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Original Article

"HEPATOPROTECTIVE ACTIVITY OF DIFFERENT EXTRACTS OF AERIAL PARTS OF *LIMNOPHILLA INDICA*" BY USING PARACETAMOL AND ETHANOL INDUCED MODELS

B.HIMAJA[1], VIJUSHA.M*[2], R.SUTHAKARAN[3]

[1]M. Pharm student, [2]Assistant Professor, Teegala Ram Reddy College of Pharmacy, Meerpet, [3] Principal, Teegala Ram Reddy College of Pharmacy, Meerpet.

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ABSTRACT

The present study was carried out to evaluate the hepatoprotective activity of leaves of *Limnophilla indica* of family *Scrophulariaceae* (Dog flower family) .The plant extract was prepared and examined against CCl_4 induced liver damage in wistar rats using silymarin as standard. The extract was screened for alkaloids, steroids, proteins, flavanoides, saponins, carbohydrates, tannins and glycosides. Enzyme activities of Serum glutamate oxaloacetate (SGOT), Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase, Total bilirubin and Total protein levels are analysed. Results indicate that the methanolic leaf extract had protective activity over paracetamol and ethanol induced hepatotoxicity when compared with toxic control and silymarin groups. Results of present investigation confirm the hepatoprotective activity of *Limnophilla indica*. The statistical analysis was carried out using one way analysis of varience (ANOVA) followed by Dunnet's t-test. P –values <0.05 were considered as significant.

Keywords: *Limnophilla indica*, SGOT, SGPT, TB, TP,ALP, CCl_4

INTRODUCTION

Nature always stands as golden mark to exemplify the outstanding phenomena of symbiosis. Nature serves humans with medicines which were used to maintain health, to treat and heal many ailments. For the treatment of human diseases a basic product from Natural products like plant, animal and minerals were used[1]. Some plants are identified as rasayanas in Indian Ayurvedic system of medicine having various pharmarcological properties such as immunostimulant, tonic, neurostimulants, antiageing, antibacterial, antiviral, antirheumatic, anticancer, adaptogen etc[2]. An entire section of Materia Medica of Ayurveda is devoted to drugs entitled as 'Rasayana' for enhancement of body resistance[3]. Rasayana, listed as a class in the texts of traditional Indian Medicine literature, consists of a number of plants reputed to promote physical and mental health, improve defense mechanisms of the body and enhance longevity. These attributes are similar to the modern concept of adaptogenic agents, which are known to afford protection of the human physiological system against diverse stressors[4].

Medicinal herbs used in liver disorders are *Silybum marianum*, *Andrograp hispaniculata, Glychyrrhiza glabra, Picrorrhiza kurroa, Phyllanthus niruri are potential candidates, Solanum nigrum, Taraxacum officinale, Cichorium intybus, Tephroia purpurea, Curcuma longa*.

The liver is the largest organ in the body weighing 1200-1500 g. It is a key organ in regulating homeostasis within the body. It regulates several important functions including protein synthesis, storage and metabolism of fats and carbohydrates, detoxification of drugs and other toxins, metabolism of hormones and excretion of bilirubin. Liver diseases are associated with distortion of these metabolic functions[5]. Although viruses are the main cause of liver diseases, the liver lesions arising from xenobioties, excessive drug therapy, environmental pollution and alcoholic intoxication are not uncommon[6].

Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase, are elevated[7,8].

MATERIALS AND METHODS

Limnophila indica (Linn.) Druce , (family: Scrophulariaceae) is originated from a *Latin* word that means pond-loving indicating its existence in aquatic environments. It is commonly known as 'Ambulia' (Asian marshweed). It is a perennial from Southeast Asia, tropical to subtropical Africa, Australia, and Pacific Islands; also finds adventive distribution in North America. *Limnophila* plants are widely distributed throughout India, and occupy a significant position in traditional systems of medicine. It is a small aquatic or aquatic submersed with 7.5-20 cm high aromatic herb, erect diffusely branched herb . It has a strong smell of turpentine resembling to camphor or oil of lemon; stems are subquadrangular and rooting at the lower nodes. Aerial parts are usually all whorled and pinnatifid, 6-20 cm long, linear-oblong and serrate-dentate. Flowers are solitary, axillary or racemed; corolla is pink in colour with about 8 mm long.

