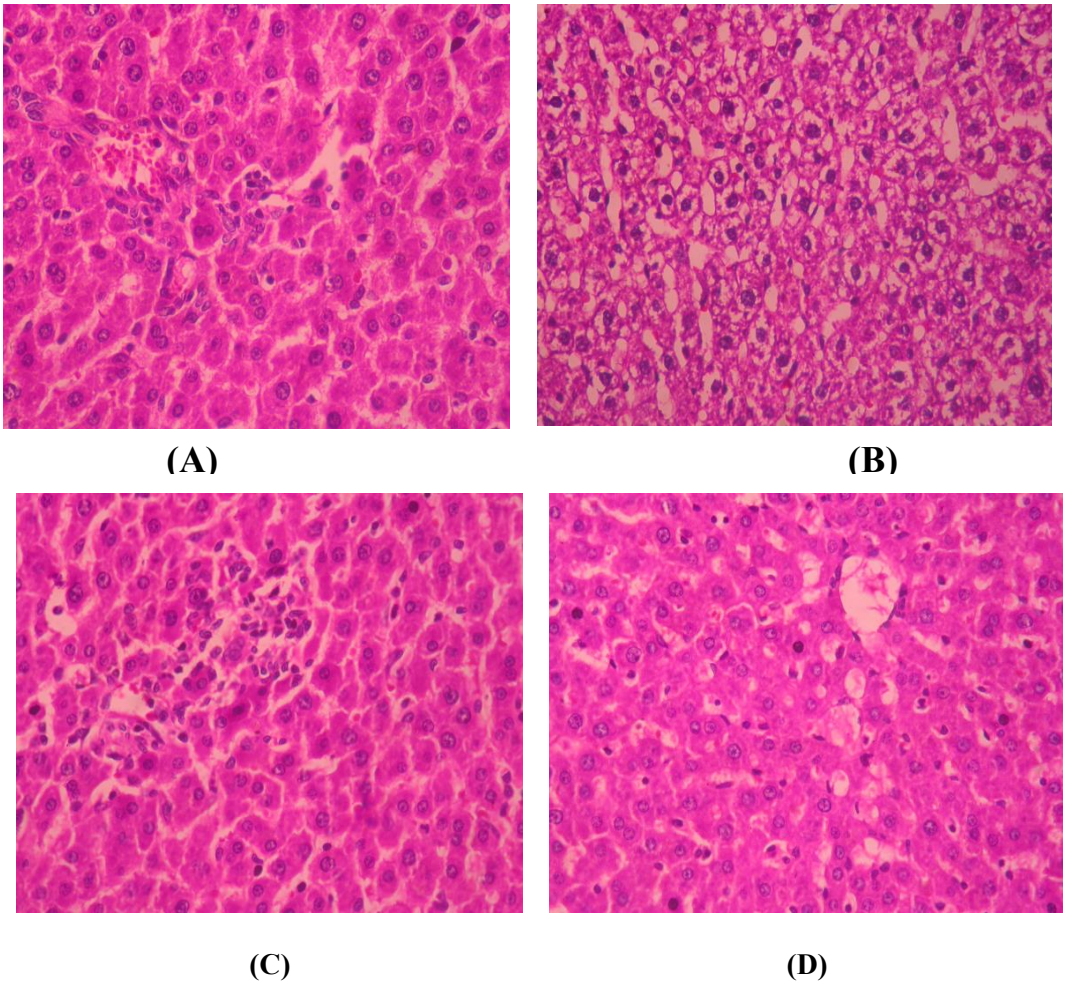

Fig.1: Histological morphology of rat livers after 10 days of treatment with ethanolic extract of *B. variegata* root. (A) Normal control rats showed well preserved cytoplasm, prominent nucleus and nucleolus and well brought out central vein while (B) CCl_4-intoxicated rats showed massive fatty changes, necrosis, hemorrhagic foci in hepatic parenchyma, ballooning degeneration and broad infiltration of the lymphocytes and the loss of cellular boundaries. Liver tissue of CCl_4-intoxicated rats treated with (C) Liv 52 syrup (1 mL kg^{-1}) and (D) ethanolic extract of *B. variegata* root (200 mg kg^{-1}) showed normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the normal control.

root (200 mg kg^{-1}) and standard Liv 52 syrup (1 mL kg^{-1}) showed normal lobular pattern with a mild degree of fatty change, necrosis and lymphocyte infiltration almost comparable to the normal control (C and D) (Fig. 1).

In vitro antioxidant –DPPH free radical scavenging activity

Several concentrations ranging from 10-1000 µg mL^{-1} of the ethanolic extract of root of *B. variegata* were tested for their antioxidant activity by DPPH model. It has been observed that free radicals were scavenged by the *B. variegata* root ethanolic extract in a concentration dependent manner in this DPPH assay (Table II). The ethanolic extract of a root of *B. variegata* showed DPPH radical scavenging activity with an IC_{50} value of 211 µg mL^{-1} when compared with standard BHT (butylated hydroxytoluene) IC_{50} value of 107 µg mL^{-1}.

