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EVALUATION OF IN VIVO HEPATOPROTECTIVE ACTIVITY OF LEAVES EXTRACTS OF *ZIZYPHUS XYLOPYRUS* (RETZ.) WILLD

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ABSTRACT

Exploring medicinal plants which are easily available and cheap and do not involve strenuous pharmaceutical production processes appears to have gained worldwide attention as alternative therapeutic agents for the diseases. Consequently, emphasis has been placed on folkloric herbs with high efficacy, low toxicity, and cost-effectiveness. Present Investigation deals with hepatoprotective effect of hydroalcoholic extract of fruit of *Zizyphus xylopyrus* (Retz.) Willd. *Zizyphus xylopyrus* fruits are an important medicinal plant which is used in traditional medicine to treat many diseases. Both the test groups i.e. low dose and high dose treated Groups shown dose dependent hepatoprotective activity. The test groups containing the plant extract alone showed an improvement in the liver activity. It clearly indicates that the plant "*Zizyphus xylopyrus* Fruits" has the hepatoprotective activity. This study showed that *Zizyphus xylopyrus* Fruits has a significant protective action against the hepatotoxicity induced by the drugs used in the treatment of tuberculosis. The hepatoprotective role of *Zizyphus xylopyrus* Fruits might be due to its antioxidant potential mechanism suggesting that the extract of plant may be useful to prevent the oxidative stress induced liver damage.

Key words: *Zizyphus xylopyrus*, Hepatoprotective activity, Fruit, Hydroalcoholic extract.

INTRODUCTION:

Hepatotoxicity implies chemical-driven liver damage. Certain medicinal agents, when taken in overdoses and sometimes even when introduced within therapeutic ranges, may injure the organ. Other chemical agents, such as those used in laboratories and industries, natural chemicals (e.g., microcystins) and herbal remedies can also induce hepatotoxicity. Chemicals that cause liver injury are called hepatotoxins. More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Chemicals often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. More than 75% of cases of idiosyncratic drug reactions result in liver transplantation or death (Ostapowicz *et al.*, 2002). On the other hand, the majority of the hepatotoxicity agents damage hepatocytes and subsequently impair

the kidney function mostly through lipid peroxidation or other oxidative forms. In cases of liver damage, the capacity of the natural antioxidant system is inadequate. ROS are generated by environmental causes such as X-rays, pollutants, ultraviolet radiation, or metabolic process in the mitochondria (Haque *et al.,* 2014).

The intracellular concentration of ROS solely depends on the rate at which they are generated by exogenous or endogenous factors as well as their elimination by several endogenous antioxidants such as enzymatic and nonenzymatic processes (Haque *et al.,* 2014). Several reports have shown that oxidative stress triggered by free radicals is the main causative agent of liver damage such as degeneration, necrosis, swelling, and apoptosis of the hepatocytes. Liver injury or damage resulting from free radicals usually occurs via the mechanisms of lipid peroxidation and covalent binding with consequent tissue injury. ROS which include peroxyl, hydroxyl, alkoxy, and superoxide radicals destruct the membrane lipids, proteins, and nucleic acid, and this has also been linked to several aging-related issues together with atherosclerosis, diabetes mellitus, lung and kidney damage, liver disorders, cancer, inflammatory diseases, and cardiovascular diseases (Singh *et al.,* 2008; Pal *et al.,* 2014). Lipid peroxidation interferes with cell membranes and consequently affects the structural integrity and functionality of the cell membrane that subsequently has a negative impact on the cell's potential to maintain constant ion gradients and transport (Madkour and Abdel‐Daim, 2013). On the other hand, liver damage can also be caused by drug abuse at high dosages and certain chemicals (Ali and Kumar, 2015). Varieties of molecules have been isolated and their physicochemical and pharmacological properties were studied. However, compounds and extracts need to be appropriately formulated to facilitate their physiological target and pharmacological activity. Factors such as low permeability and solubility could affect the absorption and delivery of bioactive molecules (Fang and Bhandari, 2011). On the other hand, the shelf life of herbal medicines should be evaluated to monitor their stability during the period of use. Degradation reactions are enhanced by temperature, humidity, pH, oxygen, and light. Herbal medicines are complex mixtures of different classes of chemical compounds, such as carbohydrates, proteins, lipids, and secondary metabolites (Bott *et al.,* 2010). Because of the negative effect allied with synthetic drugs, much effort has been made to identify novel sources of hepatoprotective agents (Mamat *et al.,* 2013). In recent times, most of the hepatoprotective drugs available in the market for use against different kinds of liver diseases have plant-based origins, either as single-plant preparations or as poly-herbal mixtures. Folkloric herbs play an essential role in improving the quality of life of rural dwellers, especially in developing countries with inadequate modern health facilities. Over 70,000 plant species have been used for therapeutic purposes. Although attention has been diverted to plants as natural treatment alternatives due to efficacy, the scientific rationale behind the plant preparation and dosage regimens has usually not well understood, and despite their efficacy and cost-effectiveness, the need to prioritize low-toxicity candidate plants becomes imperative, and although these herbal drugs are

