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IN VITRO ANTIOXIDANT ACTIVITY AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF ETHANOLIC WHOLE PLANT EXTRACT OF *NYMPHOIDES HYDROPHYLLA* IN CCL_4 INDUCED LIVER DAMAGE IN ALBINO RATS

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ABSTRACT

In the present study locally available plant *Nymphoides hydrophylla* was screened for its *in-vitro* antioxidant and *in-vivo* hepatoprotective activity. The *In vitro* antioxidant activity of *Nymphoides hydrophylla* was studied using four types of oxygen free radical systems. *Nymphoides hydrophylla* had effective scavenging abilities against free radicals which was determined using standard methods like DPPH, Reducing power method, Nitric oxide assay and Phosphomolybdenum methods against standard gallic acid. The *In-vivo* hepatoprotective effect of *Nymphoides hydrophylla* was evaluated in Carbon tetrachloride (CCl_4) induced albino rats for acute liver injury. The animals were divided into six groups, each group containing three animals and two different doses of the extract were administered orally for five days. The hepatoprotective activity was assessed by estimating various biochemical parameters and histopathological studies. CCl_4 administration caused severe hepatic damage in rats as evidenced by elevated SGPT, SGOT, alkaline phosphatase (ALP) and total bilirubin levels. The ethanolic whole plant extract of *Nymphoides hydrophylla* significantly lowered the biological indicators and the results were compared with that of standard drug Silymarin. The histopathological study of liver was carried out and observed. The present study concludes that ethanolic whole plant extract of *Nymphoides hydrophylla* has significant antioxidant activity and succeeded to restore the biochemical parameters and improved the histological alteration of the liver.

Keywords: Antioxidant Activity, Hepatoprotective Activity, Histopathology, Silymarin, *Nymphoides hydrophylla*.

INTRODUCTION

Liver disease is still a major worldwide health problem. Jaundice and hepatitis are two major hepatic disorders[1] that account for high death rate. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects[2]. The attention of pharmacologists throughout the world has been focused on findings out safer and potent hepatoprotective drug. The natural products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to humans and environment. So, people are returning to the natural product with the hope of safety and security[3]. However, so far there is no systematic study on hepatoprotective activity has been reported on the selected plant in the literature. Hence the present study focuses on evaluating the hepatoprotective activity of whole plant of *Nymphoides hydrophylla*.

Nymphoides hydrophylla is an aquatic plant with common vernacular telugu name as Antharathaarama, Chirialli, Pitta kaluva; belonging to the family of Menyanthaceae (floating heart family). *Nymphoides hydrophylla* usually flowers and fruits in the winter season. It is very common throughout India, Malaysia and South China, in India it is distributed in Andhra Pradesh, Karnataka, Kerala, Tamil Nadu, Assam, Bihar, Delhi, Goa, Madhya Pradesh, Maharashtra, Manipur, Orissa, Punjab, Rajasthan, Uttar Pradesh and West Bengal. The plant is commonly used as a substitute for chiretta in treatment of fever and jaundice. Stalks and leaves are pounded with oil and applied to ulcers and insect bites whereas decoction is used as a wash for parasitic skin infections and seeds are considered as anthelmintic. Carban tetrachloride (CCl_4), a well known hepatotoxicant; that is metabolically activated by cytochrome P450 to form CCl_3 free radicals, which initiate lipid per oxidation in the cell[4]. CCl_4 (Figure 1) induces liver necrosis, and the Kupffer cells may possibly phagocytes the necrotic cell remnants. In addition, CCl_4 metabolites react with polyunsaturated fatty acids to propagate a chain reaction leading to lipid per oxidation covalently bind with lipids and proteins; leading to the destruction of cell membrane and liver damage. Hepatotoxicity or liver damage by CCl_4 can be measured by the analysis of several biochemical parameters including serum enzymes (SGOT, SGPT, ALP), bilirubin etc. The level of serum enzymes, bilirubin, increased in blood due to the administration of CCl_4 leading to cell membrane damage and necrosis. Serum enzymes are more specific to liver and are a better marker for detecting liver injury. In the present study we have estimated and compared the antioxidant and hepatoprotective activity of whole plant extract of *Nymphoides hydrophylla*, taking the model of liver injury in albino rats with potential and well known hepatic toxicant carbon tetrachloride (CCl_4).