Collection and autentification of plant material: The plant material was collected in the month of June 2013 from Srichalam hills and a specimen was dropped in the herbarium and the leaves was authenticated by Professor Dr. Madhavachetty S. V. University, Trupathi. The collected powdered material was shade dried and pulverized.

Solvents used for extraction: Petrolium ether and methanol

Preparation of the extract: The dried powders of leaf of *Limnophila indica* were defatted with petroleum ether (60-80ºc) in a Soxhlet Apparatus by continuous hot- percolation. The defatted powder material (marc) thus obtained was further extracted with methanol with same method. The solvent was removed by distillation under low pressure and evaporation. The resulting semisolid mass was vacuum dried by using rotary flash evaporator. The resultant dried extracts were used for further study.

PHYTOCHEMICAL SCREENING : The screening was carried out in accordance with the standard protocol as described by Trease and Evans (1983).

Test for reducing sugars (Fehling's test): The aqueous ethanol extract (0.5 g in 5 ml of water) of individual plants was added to boiling Fehling's solution (A and B) in a test tube. The solution was observed for a colour reaction.

Test for anthraquinones: The individual plant extract (0.5 g) was boiled with 10 ml of sulphuric acid (H 2SO 4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipette into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for colour changes.

Test for terpenoids (Salkowski test): To 0.5 g each of the individual extract was added 2 ml of chloroform. Concentrated H 2SO 4 (3 ml) was carefully added to form a layer. A reddish brown coloration was confirmed for the presence of terpenoids.

Test for flavonoids: A portion of the individual plant extract (0.5 g) was heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration indica tes the presence of flavonoids.

Test for saponins: To 0.5 g of each plant extract was added 5 ml of distilled water in a test tube. The solution was shaken vigorously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously after which it was observed for the formation of an emulsion.

Test for tannins: About 0.5 g of the individual extract was boiledin 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride (FeCl3) was added and observed for brownish green or a blue-black coloration

Test for alkaloids: 0.5 g of each extract was diluted to 10 ml with acid alcohol, boiled and filtered. To 5 ml of the filtrate was added 2 ml of dilute ammonia. 5 ml of chloroform was added and shaken gently to extract the alkaloidal base. The chloroform layer was extracted with 10 ml of acetic acid. This was divided into two portions. Mayer's reagent was added to one portion and Draggendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Draggendorff's reagent) was regarded as positive for the presence of alkaloids.

Test for cardiac glycosides (Keller-Killiani test): To 0.5 g of individual plant extract diluted to 5 ml in water was added 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layer with 1 ml of concentrated H 2SO 4. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

S.No	Constituents	Test		Methanolic extract
1.	Alkaloids	A)	Mayer's reagent	Present
		B)	Wagner's reagent	
		C)	Dragendroff's reagent	
		D)	Hagner's reagent	
2	Carbohydrates	a)	Molisch's reagent	Present
		b)	Benedicts reagent	
		c)	Fehling' s solution	
3	Glycosides	a)	Modified borntrager's test	Present
		b)	Legal test	
4	Phytosterol	a)	Salkowski's test	Absent
		b)	Libermann Burchard's test	
5	saponins	a) Froth test		Absent
		b) Foam test		
6	Tannins	Gelatin test		Absent
7	Proteins	a)	Xanthoprotein test	Absent
		b)	Ninhydrin test	
8	Flavonoids	a)	Alkaline reagent test	Present
		b)	Lead acetate test	
		c)	Shinoda test	
9	Diterpenes	Copper acetate test		Present

ACUTE TOXICITY STUDY

Acute oral toxicity study

Acute oral toxicity test was performed as per OECD-423 guidelines (acute toxic class method). Wistar rats (n=6) of either sex selected by random sampling technique were used for the study. The animals were kept fasting for 3-4 hrs providing only water, after which the extracts were administered orally at the dose level of 5mg/kg by intra gastric tube and observed for 3 days. If mortality was observed in 2-3 animals then the dose administered was assigned as toxic dose. If mortality was observed in one animal, then the same dose

was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher doses such as 50, 300 and 2000 mg/kg.