abundant in the market and the pharmacological ingredients have not been fully identified, some of the identified bioactive ingredients have been proved to have antiviral, antioxidant, anticarcinogenic, antifibrotic, and anti-inflammatory effects (Olakunle and Ajiboye, 2011; Oloyede *et al.*, 2013). Thus, exploring medicinal plants which are easily available and cheap and do not involve strenuous pharmaceutical production processes appears to have gained worldwide attention as alternative therapeutic agents for the diseases. Consequently, emphasis has been placed on folkloric herbs with high efficacy, low toxicity, and cost-effectiveness. Present Investigation deals with hepatoprotective effect of hydroalcoholic extract of fruit of *Zizyphus xylopyrus* (Retz.) Willd.

Material and Methods

Material

INH (Isoniazid), Silymarin (Micro labs, India) were used in present study. All other chemicals and other biochemical used in the experiments were of analytical grade from different firms.

Methods

Extraction procedure

Following procedure was adopted for the preparation of extracts from the shade dried and powdered herbs.

Defatting of plant material

Fruits of *Zizyphus xylopyrus* were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place.

Extraction by maceration process

65 gm of dried powdered fruits of *Zizyphus xylopyrus* has been extracted with Hydroalcoholic solvents (Ethanol 80%) using maceration process for 48 hrs, filtered and dried using vacuum evaporator at 40^0C.

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Phytochemical Screening

Phytochemical examinations were carried out for all the extracts as per the standard methods.

1. **Detection of alkaloids:** Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: Filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Dragendroff's Test: Filtrates were treated with Dragendroff's reagent (solution of Potassium Bismuth Iodide). Formation of red precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's Test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the

junction indicates the presence of Carbohydrates.

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

5. Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

6. Detection of Phytosterols

Salkowski's Test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Libermann Burchard's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. Formation of brown ring at the junction indicates the presence of phytosterols.

7. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

8. Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

9. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

10. Detection of proteins and aminoacids

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

Ninhydrin Test: To the extract, 0.25% w/v ninhydrin reagent was added and boiled for few minutes. Formation of blue colour indicates the presence of amino acid.

11. Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

Total Phenolic content estimation

The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method [33].

Preparation of Standard: 50 mg Gallic acid was dissolved in 50 ml methanol, various aliquots of 5- 25µg/ml was prepared in methanol

Preparation of Extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Two ml (1mg/ml) of this solution was used for the estimation of phenol.

Procedure: 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15 min for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Total flavonoids content estimation

Determination of total flavonoids content was based on aluminium chloride method.

Preparation of standard: 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.

Preparation of extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Isoniazid induced hepatoprotective activity of fruits of *Zizyphus xylopyrus*

Animals: -

Wistar rats (180–250 g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55–65%). Rats received standard rodent chow and water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. All the experiments were carried in a noise-free room between 08.00 to 15.00 h. Separate group (n=6) of rats was used for each set of experiments. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute toxicity studies

Acute oral toxicity was conducted according to the method of Organisation for Economic Co-operation and Development (OECD) (OECD, 2001). Animals were kept fasting providing only water, fruits of *Zizyphus xylopyrus* (50,100,150,200,300 mg/kg/day) was administered orally for 4 days of six groups of rats (n=6) and the animals were kept under observation for mortality as well as any behavioral changes for evaluation of a possible hepatoprotective effect.

Experimental designs

Group –I: Normal control (Sterile distilled water ml/kg, p.o.).