MATERIALS AND METHODS

Plant Material Collection

The plants of *Nymphoides hydrophylla* were collected from the surrounding villages of chirala during 2013 processed into specimen number and having herbarium voucher no [R. Bharathi 0025]. The plant was authenticated by T.V. Raghava Rao, a leading Taxonomist in Maharani College, Peddapuram, AP, India[5].

Preparation of ethanolic extract

The freshly collected plants of *Nymphoides hydrophylla* were cleared from dirt then, dried under shade for about 15 days and then coarsely powered in a mechanical grinder. The powder was macerated with ethanol for 5 days, filtrate was collected and concentrated. The concentrated product was dried using desiccators with anhydrous calcium chloride. The percentage yield of the extract was 10.95 %w/w.

Chemicals

Gallic acid (Glaxo Smith Kline), DPPH (Research- Lab fine chem.), Methanol (Merck), Potassium di hydrogen phosphate (SD- fine chem. Ltd), Sodium hydroxide (SD- fine chem. Ltd), Potassium ferri cyanide (SD- fine chem. Ltd), Trichloro acetic acid (SD- fine chem. Ltd), Ferric chloride (Merck), Di ethyl ether (SD- fine chem. Ltd), Ethyl alcohol, Carbon tetrachloride (Qualigens), Silymarin (Dabur India), Olive oil, Glutamic Oxaloacetate Transaminase (ROBONIK (India) Pvt. Ltd), Glutamic Pyruvic Transaminase (ROBONIK (India) Pvt. Ltd), Bilirubin (ROBONIK (India) Pvt. Ltd), Alkaline phosphatase (ROBONIK (India) Pvt. Ltd), All the chemicals used in the study were of analytical grade.

Animals

Adult Albino rats (150-200 g) were used in the study. They were housed in well-ventilated rooms under standard conditions (23-27^{0}c, humidity 65-75 %, 12 h light/dark cycle), fed with standard rodent pellet diet and with tap water *ad libitium*. The study was performed as per the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA-regn no 1176/A/08/CPCSEA) guidelines for Laboratory Animal Facility.

Experimental schedule

***In vitro* antioxidant activity**

The antioxidant methods which were carried out to evaluate the antioxidant activity were DPPH (2, 2 Diphenyl-1-picryl-hydrazyl) free radical scavenging activity, Reducing power method, Phosphomolybdnum method and Nitric oxide method[6].

DPPH (2, 2 Diphenyl -1- picryl- hydrazyl) free radical scavenging activity

Free radical scavenging activity of *Nymphoides hydrophylla* was determined against DPPH method 0.002 %. DPPH solution in methanol was prepared and 0.1 ml of this solution was added to 1 ml of extract solution in methanol at the concentrations of 150, 300, 500 µg/mL, keep the mixtures in dark for 30 minutes, then the absorbance was measured at 517 nm using UV visible double beam spectrophotometer. A blank was prepared without adding extract. Gallic acid was used as standard at the concentrations 1, 2.5, 5 µg/mL Lower the absorbance of the reaction mixture indicates higher the free radical scavenging activity. The percentage inhibition can be calculated by the following equation[7-8].

$$\%\text{inhibition} = \frac{\text{Absorbance (blank)}_{517\text{ nm}} - \text{Absorbance (sample)}_{517\text{ nm}} \times 100}{\text{Absorbance (blank)}_{517\text{ nm}}}$$

Reducing power method

1 ml of extract solution in water at concentrations of (150, 300, 500 µg/ml) were mixed with phosphate buffer (1 ml, 0.2 molar, pH 6.6) and potassium ferricynide [$K_3Fe(CN)_6$] (1 mL, 1 %). The resulting mixture was incubated at 50°C for 20 minutes followed by the addition of 1 mL of Trichloro acetic acid (10 % w/v). The mixture was centrifuged at 3000 rpm for 10 minutes to collect the upper layer of the solution (2.5 mL), mixed with distilled water (2.5 mL) and 0.5 mL of freshly prepared $FeCl_3$ (0.1 %, w/v). The absorbance was then measured at 700 nm against blank sample. The increased absorbance of the reaction mixture indicates increased reducing power[9].

