Research Article

In vivo Anti-oxidant Screening of Bioactive Fraction of Seeds of *Strychnos potatorum* Linn

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ABSTRACT
Methanolic extract of *Strychnos potatorum* (SP) seeds were prepared by cold maceration. Methanolic extract was partitioned with different solvents. *In* vitro anti-oxidant screening of different extracts done by DPPH radical assay revealed aqueous fraction as bioactive fraction (ASP). The bioactive fraction was subjected to *in vivo* anti-oxidant screening. The liver of *Wistar* rats was intoxicated with CCl_4 which causes enormous change in the level of anti-oxidant enzymes such as superoxide dismutase, glutathione reductase, glutathione peroxidase and catalase in liver homogenate. The amount of malondialdehyde and reduced glutathione also gives account for anti-oxidant property in CCl_4 treated rat liver. The ASP was found to increase the level of the anti-oxidant enzymes and reduced glutathione content. Also found to reduce the amount of malondialdehyde in CCl_4 treated rat liver homogenate, which explains its anti-oxidant potential.

Keywords: *Strychnos potatorum*, bioactive fraction, *in vivo* anti-oxidant screening.

INTRODUCTION

Strychnos potatorum Linn (Fam: Loganiaceae) is a moderate sized tree found in southern and central parts of India, Sri Lanka and Burma. In traditional system of medicine the seeds are used for the treatment of various ailments[1] like jaundice, bronchitis, diabetes, conjunctivitis, chronic diarrhoea, dysentery etc. They are also used to clear muddy water by its coagulant action.

The useful parts of *Strychnos potatorum* is its seeds, fruits and roots. According to Ayurveda, seeds are acrid, alexipharmic, lithotriptic and cure strangury, urinary discharges, head diseases etc. Roots are Leucoderma whereas fruits are useful in eye diseases, thirst, poisoning and hallucinations. The fruits are emetic, diaphoretic alexiteric etc. According to Unani system of medicine, seeds are bitter, astringent to bowels, aphrodisiac, tonic, diuretic and good for liver, kidney complaints, gonorrhoea, colic etc[2,3,4].

The present study is focussed on the *in vivo* anti-oxidant screening of seeds of *Strychnos potatorum*. The literature reviews clearly explains the hepatoprotective effect[1,5] of the plant. The methanolic extract of the plant material was prepared by cold maceration. This extract was partitioned with different solvents and their anti-oxidant property was analysed by *in vitro* DPPH[6] free radical scavenging assay technique. The most effective fraction (bioactive fraction) was selected for the animal studies.

The animals (*Wistar* rats) were treated with CCl_4, which impairs the liver function. The liver was separated and used for anti-oxidant enzymes study. Treatment with CCl_4 decreases the level of anti-oxidant enzymes in liver. The reduced glutathione content was also reduced. As lipid peroxidation occurs in the liver, the amount of malondialdehyde, one of the by-products is increased enormously. Drugs with anti-oxidant potential can cause free radical scavenging effect and will increase the level of anti-oxidant enzymes and reduced glutathione. Also, the level of malondialdehyde will be decreased.

EXPERIMENTAL WORK

Plant Material

Strychnos potatorum seeds were collected from the suburbs of Thrissur district, Kerala and were authenticated by Dr. P Sujanapal, Scientist-B, Kerala Forest Research Institute (KFRI), Peechi, Kerala.

The plant material was defatted with petroleum ether and was macerated with methanol. The methanolic extract of SP was then partitioned (gradient) with solvents like chloroform, ethyl acetate, acetone and water.

In vitro DPPH free radical scavenging activity[6]

The different solvent extracts were screened by *in vitro* DPPH free radical scavenging assay to find out the bioactive fraction. A stock solution of DPPH (1.3 mg/ml in methanol) was prepared such that 75 µl of it in 3 ml methanol give an initial absorbance of 0.9. The decrease in the absorbance in the presence of different extracts and standard (ascorbic acid) at 100 µg/ml concentrations was noted at 517 nm after 30 minutes, using methanol as blank in UV-Visible Spectrophotometer. The one with maximum free radical scavenging activity was selected as bioactive fraction. The percentage inhibition was calculated using the formula,

$$\text{Free radical scavenged}\,(\%) = \frac{\text{A Control} - \text{A Test}}{\text{A Control}} \times 100$$

Where, A Control - Absorbance of control

A Test -Absorbance in the presence of the samples of extracts.