Group –II: INH (Isoniazid) solutions were prepared in sterile distilled water (100 mg/kg, p.o.)

Group -III: *Zizyphus xylopyrus* Extract (100mg/kg, p.o.) + INH (100 mg/kg, p.o.)
Group –IV: *Zizyphus xylopyrus* Extract (200mg/kg, p.o.) + INH (200 mg/kg, p.o.)
Group –V: Silymarin (2.5 mg/kg, p.o.) + INH (100 mg/kg, p.o.)

Animals were divided into five groups of 6 animals each. The first group received Sterile distilled water 1 ml/kg p.o. The group II received 100 mg/kg, p.o. INH (Isoniazid) solutions. The groups III, IV and V received 100 mg/kg and 200 mg/kg of Hydroalcoholic extract of *Zizyphus xylopyrus* fruits and silymarin (2.5 mg/kg p.o.) respectively once a day for 21 days. After 21st days animals was anaesthetized with ether for collection of blood from retro orbital plexus, and then sacrificed under ether anesthesia for the removal of liver. Various biochemical analysis were carried out (Jiang *et al.,* 2004; Saleem *et al.,* 2008).

Biochemical Evaluation in Serum

Serum glutamic pyruvate transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT), Alkaline phosphatase (ALP) and total bilirubin was estimated by using commercial kits as per the manufacturer instructions.

Results and Discussion

Zizyphus xylopyrus fruits are an important medicinal plant which is used in traditional medicine to treat many diseases. The liver may be considered as the most important organ in drug toxicity for two reasons: on the one hand it is functionally interposed between the site of absorption and the systemic circulation and is a major site of metabolism and elimination of foreign substances; but on the other hand these features also render it a preferred target for drug toxicity. Drug-induced liver injury (DILI) therefore poses a major clinical problem. Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds, any injury or impairment of its function may lead to several implications on one's health. Management of liver diseases is still a challenge to modern medicine (Handa and Sharma, 1990).

Increased in the level of activities of SGPT, SGOT and ALP in the blood reflect the damage of liver hepatocytes and indirectly impairment of liver functions following APAP-induced hepatotoxicity. In Table 7.6, SGPT, SGOT and ALP activities were significantly elevated ($p<0.05$) after administration of APAP. Treatments with 100 and 200 mg/ kg of *Zizyphus xylopyrus* Fruits extract significantly reduced the elevation of these enzymes ($p<0.05$). The reduction of liver enzymes was seen to be to the level of the control group and it was also similar to the level of group pretreated with silymarin. One of the hallmark signs of hepatic injury or damage is apparent leakage of cellular enzymes into plasma. In addition, the extent and type of liver injury or damage can be accessed based on the presence or absence of specific enzymes in the blood stream. In general measurement of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase are commonly used as marker enzymes in accessing APAP induced hepatotoxicity. In this study, hepatoprotective effect of *Zizyphus xylopyrus* Fruits is evidenced by the improvement SGPT, SGOT, ALP and serum bilirubin levels. Treatment with *Zizyphus xylopyrus* Fruits extract suppresses Isoniazid induced SGPT, SGOT, ALP and serum bilirubin elevations. Previous studies have reported elevations of

transaminases after Isoniazid-Rifampicin treatment (Reddy *et al.*, 2013). The increase is time dependent with significant elevation noted after 48 h ($p<0.05$) suggesting severe hepatocellular damage caused by leakage of these enzymes into circulation that is normally cytoplasmic in location (Asha, *et al.*, 2004).

Both the test groups i.e. low dose and high dose treated Groups shown dose dependent hepatoprotective activity. The test groups containing the plant extract alone showed an improvement in the liver activity. It clearly indicates that the plant "*Zizyphus xylopyrus* Fruits" has the hepatoprotective activity. This study showed that *Zizyphus xylopyrus* Fruits has a significant protective action against the hepatotoxicity induced by the drugs used in the treatment of tuberculosis. The hepatoprotective role of *Zizyphus xylopyrus* Fruits might be due to its antioxidant potential mechanism suggesting that the extract of plant may be useful to prevent the oxidative stress induced liver damage.