Phosphomolybdenum Method

Procedure

0.3 ml of test sample was taken in a tube and mixed with 3 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate and incubated at 95°C for 90 minutes. Gallic acid was utilized as a reference standard. The absorbance of the mixture was then measured at 695 nm with methanol blank. The antioxidant activity was expressed as the number of gram equivalents of Gallic acid[10].

Nitric oxide scavenging activity

Procedure

Two (2) mL of 10 mM sodium nitroprusside dissolved in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of sample at various concentrations (0.2–0.8 mg/mL). The mixture was then incubated at 25^{0}C. After 150 minutes of incubation, 0.5 mL of the incubated solution was withdrawn and mixed with 0.5 mL of Griess reagent [(1.0 mL sulfanilic acid reagent (0.33 % in 20 % glacial acetic acid at room temperature for 5 minutes with 1 mL of naphthylethylenediamine dichloride (0.1 % w/v)]. The mixture was then incubated at room temperature for 30 minutes and its absorbance pouring into a curette was measured at 546 nm[11]. The amount of nitric oxide radical inhibition was calculated by following this equation:

$$\%\text{ inhibition of NO radical} = [A_0 - A_1] / A_0 \times 100$$

Where A_0 is the absorbance before reaction A_1 is the absorbance after reaction has taken place with Griess reagent

Toxicity studies

Acute Toxicity (LD_{50}) Studies

Acute toxicity studies for Ethanolic extract of *Nymphoides hydrophylla* belonging to mentyhacea were conducted as per OECD guidelines using albino rats. Each animal was administered ethanolic extract by oral route. The animals were observed for any changes continuously for the first two hours and up to 24 h for mortality. There were no mortality and noticeable behavioral changes in all the group's tested[12].

Hepatoprotective activity

Evaluation of Hepatoprotective activity in CCl_4 induced Hepatotoxicity

The animals were divided into six groups, each group with six animals of either sex. Group I: Served as normal

control and received 10 % acacia suspension (1 ml/kg, p. o) daily for five days with olive oil (1 ml/kg, i. p) on days 2 and 3. Group II: Served as CCl_4 control and received 10 % acacia suspension (1 ml/kg, p. o) daily for five days along with CCl_4: olive oil (1:1, 2 ml/kg, i. p) on 2 and 3 days respectively. Group III: Was treated with reference drug Silymarin (50 mg/kg, i. p.) daily for five days and also receive CCl_4: olive oil (1:1, 2 ml /kg, i. p.) on 2 and 3, days respectively, 30 minutes after administration of reference drug. Group IV and V: Were treated with extract of (300 mg and 500 mg/kg, p. o. respectively) daily for five days and they also received CCl_4: olive oil (1:1, 2 ml /kg, i. p.) On days 2 and 3, respectively, 30 minutes after test dose. During this period of treatment the rats were maintained under normal diet and water. After the experimental period the overnight fasted rats were sacrificed. Blood samples were collected and liver tissue was excised for the determination of reduced glutathione and a part was fixed in buffered formalin for histopathological assessment of liver damage. Liver damage was assessed by the estimation of serum activities of SGOT (Aspartate transaminase), SGPT (Alanine transaminase) and TB (Total bilirubin) using commercial kits. Histopathological assessment of liver damage was done by studying haematoxylin and eosin stained slides of liver tissue[13-15].

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dennett's multiple tests. Results are expressed as mean ± SD for six rats in each group. Differences among groups were considered significant at $P < 0.001$ level.