Table II: DPPH scavenging activity of ethanolic extract of *B. variegata* root

Concentration	DPPH scavenging (% inhibition)	
(µg mL^{-1})	Ethanolic *B. variegata root* extract	BHT (butylated hydroxytolune)
10	2.18	20.07
50	12.92	38.17
100	20.87	47.71
250	59.76	90.25
500	67.45	97.21
1000	75.54	95.22
IC_{50}	211 µg mL^{-1}	107 µg mL^{-1}
Data represents mean ± S.E.M. of triplicate analysis		

DISCUSSION

Herbal medicine has potential to treat many diseases that are obstinate and incurable in other systems of medicine. Currently, marketed synthetic and semisynthetic drugs have side effects and adverse effects. In comparison, natural herbal drugs have several advantages likes often fewer side effects as compared to synthetic drugs, better patient tolerance, relatively less expensive and more acceptance by patients due to long history of use. Hence there is arise in the popularity of natural herbal drugs. Plants are often less prone to the emergence of drug resistance.

The 1, 1-diphenyl -2-picryl hydrazyl (DPPH) radical is widely used as the model system to investigate the scavenging activities of several natural compounds. Plants provide a rich source of antioxidants, which include tocopherols, Vit.C, phenolic compounds, carotenoids[15], flavonoids, terpenoids, anthraquinones, steroids, strychnine and eugenol alkaloids[16]. From the present results, it may be postulated that *B. variegata* root ethanolic extract reduces the radical to corresponding hydrazine when it reacts with hydrogen donors in antioxidant principals. So it can be concluded that the *B. variegata* root ethanolic extract has potent *in vitro* antioxidant potential which is attributed due to the presence of flavonoids, quinones and tannin - like constituents present therein. Elevation in hepatospecific enzymes is the indication of damage of liver cells, these enzymes are cytoplasmic enzymes and are released into systemic circulation after cellular damage and destruction of the cell.

In this study, significant elevation in the total bilirubin content as well as SGOT, SGPT and ALP activities in the CCl_4 treated group could be taken as an index of liver damage. Treatment with *B. variegata* root ethanolic extract inhibited CCl_4 induced increase in total bilirubin and SGOT, SGPT and ALP activities as compared with CCl_4 treated group[17]. The mechanism of hepatic damage by CCl_4 is well documented. CCl_4 is metabolized by CYP 450 enzyme system to trichlormethyl radical (CCl_3). This in turn reacts with molecular oxygen and gets converted to trichloromethyl peroxy radical. This radical forms covalent bonds with sulfhydryl groups of several membrane molecules like GSH leading to their depletion and causes lipid peroxidation. The lipid peroxidation initiates a cascade of reactions leading to tissue necrosis[18]. Amino transferases are important classes of enzymes linking carbohydrates and amino acid metabolism that are present in high concentrations in the liver. For estimation and diagnosis of liver disease alanine amino transferase and aspartate amino transferase are well known diagnostic indicators e.g. for parenchymal cell necrosis and hepatocellular lesions, these biomarker enzymes are released during damage of tissue and released from damaged tissues into blood stream.

Alkaline phosphatase is a membrane bound enzyme and its elevations in plasma indicates membrane disruption in the organ. Alkaline phosphatase, although not a liver specific enzyme, the liver is the major source of this enzyme. The level of this enzyme increases in cholestasis. Hepatotoxicity is characterized by cirrhotic liver condition which in turn increases the bilirubin release[19]. Ethanolic extract of *B. variegata* root has significantly scavenged reactive oxygen species as indicated in Table II. Similarly, the test extract significantly reduced the elevated serum biochemical markers of hepatic injury. It is apparent

from the present results that the antioxidant property of ethanolic extract of *B. variegata* root prevented the formation of trichloromethyl peroxy radical, thereby reducing tissue damage. This is further confirmed by the histopathological study. Therefore, the hepatoprotective activity of ethanolic extract of *B. variegata* root may be due to its antioxidant potential. Since there are reports that the plants containing flavonoids possess antioxidant properties, the hepatoprotective and antioxidant properties of the test plant may be attributed to the presence of flavonoids[20-22].

CONCLUSION

B. variegata root ethanolic extract is shown to have hepatoprotective and antioxidant action. It is conceivable that antioxidant/ free radical scavenging activity of *B. variegata* root ethanolic extract is one of the mechanisms associated with hepatoprotective effect. The other mechanism is presence of flavonoids in ethanolic extracts of *B. variegata* root which significantly reduced the activities of SGPT, SGOT, ALP and total bilirubin enzymes as compared to that of toxicant rats. However, the extract should further be subjected to bioactivity-guided drug discovery to isolate the lead compound responsible for hepatoprotective activity and possible mechanisms(s) of action.

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