Table 1: % Yield of fruit extracts of *Zizyphus xylopyrus*

S. No.	Solvents	% Yield
1	Pet ether	0.254
2.	Hydroalcoholic	6.321

Table 2: Phytochemical screening of extracts *of Zizyphus xylopyrus*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Mayer's Test	-ve
	Wagner's Test	-ve
	Dragendroff's test	-ve
2.	**Glycosides**	
	Modified Borntrager's Test	-ve
	Legal's test	-ve
3.	**Flavonoids**	
	Lead acetate	-ve
	Alkaline test	+ve
4.	**Phenolics**	
	Ferric Chloride Test	+ve
5.	**Proteins and Amino acids**	
	Xanthoproteic test	+ve
	Ninhydrin Test	-ve
6.	**Carbohydrates**	
	Molisch's Test	-ve
	Benedict's Test	-ve
	Fehling's test	-ve
7.	**Saponins**	
	Froth Test	+ve
	Foam test	+ve
8.	**Diterpins**	
	Copper acetate test	-ve

Table 3: Total phenolic and total flavonoid content of *Zizyphus xylopyrus* extract

S. No.	Extract	Total Phenol (mg/100mg)	Total flavonoid (mg/100mg)
1.	Hydroalcoholic *extract*	0.323	0.985

SGOT levels in Isoniazid induced hepatotoxicity in rats.

Treatment	Dose	SGOT (%)
Normal	1 ml/kg, p.o.	159 ± 2.5
INH	100 mg/kg, p.o.	329.45 ± 6.5
Zizyphus xylopyrus Extract	100 mg/kg p.o.	219.0 ± 3.5***
Zizyphus xylopyrus Extract	200 mg/kg p.o.	198.0 ± 3.9***
Silymarin	2.5 mg/kg p.o.	163.0 ± 2.6***

Values are expressed as the mean ± SEM of six observations. *** $P<0.001$ vs. control treatment (One-way ANOVA followed by Dunnett's test)

Table 5: Effect of Hydroalcoholic extract of *Zizyphus xylopyrus* Fruits and Silymarin on %SGPT levels in Isoniazid induced hepatotoxicity in rats.

Treatment	Dose	SGPT (%)
Normal	1 ml/kg, p.o.	155.0 ± 2.50
INH	100 mg/kg, p.o.	315.0 ± 3.60
Zizyphus xylopyrus Extract	100 mg/kg p.o.	205.0 ± 4.20***
Zizyphus xylopyrus Extract	200 mg/kg p.o.	192.0 ± 3.40***
Silymarin	2.5 mg/kg p.o.	148.0 ± 3.70***

Values are expressed as the mean ± SEM of six observations. *** $P<0.001$ vs. control treatment (One-way ANOVA followed by Dunnett's test)

Table 6: Effect of *Zizyphus xylopyrus* Fruits and Silymarin on % serum bilirubin levels in Isoniazid induced hepatotoxicity in rats.

Treatment	Dose	Serum Bilirubin (%)
Normal	1 ml/kg, p.o.	120.0 ± 5.50
INH	100 mg/kg, p.o.	278.0 ± 3.50
Zizyphus xylopyrus Extract	100 mg/kg p.o.	163.0 ± 4.51***
Zizyphus xylopyrus Extract	200 mg/kg p.o.	142.0 ± 1.60***
Silymarin	2.5 mg/kg p.o.	120.0 ± 4.50***

Values are expressed as the mean ± SEM of six observations. *** $P<0.001$ vs. control treatment (One-way ANOVA followed by Dunnett's test)

Table 7: Effect of *Zizyphus xylopyrus* Fruits and Silymarin on % ALP levels in Isoniazid induced hepatotoxicity in rats.

Treatment	Dose	ALP (%)
Normal	1 ml/kg, p.o.	158.0 ± 3.20
INH	100 mg/kg, p.o.	320.0 ± 5.57
Zizyphus xylopyrus Extract	100 mg/kg p.o.	223.0 ± 4.30***
Zizyphus xylopyrus Extract	200 mg/kg p.o.	193.0 ± 3.78***
Silymarin	2.5 mg/kg p.o.	156.0 ± 5.40***

Conclusion

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. A large number of plants and formulations have been claimed to have hepatoprotective activity. The present study concluded that the ethanolic extract of *Zizyphus xylopyrus* Fruits may be used as an effective hepatoprotective agent. Further studies on isolation and structural determination of active principles might be worthy.

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