Research paper

International Journal of Chemical and Pharmaceutical Sciences
2011, Sep., Vol.2 (3)

Hepatoprotective Effect of Hydroalcoholic Extract of Calycoperis floribunda Leaves on Rifampicin-isoniazid Induced Rats

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ABSTRACT

Calycoperis floribunda leaves used for treatment of jaundice in Ayurvedic medicine. Adult male wistar rats were rendered hepatotoxicity by Rifampicin-Isoniazid(50mg/kg each) by intraperitonially for 14days while leaf extract (100mg/kg and 200mg/kg) was administered orally for 14 days. Administration of leaf extract (100mg/kg and 200mg/kg) resulted in a significant (p<0.01) increased in plasma and hepatic lipid profiles. The extract suppresses cytochrome P-450 activity. Decreased levels of transaminases indicate stabilization of plasma membrane and protection of hepatocytes against damage caused by hepatotoxic. The levels of SOD, catalase, GSH significantly decreased along with concentration of malondialdehyde in these groups indicating increased lipid peroxidation. Histomorphological findings also supported the biochemical findings. The present study demonstrates that administration of leaf extract (100mg/kg and 200mg/kg) has significant hepatoprotective activity as evidenced by the biochemical, functional and histopathological parameters.

KEY WORDS: Hepatotoxicity, Calycoperis floribunda, AST, ALT, GSH.

1. INTRODUCTION

The world Health Organization (WHO) estimates that 4 billion people, 80% of the world population, presently use herbal medicine for some aspect of primary health care. Herbal medicine is a major component in all indigenous people’s traditional medicine and a common element in ayurvedic, homeopathic, naturopathic, traditional oriental and Native American Indian medicine. WHO notes that 119 plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses as plant medicines by native cultures. Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicinal value[1]. Over thousands of years; traditional Chinese medicine has developed a theoretical and practical approach to the treatment and prevention of diseases. The first documented source of Chinese medical theory, the Huangdi Nei Jing (“Inner Classic of the Yellow Emperor”) was written between 300 and 100 BC. It describes the diagnosis and treatment of a huge range of disorders and gives advice about healthy lifestyles, exercise, and diet, which conforms remarkably well to current recommendations for the prevention of chronic diseases.

The liver is a key organ regulating homeostasis within the body. It has wide range of functions, including detoxification, protein synthesis and production of biochemicals necessary for digestion. This organ plays a major role in metabolism and has a number of functions in the body including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production and detoxification. It lies below the diaphragm in the thoracic region of the abdomen. It produces bile, an alkaline compound which plays in the digestion via emulsification of lipids. It is also performs and regulates a wide variety of high-volume biochemical reactions requiring highly specialized tissues including the synthesis and breakdown of small and complex molecules many of which are necessary for normal vital functions. Substances derived from the plants remain the basis for a large proportion of the commercial medications used today for the treatment of heart disease, high blood pressure, pain, asthma and other problems. Inspite of phenomenal growth of allopathic system of medicine the synthetic drug to prevent or cure
the hepatic damage due to various hepatotoxins is not available. Treatment of hepatotoxicity with plants or plant preparations and medicaments has been mentioned in the ancient indigenous systems of medicine of many countries. Even today, rural folks and aboriginal tribes all over the world, including India, are using many plants in the treatment of liver damage [2].

Up-regulation of hepatic metabolism of hormones decreases their levels, and Rifampicin can also in similar fashion reduce the efficacy of hormonal contraception, to the extent the unintended pregnancies have been reported among users of oral contraceptives taking Rifampicin in even short courses (for example, as prophylaxis against exposure to bacterial meningitis). Calycoperis floribunda (Combretaceae) commonly known as Ukshi belongs to family Combretaceae. It is a large scandent shrub or liana, which can reach up to 10 m long. Ukshi is found extensively in the low-lying tropical evergreen forests of the Western Ghats.Ukshi containing alkaloids, flavonoids, volatile oils, steroids.Traditionally the plant Calycoperis floribunda is claimed to treat jaundice. So we can use Calycoperis floribunda as hepatoprotective drug which is caused by Rifampicin - Isoniazid induced liver toxicity [3]. Keeping in view all the literature survey the present study was planned to evaluate the activity of Ukshi for hepatoprotective. An attempt is also made to evaluate its effect on liver metabolic functions.