The one with maximum free radical scavenging activity was selected as bioactive fraction.

In vivo anti-oxidant screening

Studies related to free radical scavenging activity using rat liver

Test animals

Antioxidant enzyme activity was investigated in *Wistar* strains of rats. They were bred in colony and brought up in our own animal house. They were kept in standard environmental conditions (Temperature- 25-28^{0}C and 12 hours light/dark cycle). Rats of either sex, weighing 150-250g were used for animal experiments. (All ethical formalities were cleared for the conduct of animal experiments using albino rats.)

Acute toxicity studies

Wistar rats of either sex weighing 150 - 250g were used for carrying out acute toxicity studies following OECD guidelines No. 425 of CPCSEA. After oral administration of the drug, the animals were observed continuously for 2 hours and then intermittently for another 4 hours. After 24 hours, the deaths if any were noted to calculate the LD_{50}. 1/10th & 1/5th of the LD_{50} dose was selected for the pharmacological activity.

Impairment of liver function by CCl_4

CCl_4 was mixed with olive oil in the ratio 1:1 and given intraperitoneally at a dose of 0.5ml/kg body wt to intoxicate the rat liver.

Grouping of animals and different treatments

Male *Wistar* albino rats weighing 100-120g were used for hepatic anti-oxidant enzyme studies. The animals were divided into four groups of six each.

Anti-oxidant activity of *Strychnos potatorum* in Carbon tetrachloride treated rats.

Group I – Control

Group II – CCl_4 treated (0.5 ml / kg body wt)

Group III – CCl_4 + aqueous fraction of *Strychnos potatorum* (orally, 200 mg /kg)

Group IV – CCl_4 + aqueous fraction of *Strychnos potatorum* (orally, 400 mg /kg).

This treatment was repeated for 5 consecutive days and on the 6th day animals were sacrificed. The liver homogenate was prepared and preserved for the studies.

Activity of Scavenging Enzymes

1. Assay of Superoxide dismutase (SOD)

SOD activity was determined by the method of Kakkar et al[7]. The liver tissue was homogenized in 0.25M sucrose and differentially centrifuged at 10,000 rpm under cold conditions to get the cytosol fraction. The initial purification was done by precipitating the protein from the supernatant with 90% ammonium sulphate and after dialysis against 0.0025M Tris-HCl buffer, pH 7.4. The supernatant was used as the enzyme source.

The assay mixture contained 1.2ml Sod.pyrophosphate buffer (0.052M, pH8.3), 0.1ml 186µM phenazine methosulphate (PMS), 0.3ml 300µm nitroblue tetrazolium (NBT), 0.2ml 780µM NADH, appropriately diluted enzyme preparation and water in a total volume of 3ml. Reaction was started by the addition of NADH. After incubation at 30^{0}C for 90 seconds reaction was stopped by the addition of 1ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4ml of n-butanol. Mixture was then allowed to stand for ten minutes. Centrifuged and butanol

layer was taken out. Colour intensity of the chromogen in the butanol fraction was measured at 560nm against butanol. A system devoid of enzyme served as control.
One unit of enzyme activity is defined as the enzyme concentration required inhibiting chromogen production (OD at 560 nm) 50% in one minute under the assay conditions. The specific activity is expressed in units/ mg protein. Unit is defined as the velocity constant per second.