Hepatoprotective activity

Screening methods for hepatoprotective activity

Paracetamol induced hepatotoxicity model.
Arsenic induced hepatotoxicity model.
Isoniazide induced hepatotoxicity model.
Carbon tetrachloride induced hepatotoxicity model.
Glucocorticoids induced hepatotoxicity model.
NSAIDS associated toxicity of liver.

Models chosen for the study: paracetamol and ethanol induced hepatotoxicity model.

Paracetamol induced hepatotoxicity in rats

Animals were divided into 5 groups of six animals each

Group I : Treated as Normal control and receives 5% Gum Acacia.

Group II: Treated as toxic control and is administered with paracetamol to induce hepatotoxicity.

Group III : Administered with hepatotoxin and standard drug silymarin at a dose of 25mg/kg.

Group IV: Administered with hepatotoxin and MELI extract at a dose of 250 mg/kg.

Group V: Administered with hepatotoxin and MELI extract at a dose of 500mg/kg.

After thirty minutes of paracetamol and ethanol administration Group III , IV and Group V rats were treated with Silymarin (25 mg/kg/day),MELI 250mg/kg/day and MELI 500 mg/kg/day respectively. All the animals were sacrificed by cervical decapitation under light ether anesthesia on the Ninth day. Blood was collected by heart puncture and centrifuged (300 rpm for 10 mins) to obtain

serum. The serum was used for the assay of total bilirubin, alkaline phosphatase (ALP), serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and total protein.

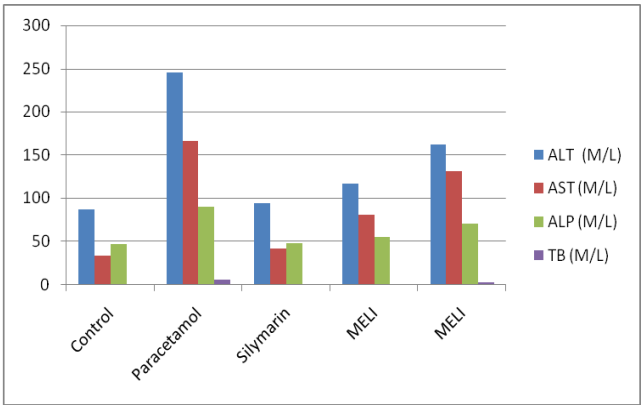
Statistical analysis

The results obtained in biochemical assays were given in terms of mean ± SEM. The statistical significance of the data was assessed by one way analysis of variance (ANOVA) followed by Dunnett's 't' test between different groups. Toxic, Standard and Test groups were compared with normal group. Standard and all Test groups were compared with the toxic group. $p<0.05$ was considered as statistically significant.

RESULT AND DISCUSSION

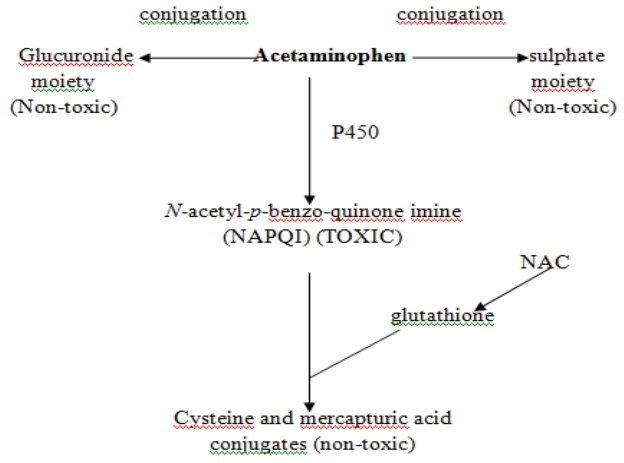
Groups	Treatment	ALT (M/L)	AST (M/L)	ALP (M/L)	TB (M/L)
Group I	Control	86.66± 2.801	33.33±1.89	46.83±1.64	0.268±0.02
Group II	Paracetamol	245.33±2.985	165.5± 3.38	90.166±2.509	5.378±0.03
Group III	Pracetamol +Silymarin	93.66±2.108***	41.5±1.995***	47.83±1.641***	0.276±0.019***
Group IV	Paracetamol+ MELI 250mg/kg	116.33±3.48***	80.83±2.45***	54.66±2.27***	0.75±0.036***
Group V	Paracetamol+ MELI 500mg/kg	162.16±3.42***	130.83±2.006***	69.66±2.81***	1.93±0.227***