2. MATERIALS AND METHODS

2.1. Animals

Male Wistar rats weighing (150-200g) were obtained from Kings Institute, Guindy. They were maintained in animal house of SRM College of pharmacy as per IAEC guidelines. Animals were access to standard pellet diet and water given ad libitum. The study was approved by Institutional animal ethical committee, CPCSEA. The proposal number is IAEC/131/2010.

2.2. Plant material

The leaves of Calycoperis floribunda used in the present study was collected from the natural habitat in and around Chennai, Tamilnadu and the plant material was authenticated by Dr.P.Jayaraman Ph.D., Plant Anatomy Research Centre(PARC),Tambaram. Voucher number is PARC/2010/803.

2.3. Plant extraction

The fresh leaves of Calycoperis floribunda were collected. It was defatted using petroleum ether. The marc obtained was dried and subjected to extraction by adding dried leaf powder of distilled water (1:10), heated to 50-60°C under constant stirring conditions for 1hour and filtered. The methanol extract was prepared by using Soxhlet’s apparatus [4].

2.4. Phytochemical screening

The alcoholic extracts obtained were subjected to preliminary phytochemical screening (Kokate C.K.,et al.,) to identify the chemical constituents. The methods of analysis employed were those described by Harbone & Baxter [5,6].

2.5. Induction of hepatotoxicity

Hepatotoxicity was induced by administering the Rifampicin-Isoniazid (50mg/kg each, i.p) for 14 days in Albino Wistar rats [7].

2.6. Experimental design

In experiment, totally 24 rats used. The rats were divided into 4 groups of six animals each [8]

- Group I: Control (saline) 2ml/kg, p.o
- Group II: Inducing agent (Rifampicin-Isoniazid), each 50mg/kg, i.p
- Group III: Inducing agent 50mg/kg, i.p and hydroalcoholic leaf extract, 100mg/kg, p.o
- Group IV: Inducing agent 50mg/kg, i.p and hydroalcoholic leaf extract, 200mg/kg, p.o

Animals were fasted overnight, the hydroalcoholic extract of Calycoperis floribunda (100mg/kg, 200mg/kg) was given orally for 14 days to the third and fourth group of animals with liver damage induced by Rifampicin-Isoniazid (50mg/kg) was administered intraperitoneally for all 14days. At 15th day 1ml Blood was collected from all animals by Retro-orbital bleeding for the evaluation of serum parameters like Aspartate transaminase (AST), Alanine transaminase (ALT), Alkaline phosphatase (ALP), Total serum proteins, Bilirubin. Then animals were sacrificed and liver tissues was used for histopathological study [9].

2.7. Preparation of rat liver homogenate

Tissue homogenate was prepared in a ratio of 1 g of wet tissue to 10 times (w/v) 0.05M-ice cold phosphate buffer (pH 7.4) and homogenized by using a Teflon homogenizer. 0.2 ml of sample homogenate was used for estimation of thiobarbituric acid reactive substance (TBARS). The remaining part of the homogenate was centrifuged at 15,000 g at 4°C for 60 minutes and the supernatant was used for
superoxide dismutase, catalase and HMG-CoA estimation