2. Assay of Catalase
The catalase activity was assayed by the method of Maehly and Chance[8]. The tissue was homogenized with 0.91M-phosphate buffer (pH 7.0) at 1- 4^0C and centrifuged at 5000 rpm. The estimation was done spectrophotometrically following the decrease in absorbance at 240nm. The reaction mixture contained 0.91 M phosphate buffer pH 7.0, 2mM H_2O_2 (diluted 0.1ml H_2O_2 to 100ml using buffer) and 50µl enzyme extract. Specific activity is expressed in terms of units / mg protein. Unit is defined as the velocity constant per second.

3. Assay of Glutathione peroxidase
The activity of glutathione peroxidase was determined by the method of Lawrence and Burk[9] as modified by Agerguard and Jense[10]. Tissue homogenate (10%) was prepared in 0.25M sucrose, centrifuged at 10,000 rpm for 30 minutes and the supernatant fraction was used for the assay. Activity was determined in phosphate buffer (50mM pH7.0) containing EDTA (1.5mM), sodium azide (1.0mM), reduced glutathione (1.0mM), NADPH (0.1mM) and glutathione reductase (1.0µM/ml). Absorbance was measured at 340nm at 20 seconds interval. Enzyme activity is defined as µM of NADPH oxidized/min /mg protein using 0.25mM H_2O_2 as substrate.

4. Assay of Glutathione reductase
Glutathione reductase activity was determined by the method described by Bergmeyer[11]. Tissue homogenate (10%) was prepared in 0.25 M sucrose, centrifuged at 10,000 rpm for 30 minutes and supernatant fraction was used for the enzyme assay. The assay system contained 1ml phosphate buffer (0.12M, PH 7.2) 0.1ml EDTA, 0.1ml sodium azide (10mM/l), 0.1ml oxidized glutathione (6.3mM) and 0.1 ml enzyme source. It was kept for 3 minutes. Then 0.1 ml NADPH (9.6 mM/l) was added. The absorbance at 340 nm was measured at an interval of 15 seconds for 2 minutes. The activity is expressed as µM NADPH oxidized per minute / mg protein.

5. Estimation of Glutathione content
Glutathione content was estimated by the method of Benke et al[12]. 20% tissue homogenate prepared in 5% TCA containing 0.001M EDTA, was centrifuged at 2000 rpm for 5 minutes; 0.2ml aliquot of each supernatant fraction was transferred to another tube containing 4.75ml of 0.1M sodium phosphate buffer (pH 8) and to it 0.05ml of 0.01m 5,5 dithiobis-2-nitrobenzoic acid (DTNB) was added. The absorbance was read at 412 nm within 4 minutes.

6. Estimation of Malondialdehyde
Determination of Malondialdehyde was based up on Nichans and Samuelson method[13]. To 1ml of liver homogenate in a test tube, 2ml of freshly prepared TBA-TCA-HCl mixture was added and thoroughly mixed. Kept in boiling water bath for 15 minutes. The mixture was cooled and centrifuged at 1000 rpm for 10 minutes to remove the flocculent precipitate. The supernatant was recovered and absorbance was recorded at 535 nm in UV-VIS spectrophotometer against reagent blank. A Standard calibration curve was obtained by plotting absorbance values against various concentrations (0.5nm/ml, 1.0 nm/ml, 2 nm/ml, 2.5 nm/ml, 3 nm/ml and 3.5 nm/ml). The effect on lipid peroxidation was expressed in nM of malondialdehyde/gram of tissue.

RESULT AND DISCUSSION

Determination of bioactive fraction by *in vitro* DPPH free radical scavenging assay
The *in vitro* DPPH free radical scavenging assay was carried out for different extracts of SP. The aqueous fraction showed maximum free radical scavenging activity (70.32 %) when compared to ascorbic acid (75.77 %). The results are tabulated in Table 1.

***In vivo* anti-oxidant enzyme studies in CCl_4 treated *Wistar* rats**
After treating the animals with CCl_4, the liver homogenate was examined for the anti-oxidant enzyme activities. The content of malondialdehyde and reduced glutathione was also calculated. Bioactive fraction of SP showed increased levels of anti-oxidant enzymes and reduced glutathione and there was a decrease in the level of malondialdehyde. (Table 2 and Table 3)
Graphical representation of the results was done to compare the activities of different groups under the study. (Figure 1 and Figure 2).