RESULTS

Acute toxicity results

The extracts were found to be safe up to 1000 mg/kg body weight. Since no mortality was observed at 1000 mg/kg, it was thought that 1000 mg/kg was the cut off dose. Therefore $1/6^{th}$ and $1/4^{th}$ dose (i.e. 300 mg/kg and 500 mg/kg) were selected for all *in vivo* studies.

In-Vitro Antioxidant Activity

Table 1: Effect of Ethanolic extract of *Nymphoides hydrophylla* on DPPH Free radical scavenging activity and Oxide Scavenging Activity

DPPH Free radical scavenging activity				Nitric Oxide Scavenging Activity	
Tested Material	**Concentration (µg /mL)**	**Inhibition ± SEM**	**IC_{50} (µg/mL)**	**Inhibition ± SEM**	**IC_{50} (µg/mL)**
Sample Extract	150	74 ± 0.121	6.35	17 ± 0.228	432
	300	80 ± 0.098		39 ± 0.189	
	500	86 ± 0.202		53 ± 0.252	
Gallic Acid	1.0	78 ± 0.231	0.01	42 ± 0.241	1.73
	2.5	86 ± 0.107		55 ± 0.207	
	5	92 ± 0.106		71 ± 0.206	

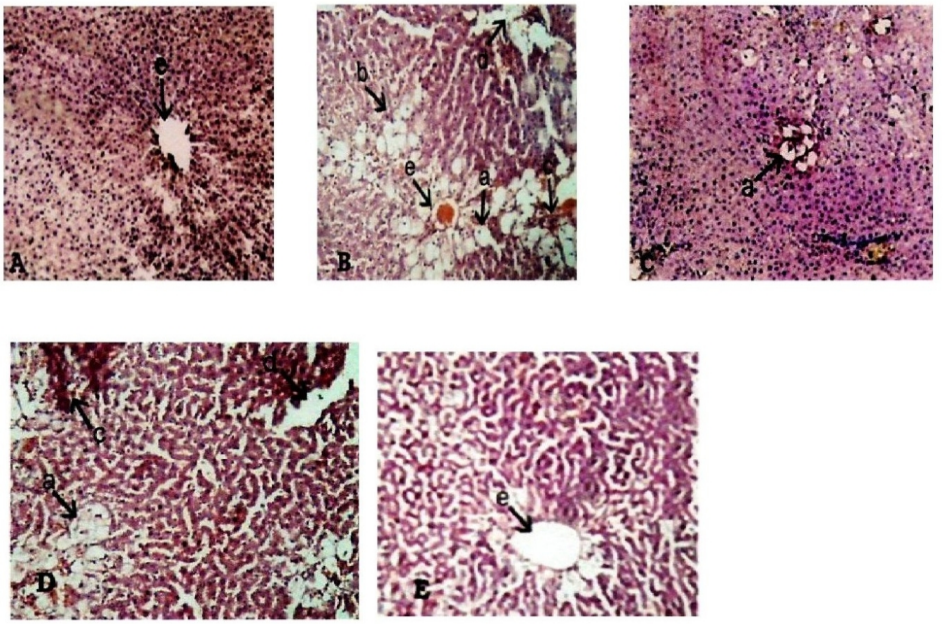
Table 2: Effect of Ethanolic Extract of *Nymphoides hydrophylla* on Reducing Power and Phosphomolybdnum Assay

Reducing power assay			Phosphomolybdnum Assay	
Tested Material	**Concentration (µg /mL)**	**Absorbance ± SEM**	**Tested Material**	**Absorbance ± SEM**
Sample Extract	150	0.1473 ± 0.01	Sample Extract	0.1016 ± 0.02
	300	0.2582 ± 0.08		0.1669 ± 0.03
	500	0.2738 ± 0.05		0.2420 ± 0.08
Gallic Acid	1.0	0.1646 ± 0.04	Gallic Acid	0.0413 ± 0.02
	2.5	0.1833 ± 0.01		0.0527 ± 0.01
	5	0.261 ± 0.03		0.0630 ± 0.05