2.8. Histopathological study of liver

Liver was collected after the rats were sacrificed in 10% formalin solution and utilized for the histopathological studies. Liver was separated from all the groups and blotted free of blood and tissue fluids. They were fixed in bovine’s fluid (picric acid: Formalin:Acetic acid in the ratio of 75:52:5). After 24 hours the tissues were washed thoroughly in 70% alcohol and then dehydrated in ascending grades of alcohol (70,100%). Dehydration in absolute alcohol was followed by treatment of tissue with toluene:xylin (50:50) successively by 10%, 50%, 70%, 90% paraffin wax in toluene and finally to 100 % paraffin wax, at 60-62º C followed by embedding of tissue in wax.5-15 micro-meter thick sections were serially cut in leitz microtome in horizontal plane and mounted on glass slides with the help of egg albumin in glycerin solution (50% v/v). The sections were deparaffinated in xylene and downgraded through 100, 90, 50 and 30% alcohol and then finally in water. They were then stained with105 hematoxylin for 3-5 minutes and staining was intensified by running water. The hematoxylin stained section was stained with 10% eosin for two minutes and were then quickly passed through ascending grades of alcohol and finally treated with xylene followed by mounting in DPX. The sections were observed and desired area was photographed in an Olympus microscope. The sections were observed under 40X magnifications [12].

2.9. Statistical analysis

All the data were expressed as mean ± SEM. Statistical significance was tested using one way ANOVA followed by the Dunner’s t test using computer based fitting program (Prism, Graph pad.). Statistical significance was determined at P < 0.05.

3. RESULTS AND DISCUSSION

Preliminary phytochemical screening of the plant extract of Calycoperis floribunda reveals the presence of alkaloids, carbohydrates, phytosterols, glycosides, saponins, tannins and phenolic compounds[10],

3.2. Histopathology of liver:

![Liver histology images]

The use of rats as experimental animals for hepatoprotective activity is mainly because of the structural homology of rat CYP 450 enzymes with that of humans (Burke et al., 1994) and moreover female rats are less susceptible to chemical induced liver damage, especially hydroxyproline accumulation. So, we had used male rats in our study. Rifampicin acts directly on messenger RNA synthesis. Isoniazid inhibits the mycolic acid synthesis. Necrosis or membrane damage releases the enzymes into circulation and hence it can be measured in the serum. The reversal of increased serum enzymes in Rifampicin-Isoniazid induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilising activity[11]. (Serum biochemical parameters)
Table 1. Effect of Calycophyllum floribunda on serum biochemical parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (KA units)</th>
<th>TB (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>14.48±0.53</td>
<td>15.71±0.63</td>
<td>17.03±1.03</td>
<td>0.4083±0.01641</td>
</tr>
<tr>
<td>Rifampicin-Isoniazid treated</td>
<td>41.62±0.90**</td>
<td>32.52±0.65***</td>
<td>32.12±0.62***</td>
<td>0.9433±0.03904***</td>
</tr>
<tr>
<td>Rifampicin + Isoniazid + Test extract (100 mg/kg PO)</td>
<td>32.28±0.62***</td>
<td>23.1±0.77***</td>
<td>29.12±0.76</td>
<td>0.785±0.02291***</td>
</tr>
<tr>
<td>Rifampicin + Isoniazid + Test extract (200 mg/kg PO)</td>
<td>19.28±0.76***</td>
<td>13.7±1.5***</td>
<td>24.68±1.02***</td>
<td>0.567±0.01352***</td>
</tr>
</tbody>
</table>

All values are shown as mean ± SEM and n=6.

* indicate p<0.05, ** indicate p<0.01, *** indicate p<0.001 when compared to control group.

