



PHYTOCHEMICAL EVALUATION AND HEPATOPROTECTIVE ACTIVITY OF FRESH JUICE OF YOUNG STEM (TENDER) BARK OF *AZADIRACHTA INDICA* A. JUSS

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ABSTRACT

The antioxidant and hepatoprotective activity of fresh juice of young (tender) stem bark of *Azadirachta indica* A. Juss, were evaluated against carbon tetrachloride (CCl₄)-induced hepatic damage in albino rats. The hepatoprotective activity of *A. indica* was evaluated by measuring levels of serum marker enzymes like serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP). The serum levels of total proteins and bilirubin were also estimated. Silymarin was used as standard drug. Administration of fresh juice *A. indica* (200 and 500 mg/kg, p.o.) markedly decrease in the elevated level of the above enzymes would indicate reversal of the induced toxicity of the liver. The perusal pharmacological assay shows that fresh juice of young stem bark extract of *A. indica* was good hepatotoxic agent at a dose level of 500mg/kg. the plant extract has decreased the enzyme level of SGOT, SGPT, ALP, Bilirubin by the dose of 500mg/kg and these result are statistically significant P < 0.01 when compared with CCl₄ group, while juice extract increases the proteins serum level to. The results are comparable to that of silymarin as a standard. The results suggest that antioxidant and hepatoprotective effect of fresh juice of *A. Indica* is possible related to the free radical scavenging activity.

Keyword: Hepatoprotective activity, antioxidant, carbon tetrachloride, *A. indica*. Silymarin.

INTRODUCTION

Liver is the most important organ concerned with the biochemical activities in the human body. It has great capacity to detoxicate toxic substances and synthesize useful principles. Therefore, damage to the liver inflicted by hepatotoxic agents is of grave consequences. There is an ever increasing need of an agent which could protect it from such damage. In view of severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines which are claimed to possess hepatoprotective activity.¹

Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical disease. More attention has been paid to the protective effects of natural antioxidants against drug-induced toxicities especially whenever free radical generation is involved. Flavonoids have been found to play important roles in the non-enzymatic protection against oxidative stress, especially in case of cancer. Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, neem, cocoas and red wine.²

Neem has been found to possess several types of chemicals that could be exploited for the pest management. Neem seeds mostly contain the complex tetranortriterpenoid lactones azadirachtin, Nimbin, nimbidin, salanin and nimbolin B out of which azadirachtin is the most active component. The leaves also contains azadirachtin, meliantrol, salanin, β -sitosterol, stimerol and flavonoides.³ The fresh stem bark yielded the bitter principles, nimbin, 0.04%; nimbinin, 0.002%; and nimbidin, 0.4%. Another terpenic constituent, identical with Sugiol is reported to be present in the stem bark.⁴

In this communication, we report the in vitro antioxidant activity and hepatoprotective activity of the fresh juice of young stem (tender) bark of *A.indica* was assayed by standard DPPH method. The hepatoprotective activity was determined by CCl₄ induced acute hepatotoxicity in rats and Silymarin is use as a standard hepatoprotective agent. The extent of liver damage was assessed by biochemical studies examination.

MATERIAL AND METHOD

Plant materials

Young (tender) stem barks of *A.indica* A. Juss tree were collected from Nagpur department, India. A qualified botanist of Go-Vigyan

Anusandhan Kendra, Nagpur, India, authenticated raw plant material used in the activity.

Preparation of fresh Juice

The authenticated plant parts i.e. young (tender) stem bark of *Azadirachta indica* A. Juss was collected and scrap by knives. The pieces of young stem bark were weighed and to that measured quantity of water were added and juice was made in mixer. Juice was separated by squeezing the material through clean muslin cloth and filtered; this clear liquid was allowed to dry in Lyophilizer (CAT NO. MSW 137) at reduce pressure for freeze drying. So that it stop the degradation of sensitive constituents, that may be present in the juice, till all the water got evaporate and complete dry powder was formed. The dry juice was transferred to air tight glass or plastic container. This container was placed inside a vacuum container to avoid attack of moisture.

Phytochemical evaluation

Phytochemical screening of fresh juice extract of *A. indica* A. Juss family, *Meliaceae* for the presence of these secondary metabolite⁵; Alkaloids (Draggendorffs), flavonoides (Shinoda test), saponins (Frothing test), tannins (5 % Ferric chloride), terpenoides (2, 4-dinitro-phenyl hydrazine), carbohydrates (Molish's test) were evaluated according to the methods described by Khandelwal. 2000⁵.

Test Animal

The experimental protocol was submitted and approved by Institutional Ethical Committee (IAEC No. 648/02/C/CPCSEA), J. L. C. College pharmacy, Nagpur, India. Wister albino rats (150-200 g) of approximate same age were employed in this investigation. The animals were fed with standard pellet diet and water and ad libitum. They were housed under standard conditions of temperature 22^o C (\pm 3^o C) humidity 35 % to 60 %, and light (12:12 hr light/dark cycle) in polypropylene mice cage. The animals received the drug treatments by oral gavages tube.

Chemicals

Silymarin was obtained as a gift sample from German Remedies Ltd., Mumbai for research, carbon tetrachloride (CCl₄) from Merck, DPPH from Sigma Chemical Co., India. The other chemicals and reagents used were of analytical grade.