Each value is the mean ± SEM for 6 rats, *P<0.5, **P<0.01, ***P<0.001. Compared with control, data were analysed by using one-way ANOVA followed by Dunnet's test, standard Silymarin (5 ml/kg; p.o) MELI- Methanolic extract of *Limnophila indica*, dose (250mg/kg of B.W) MELI- Methanolic extract of *Limnophila indica* (500mg/kg of B.W)



Effect of MELI on serum markers (ALT,AST,ALP,and TB) in Paracetamol induced rats

RESULT



Metabolism of Acetaminophen (Paracetamol) by the hepatocyte

PHYTOCHEMICAL INVESTIGATION

RESULT

In the preliminary phytochemical investigation on the *Limnophila indica* it reveals the presence of alkaloids, flavonoids, tannins, terpenoids, glycosides and sugars. The result of chemical test of *Limnophila indica* was shown

DISCUSSION

Preliminary phytochemical investigation of the Methanolic extracts of *Limnophila indica*. Showed positive results for alkaloids, flavonoids, glycosides and tannins.

Hepatoprotective activity of methanolic extracts *Limnophila indica* on Serum marker enzymes (ALT,AST,ALP) and Total bilirubin on Paracetamol induced hepatotoxicity, in rats.

Paracetamol-treated group had ALT, AST, ALP and bilirubin level which was significantly higher than control . The methanolic extract, and silymarin pretreatment inhibited the rise of serum ALT, AST, ALP and bilirubin levels effectively as their values were significantly decrease as compare to paracetamol treated group. The lower doses of *Limnophila indica* extract was less significantly affect the Paracetamol- induced rise of serum ALT enzyme and bilirubin whereas the higher doses show moderate effect while when compare to control in rise of serum enzymes level .

DISCUSSION

Acetaminophen is an antipyretic and analgesic drug, which is activated and converted by cytochrome P450 enzyme to toxic metabolite N-acetyl-para-benzoquineimine (NAPQI) at high doses. The reactive metabolite can be conjugated with cellular glutathione (GSH) to cause extensive GSH depletion which leads to the cellular necrosis[9,10]. GSH removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thioles. The GSH depletion in hepatic mitochondria is considered the most important mechanism in the paracetamol induced hepatotoxicity. Reduced GSH level was depleted in paracetamol treated group may be due to conjugation of GSH with NAPQI to form mercapturic acid. Paracetamol toxicity is due to the formation of toxic metabolites when a part of it is metabolized by cytochrome P-450. Introduction of cytochrome[11] or depletion of hepatic glutathione is a prerequisite for paracetamol induced hepatotoxicity[12,13].

Normally, AST and ALP are present in high concentration in liver. Due to hepatocyte necrosis or abnormal membrane permeability, these enzymes are released from the cells and their levels in the blood increases. ALT is a sensitive indicator of acute liver damage and elevation of this enzyme in non hepatic diseases is unusual. ALT is more selectively a liver paranchymal enzyme than AST[14].Assessment of liver function can be made by estimating the activities of serum ALT, AST, ALP and Bilirubin which are enzymes originally present higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage[15]. The elevated level of these entire marker enzymes observed in the group- II, paracetamol treated rats in this present study corresponded to the extensive liver damage induced by toxin. The reduced concentration of ALT, AST and ALP as a result of plant extract administration observed during the present study might probably be due in part to the presence of flavonoids. Liver protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotinoids, glycosides, flavanoids, organic acids, lipids, alkaloids and xanthenes[16]. Bilirubin is one of the most useful clinical clues to the severity of necrosis and its accumulation is a measure of binding, conjugation