CONCLUSION

The bioactive fraction of seeds of *Strychnos potatorum* showed a good *in vivo* anti-oxidant activity. After treating the animals with CCl_4, there was a decrease in the amount of anti-oxidant enzymes (group II). The level of reduced glutathione was decreased and the malondialdehyde level was also increased. When the animals (previously treated with CCl_4) were treated with ASP at a dose of 200 mg/kg and 400 mg/kg (Group III and Group IV), there was an increase in the level of anti-oxidant enzymes and glutathione content. The malondialdehyde level was declined. All these explain that *Strychnos potatorum* is having significant anti-oxidant activity.

Statistical analysis

The experiment results were represented as the Mean±SD. Data were assessed by ANOVA method followed by student's t-test. $P<0.05$ was considered as statistically significant. (Table 2a and Table 3a).

Table 1: Results showing *in vitro* DPPH free radical scavenging activity by different extracts of SP

Extracts of SP (100µg/ml)	Percentage inhibition
Chloroform extract	15.45±0.47
Ethyl acetate extract	20.51±0.18
Acetone extract	18.99±0.25
Aqueous extract	70.32±0.34
Standard (ascorbic acid)	75.77±0.88

Value are expressed as Mean±SD (n=3)

Table 2: Results showing effect of ASP on the activity of Malondialdehyde, Glutathione and Superoxide dismutase in liver homogenate of CCl_4 treated *Wistar* rats

Groups	Malondialdehyde (MDA) (nmols / g wet tissue)	Glutathione (GSH) (mg / g)	Superoxide dismutase (SOD) (Units / mg)
I	0.75 ± 0.16^{a}	3.79 ± 1.14^{a}	7.39 ± 0.93^{c}
II	3.11 ± 0.62^{b}	2.97 ± 0.41^{a}	2.46 ± 0.73^{a}
III	1.27 ± 0.28^{a} ***	3.38 ± 0.72^{a}	4.55 ± 0.92^{b} *
IV	1.07 ± 0.48^{a} ***	3.84 ± 0.66^{a}	5.60 ± 1.19^{b}***

Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + Strychnos potatorum aqueous extract (200 mg / kg);
Group IV – CCl_4 + Strychnos potatorum aqueous extract (400 mg / kg).
Means in the same column scored by the same alphabet are not significantly different at 1% level.
MDA- ***p<0.001 compared to group II ;
GSH- Comparison between treatments are non-significant (p > 0.05).
SOD- *p<0.05 compared to group II ; ***p<0.001 *compared to group II*
Values are Mean±SD (n=6)

Table 2a: Statistical analysis (One-Way ANOVA) of data

Groups compared	F value (2, 15)		
	MDA	GSH	SOD
I, III & IV	3.88	0.51	11.80
II, III & IV	33.22	3.05	16.57

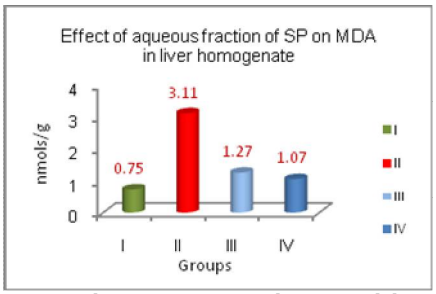
Table 3: Results showing effect of ASP on the activity of Glutathione reductase, Glutathione peroxidase and Catalase in liver homogenate of CCl_4 treated *Wistar* rats

Groups	Glutathione reductase(GR) (nmol of NADPH oxidized / min / mg protein)	Glutathione peroxidase(GPx) (nmol of NADPH oxidized / min / mg protein)	Catalase (moles of H_2O_2 decomposed / min / mg protein)
I	59.20±1.45[d]	187.35±1.18[d]	69.22±1.09[d]
II	43.84±1.86[a]	96.76±0.98[a]	38.32±1.08[a]
III	51.76±1.70[b]***	121.32±1.18[b]***	59.93±1.17[b]***
IV	56.29±1.57[c]***	147.58±1.08[c]***	62.47±1.83[c]***