Table 3: Effect of Ethanolic Plant Extract of *Nymphoides hydrophylla* on Serum Liver Enzymes

Serum parameters	Group I Normal control	Group II CCl_4 control (2 ml/kg)	Group III Standard (50 mg/kg) + CCl_4	Group IV Extract (300 mg/kg) + CCl_4	Group V Extract (500 mg/kg) + CCl_4
Bilirubin Mg/dl	0.923 ± 0.012	6.036 ± 0.035	2.03 ± 0.026	3.25 ± 0.021	1.20 ± 0.30
SGPT(U/L)	48 ± 1.27	287 ± 4.05	78 ± 2.46	186 ± 2.86	130 ± 8.49
SGOT(U/L)	87 ± 3.50	355 ± 4.95	85.5 ± 2.25	250 ± 2.82	150 ± 2.671
ALP(U/L)	218 ± 5.59	526 ± 2.92	270 ± 6.56	298 ± 4.29	235 ± 3.06

Values are expressed as mean ± SEM (n = 6) Groups III, IV and V are compared with group II ***p < 0.001 when compare to CCl_4 Control



Sections are Stained With Haemotoxylin And Eosin, 100 X Magnifications.

(A) Normal control,(B) CCl_4 control,(C) Silymarin (50 mg/kg) + CCl_4, (D) Ethanolic whole plant extract of *Nymphoides hydrophylla* (300 mg/kg) + CCl_4 ,(E) Ethanolic whole plant extract of *Nymphoides hydrophylla* (500 mg/kg) + CCl_4.

(B) Small arrowheads represent

(a) Macro, (b) Micro vesicular Infiltration,(c) Necrosis,(d)Sinusoidal dilation,(e) Central vein.

Figure 1: Effect of *Nymphoides hydrophylla* Extract on Histopathological Changes in CCl_4 Induced Hepatotoxicity in Rats

DISCUSSION

In the present study one of the locally available plant *Nymphoides hydrophylla* was screened for the antioxidant and the Hepatoprotective activity. Antioxidant activity is considered as an important mechanism by which many of the medical practitioners's used for the treatment of liver diseases. In the present study the plant extract contains phytochemical constituents such as flavonoids and phenols which are responsible for the Hepatoprotective action[16]. The Ethanolic plant extract of *Nymphoides hydrophylla* was subjected to *in vitro* antioxidant studies, the methods followed were DPPH, reducing power, phosphomolybdenum and nitric oxide assay. From the results it is evident that the extracts were acting as hydrogen donors[17] and thus were able to scavenge DPPH free radical. Results also indicate that the DPPH scavenging activity[18] was found to be concentration dependent. The evaluation of reducing power was based on the principle increase in the absorbance of the reaction mixture by the plant extract. Increase in the absorbance indicates increase in the reductive power. For the measurement of reductive ability, Fe^{3+} - Fe^{2+} transformation in the presence of sample was selected. The extract was found to increase in absorbance in a linear concentration. The plant extract was subjected to Phosphomolybdenum assay for the study of the antioxidant activity. The assay was based on the reduction of Mo (VI)-Mo (V) by the extract by the formation of green phosphate /Mo (V) complex at acidic pH. The extract was found to increase in absorbance in a linear concentration. Nitric oxide[19] is a potent pleitropic inhibitor of physiological process such as smooth muscle relaxation, neuron signaling, regulation of cell mediated toxicity. Preliminary phytochemical studies of Ethanolic whole plant extract of *Nymphoides hydrophylla* plant shows the presence of alkaloids, saponins, flavonoids and phenols. As the extract of *Nymphoides hydrophylla* decreases the amount of nitrite generated. The scavenging of NO by the extract was dose dependent. The IC_{50} values were found to be 432. The extract was subjected to screen for Hepatoprotective activity against CCl_4 induced Hepatotoxicity in rats. Administration of CCl_4 has caused the Hepatotoxicity as indicated by the enhanced levels of biochemical parameters like e.g. SGPT, SGOT, ALP and Bilirubin[20]. Histopathology reports reveal that administration of CCl_4 has shown macro and micro vesicular fatty infiltration, necrosis, sinusoidal dilatation and congestion of central vein. This further confirms that CCl_4 administration cause hepatotoxicity. Upon pre-treatment with Ethanolic extract of *Nymphoides hydrophylla* has decreased the elevated levels of biochemical markers like SGPT, SGOT, ALP and