Amino transferases contribute a group of enzymes that catalyse the interconversion of amino acids and α-keto acids by the transfer of amino groups. These are liver specific enzymes and are considered to be very sensitive and reliable indices for necessary hepatotoxic as well as hepatoprotective or curative effect of various compounds. Both AST and ALT levels increase due to toxic compounds that affect the integrity of liver cells. Decreased levels of transaminases indicate stabilisation of plasma membrane and protection of hepatocytes against damage caused by hepatotoxic. Both the test groups could significantly lower the elevated amino transferase levels when compared to Rifampicin-Isoniazid group. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes. Moreover, the Test extract (200mg/kg.p.o) showed a remarkable decrease in the enzyme levels than the Test extract (100 mg/kg,p.o) indicating the greater hepatoprotective activity.[12]

Alkaline phosphatase is a membrane bound glycoprotein enzyme with a high concentration in sinusoids and endothelium. This enzyme reaches the liver mainly from the bone. It is excreted into the bile; therefore its elevation in serum occurs in hepatobiliary diseases. Serum alkaline phosphatase is related to the functioning of hepatocytes and increase in its activity is due to the increased synthesis in presence of biliary pressure. The results of the present study indicate that both Test groups probably stabilize the hepatic plasma membrane

Table 2. Effect of Calycophyllum floribunda on tissue and functional parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD(U/mg protein)</th>
<th>CAT(µM H₂O₂consumed/mg protein)</th>
<th>Reduced GSH(µg of GSH/mg protein)</th>
<th>MDA (nM of MDA/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline)</td>
<td>8±0.80</td>
<td>10.68±0.54</td>
<td>9.854±1.002</td>
<td>0.448±0.07</td>
</tr>
<tr>
<td>Rifampicin-Isoniazid treated</td>
<td>4.394±0.51**</td>
<td>6.136±0.45**</td>
<td>5.35±0.64**</td>
<td>1.288±0.14**</td>
</tr>
<tr>
<td>Rifampicin + Isoniazid + Test extract (100 mg/kg PO) treated</td>
<td>9.808±0.83***</td>
<td>10.99±0.50</td>
<td>10.52±0.64</td>
<td>0.4384±0.05</td>
</tr>
<tr>
<td>Rifampicin + Isoniazid + Test extract (200 mg/kg PO) treated</td>
<td>7.658±0.73</td>
<td>10.73±0.87</td>
<td>9.516±0.41**</td>
<td>0.718±0.059**</td>
</tr>
</tbody>
</table>

All values are shown as mean ± SEM and n=6.

* indicate p<0.05, ** indicate p<0.01, *** indicate p<0.001 when compared to control group.

SOD: Superoxide dismutase, CAT: Catalase, GSH: Glutathione, MDA: Malondialdehyde
from Rifampicin-Isoniazid induced damage. Reduction of alkaline phosphatase levels with concurrent depletion of raised bilirubin levels suggests the stability of biliary function during injury with Rifampicin-Isoniazid. There was a remarkable reduction in the bilirubin levels of both Test groups implying its potential as hepatoprotective agent [13].

The liver was also known to play a significant role in the serum protein synthesis, being the source of plasma albumin, fibrinogen and also the other important components like α and β-globulin. The liver is also concerned with the synthesis of γ-globulin. The serum albumin level is low in hepatic diseases. The metabolic biotransformation of amino acids in liver by synthesis, transamination, etc., may be impaired due to the escape of both non-proteins and protein nitrogenous substances from injured cells as mediated by raise in the serum enzyme levels of ALP, AST and ALT. The reduction in the total protein (TP) is attributed to the initial damage produced and localised in the endoplasmic reticulum which results in the loss of CYP 450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver. The Test groups considerably enhanced the synthesis of TP which may be by accelerating the regeneration process and protecting the liver cells. The increased levels of total protein in serum are indicative of the hepatoprotective activity [14].

Inhibition of bile acid synthesis from cholesterol which is synthesized in liver or derived from plasma lipids leading to an increase in cholesterol levels also results during Rifampicin-Isoniazid intoxication. Significant suppression of cholesterol levels by both the Test groups suggests that bile acid synthesis inhibition was reversed. Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index of hepatocellular damage and is the most sensitive enzymatic index in liver injury. Curtis and Mortiz (Curtis et al., 1972) reported SOD as one of the most important enzymes in the enzymatic antioxidant defence system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishes the toxic effect caused by this radical. The Test groups showed a markable increase in the SOD levels when compared to the Rifampicin-Isoniazid treated group [16]. (Tissue and functional parameters present in Table 2.)