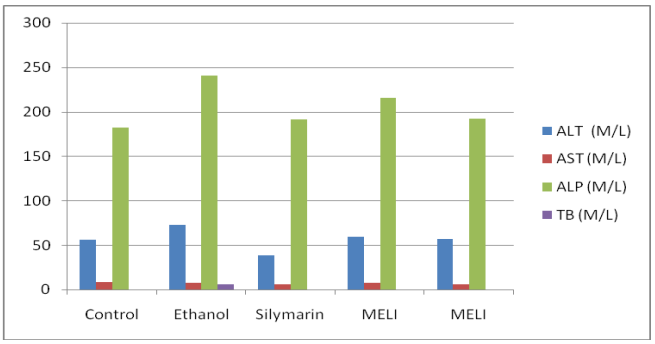
Groups	Treatment	ALT (M/L)	AST (M/L)	ALP (M/L)	TB (M/L)
Group I	Control	56.00 ± 1.527	8.33 ±0.33	182.16±5.04	0.275±0
Group II	ethanol	73 ±1.390	7.33± 0.66	241± 3.18	6.417± 0.166
Group III	Silymarin + ethanol	38.66 ±2.49***	5.66±0.763**	191.5±1.89***	0.38±0*
Group IV	MELI 250mg/kg + ethanol	59.33±1.429***	7.5 ± 0.428**	215.33±1.68***	0.612 ±0*
Group V	MELI 500mg/kg + ethanol	56.66±1.563***	5.5± 0.726**	191.83 ±0.90***	0.492±0*

and excretory capacity of hepatocyte. Decrease in serum bilirubin after treatment with extract in liver damage induced by paracetamol, indicated the effectiveness of the extract in normal functional status of the liver.

In the present study, both the methanolic extracts *Limnophila indica*, have the ability to reduce oxidative stress by increasing glutathione level and preventing lipid peroxidation.

Hepatoprotective activity of methanolic extracts *Limnophila indica* on Serum marker enzymes (ALT,AST,ALP) and Total bilirubin on Ethanol induced hepatotoxicity, in rats.

Each value is the mean ± SEM for 6 rats, *P<0.5, **P<0.01, ***P<0.001. Compared with control, data were analysed by using one-way ANOVA followed by Dunnet's test, standard Silymarin (5 ml/kg; p.o) MELI- Methanolic extract of *Limnophila indica*, dose (250mg/kg of B.W) MELI- Methanolic extract of *Limnophila indica* (500mg/kg of B.W)



Effect of MELI on Serum Enzymes (ALT, AST, ALP, TB) on Ethanol induced rats

RESULT

On the other hand suppression of elevated ALP activities with concur-rent depletion of raised bilirubin level and an increase in the total plasma protein content suggests the stability of biliary dysfunction in rat liver during hepatic injuries with toxicants

DISCUSSION

There are many factors which are responsible for the liver damage or injuries such as chemicals and drugs. In the present study ethanol was used to induce hepatotoxicity, since it is clinically relevant. Ethanol produces a constellation of dose related deleterious effects in the liver. The majority of ethanol is metabolized in the liver and individuals who abuse alcohol by routinely drink-ing 50-60 g (about 4 to 5 drinks) of ethanol per day are at risk for developing alcoholic liver disease.In addition, both acute and chronic ethanol administration cause enhanced formation of cytokines, especially TNF-alpha by hepatic Kupffer cells, which have a significant role in liver injury Besides the develop-ment of fatty liver (steatosis), another early sign of excessive ethanol consumption is liver enlargement and protein accumulation, both of which are common findings in alcoholics and heavy drinkers.

CONCLUSION

The preliminary phytochemical studies reveal the presence of flavanoids. The flavanoids are known as hepatoprotectives, so the observed hepatoprotective activity of *Limnophila indica* may be due to the presence of flavanoids. The mechanism of hepatoprotective activity may be due to activation of regeneration of hepatocytes that restores the structural and functional integrity of liver. Further studies to characterize the active principles and to elucidate the mechanism are in progress.

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