Bilirubin, in a dose dependent manner[21]. Similarly, histopapathological observations show that hepatic globular architecture was normalized: only fewer macro infiltrations were seen. These observations suggest that the Ethanolic whole plant extract of plant *Nymphoides hydrophylla* possess hepatoprotective activity against CCl_4 induced hepatotoxicity. The lowering of enzyme level is a definite indication of the hepatoprotective action of the drug. Hepatic damage caused by CCl_4 administration was observed by recording SGOT, SGPT, ALP and Bilirubin levels in different groups, although the low dose (300 mg/kg) of *Nymphoides hydrophylla* exhibits hepatoprotection, the high dose (500 mg/kg) has showed more significant activity (values shown in Table 3). Depending on the type of cell and the membrane involved, lipid peroxidation due to CCl_4 results in haemolysis, which increases the serum Bilirubin level[22-24]. The 500 mg/kg dose of extract showed an effective decrease in the elevated serum Bilirubin level, as compared to the low dose and which was very close to Silymarin (50 mg/kg) shown in Tables 3. A possible mechanism of the extract on Bilirubin levels may be interference with cytochrome P-450, result in hindrance of the formation of hepatotoxic free radicals, thereby protecting the integrity of the membrane. The histopathological studies also exhibit the efficacy of drug as a hepatoprotective. Simultaneous treatment of Ethanolic extract with CCl_4 produces lesser degree of damage to the liver cells as compared to the animals treated with CCl_4 alone. The sections of the liver treated with extract (500 mg/kg) and CCl_4 reveal better hepatoprotective activity with no areas of necrosis and sinusoidal dilatations, which was almost similar to the standard (Silymarin) group. Thus, the administration of Ethanolic plant extract of *Nymphoides hydrophylla* revealed hepatoprotective activity against the toxic effect of CCl_4. In conclusion *Nymphoides hydrophylla* inhibited and reduced the CCl_4- induced hepatotoxicity in rats possibly by scavenging or blocking the formation of free radical generated during CCl_4 metabolism. Improving effect exhibited by *Nymphoides hydrophylla* could be attributed to the bioactive constituents that alleviated the deleterious effect of CCl_4 either by well known scavenging action or the antioxidant properties that inhibited lipid per oxidation, stabilized the reactive radicals preserve the cellular integrity and restrain the severity of CCl_4.

CONCLUSION

The study was taken up to evaluate ethanolic whole plant extracts of *Nymphoides hydrophylla* for antioxidant and hepatoprotective activities. The acute toxicity study conducted for ethanolic extracts indicates that they are safe up to 1000 mg/kg body weight. Ethanolic extracts of plant *Nymphoides hydrophylla* has demonstrated dose dependent results for DPPH radical scavenging, reducing power method, Phosphomolybdenum assay and nitric oxide scavenging assay. The extract of *Nymphoides hydrophylla* has antioxidant activity comparable to standard Gallic acid. The Hepatoprotective activity of *Nymphoides hydrophylla* extract is comparable with that of standard drug Silymarin ($p < 0.001$). Treatment with ethanol extract has lowered the elevated levels of SGPT, SGOT, ALP and Bilirubin, in CCl_4 induced hepatotoxicity in rats. Histopathological observation revealed that treatment with ethanolic extract has reversed the hepatic damage by CCl_4. The ethanolic extract of plant of *Nymphoides hydrophylla* possesses hepatoprotective activity and this may be due to the presence of flavonoids and antioxidant principles. These results are indicating that antioxidant principles are having a role in this plant. The results of the study justify that further extension of study on this extract would throw more importance for explaining its potential use in human beings.

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