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues and its highest activity is found in the red blood cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals. Therefore, reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. The Test groups showed a drastic increase in the catalase levels when compared to the Rifampicin-Isoniazid treated group. This clearly implies the antioxidant ability of Calycoperis floribunda. Glutathione (GSH) is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is a substrate for glutathione peroxidase (GPx). Decreased levels of GSH are associated with an enhanced lipid peroxidation in Rifampicin-Isoniazid treated rats. Test group was found to produce a rise in the collapsed GSH levels when compared to Rifampicin-Isoniazid group. This demonstrate an increase in the liver tissue GSH levels [18].

The activated radicals bind covalently to the macro molecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to the formation of lipid peroxides, which in turn give products like malondialdehyde (MDA) that cause damage to the membrane. MDA levels have seen a dramatic reduction in Test groups. This may be attributed due to the anti lipid peroxidative potential of Test groups which could considerably decrease MDA levels. This might be due to the presence of alkaloids in our plant which are responsible for antioxidative activity. The extent of hepatic damage is assessed by histological evaluation along with the levels of various biochemical parameters in circulation. The animals in the Rifampicin-Isoniazid group showed severe hepatotoxicity evidenced by profound steatosis, centrilobular necrosis, ballooning degeneration, nodal formation and fibrosis as compared to the normal hepatic architecture of the control group animals. Test groups showed the healing of damaged parenchyma. The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been disturbed by a hepatotoxin. Both the Test groups decreased Rifampicin-Isoniazid induced elevated enzyme levels, indicating the protection of structural integrity of hepatocytic cell membrane or the regeneration of damaged liver cells.

Both the test groups showed hepatoprotective activity. The Test group containing the plant extract showed an
improvement in the liver activity. It clearly indicates that the plant “Calycopteris floribunda” has the hepatoprotective potential which is independent. Thus, the drastic improvement in liver functions may be due phytoconstituents present in Calycopteris floribunda. The activity of extract is due to the chemical constituents present in it. Phytoconstituents like the flavonoids, triterpenoids, saponins and alkaloids are known to possess hepatoprotective activity. Along with these, the antioxidants and prooxidants like caryophyllene oxide, calycoperonones, caryopterones, ß-caryophellene, n-hexadecanoic acid, linolic acid, pachypedol, in our plant i.e. Calycopteris floribunda might be responsible for its antioxidant and thus hepatoprotective activity.

In summary, this study suggests that the oral administration of Calycopteris floribunda alone significantly ameliorates Rifampicin-Isoniazid induced hepatotoxicity in rats. The extract may be protecting the liver by free radical scavenging activity and thus preventing peroxidation of lipids of the endoplasmic reticulum. And this may be due to the presence of flavonoids and alkaloidal pigments in our extract. However, the possibility that Calycopteris floribunda might suppress the cytochrome P-450 mediated metabolic activation of Rifampicin-Isoniazid cannot be ruled out.

4. CONCLUSION

In our study we have made an attempt to study the hepatoprotective activity of a locally available plant which is in use by the local tribal people but lacks its mention in scientific literature. Our work aims to study the therapeutic effect of the hydroalcoholic extract of the plant Calycopteris floribunda by examining the prevention of Rifampicin-Isoniazid induced hepatotoxicity in rats. From all these findings we can conclude that the plant Calycopteris floribunda has significant hepatoprotective activity as evidenced by the biochemical, functional and histological parameters.

The present findings provide scientific evidence to the ethno medicinal use of this plant genetic resource by the tribal people in treating jaundice. The potential usefulness of the extract in clinical conditions associated with liver damage is still to be demonstrated. Further studies are needed to be carried out with regard to the isolation of active principles responsible for hepatoprotective activity and also for the intoxication with other models such as iron, alcohol etc to prove its efficacy.

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