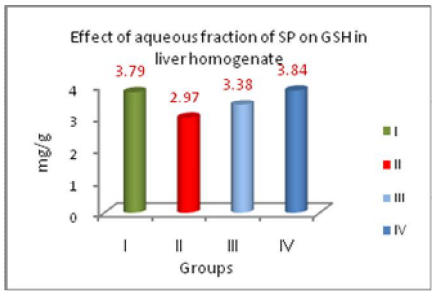
Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + Strychnos potatorum aqueous extract (200 mg / kg);
Group IV – CCl_4 + Strychnos potatorum aqueous extract (400 mg / kg).
Means in the same column scored by the same alphabet are not significantly different at 1% level.
***p<0.001 compared to group II in GR, GPx and Catalase.
Values are Mean±SD (n=6)

Table 3a: Statistical analysis (One-Way ANOVA) of data

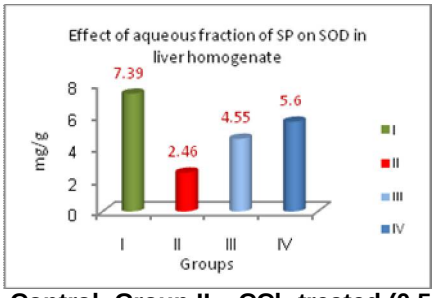
Groups compared	F value (2, 15)		
	GR	GPx	Catalase
I, III & IV	33.88	5013.36	70.15
II, III & IV	81.11	3292.56	538.74



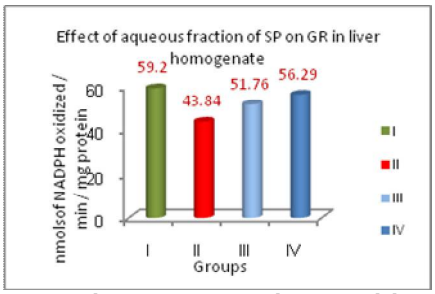
Group I– Control; Group II – CCl_4 treated (0.5 ml / kg); Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg); Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 1: Result showing effect of ASP on malondialdehyde in rat liver homogenate



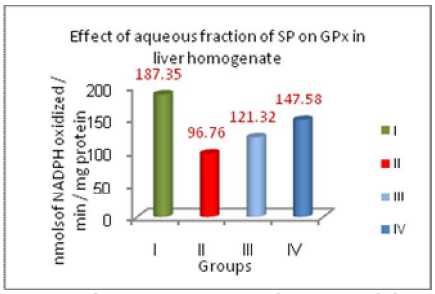
Group I– Control; Group II – CCl_4 treated (0.5 ml / kg); Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg); Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 2: Result showing effect of ASP on glutathione in rat liver homogenate



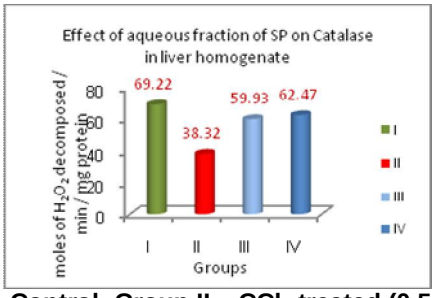
Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg);
Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 3: Result showing effect of ASP on superoxide dismutase in rat liver homogenate



Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg);
Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 4: Result showing effect of ASP on glutathione reductase in rat liver homogenate



Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg);
Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 5: Result showing effect of ASP on glutathione peroxidase in rat liver homogenate



Group I– Control; Group II – CCl_4 treated (0.5 ml / kg);
Group III – CCl_4 + aqueous extract of Strychnos potatorum (200 mg / kg);
Group IV – CCl_4 + aqueous extract of Strychnos potatorum (400 mg / kg).
Fig. 6: Result showing effect of ASP on catalase in rat liver homogenate

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