Determination of total polyphenolic contents**Standard stock solution**

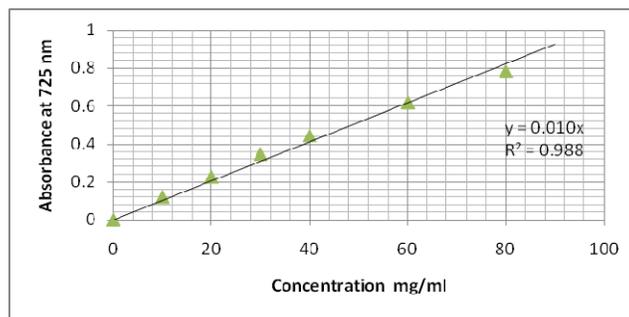
An accurately weighed quantity of gallic acid (~500mg) was dissolved in methanol and volume was made up to 100 ml with methanol (5 mg/ml).^{6,7,8}

Standard working solution

The aliquot portions of standard stock solution of gallic acid were diluted appropriately with methanol to obtain a concentration range of 10 – 90 mg/ml.

Procedure for calibration curve

Total polyphenolic content was estimated by the Folin-Ciocalteu method, 1mg/ml of diluted sample were added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min 800 μ l of saturated sodium carbonate (75 g/l) was added. After 2 hr incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid was used for the slandered calibration curve. The result were expressed as Gallic acid equivalence (GAE)/g, calculate as mean value \pm SD (n=3).



Graph 1: Shows Calibration curve of Gallic acid

Table 1: Table Shows Polyphenolic contents of aqueous crude extract of *A. indica* A. Juss

Sr. No.	Crude Aqueous extract (1mg/ml)		GAE mg/g
	Abs	Mean Abs	
1	0.706	0.709	70.66 \pm 0.34
2	0.711		
3	0.712		

Determination of total flavonoids**Standard stock solution**

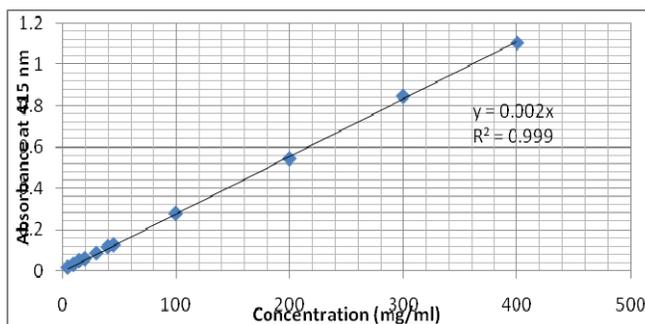
An accurately weighed quantity of rutin (~250mg) was dissolved in methanol and volume was made up to 100 ml with methanol^{6,7,8}

Standard working solution

The aliquot portions of standard stock solution of rutin were diluted appropriately with methanol to obtain the concentration range of 5 mg to 100 mg/ml.

Procedure for calibration curve

0.5 ml of extract was taken; to this 1.5 ml of 95 % alcohol, 0.1 ml of 10 % aluminium trichloride in methanol (100g/l) and 0.1 ml of sodium acetate was added and finally volume was adjusted to 5 ml with deminorized water. The mixture was kept aside at 20° C for 40 min. then the absorption was read at 415 nm. The graph was plotted as absorbent verses concentration. All the determination were carried out in triplicate the amt of flavonoides in plant extract in rutin equivalents (RE) was calculated^{6,7,8}.



Graph 2: Shows Calibration curve of rutin

Table 2: Table shows the flavonoids contents of aqueous crude extract of *A. indica* A. Juss

Sr. no.	Crude Aqueous extract (1mg/ml)		RE mg/g
	Abs	Mean abs	
1	0.22	0.236	77.66 \pm 2.34
2	0.24		
3	0.25		

Assay using DPPH**Preparation of standard DPPH solution**

2, 2-Diphenyl - 1picrylhydrazil (DPPH) was used as stable radical. 0.0098 g of DPPH was dissolved in methanol buffered with acetic acid buffer (0.1 M, pH 5.5) and the volume is made up to 100 ml. (500µM)^{6,7,8}

Preparation of working DPPH solution

20 ml of Std. DPPH was taken and volume was adjusted by buffered methanol to 100 ml. This served as working DPPH solution (50 µM).

Test solution and assay

4.9 ml. of the DPPH working solution was added to the 0.1 ml of test solution at different concentrations in methanol. Each mixture was

then shaken vigorously and held for 30 min at room temperature in the dark. The decrease in the absorbance of DPPH at 515 nm was measured. Water and methanol was used as blank solution. 4.9 ml DPPH solution in 0.1ml water served as control. All tests were performed at in triplicate. The radical scavenging activity of the samples (antioxidant) was expressed in terms of IC₅₀ (concentration in mcg required for a 50% decrease in absorbance of DPPH radical) and as % inhibition of DPPH absorbance which was calculated with following formula

$$\% \text{ Inhibition} = [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100, \text{ where}$$

A_{control} = Absorbance of control,

A_{test} = Absorbance of test sample.

From the graph it observes that the IC₅₀ is at 18 µg.

Table 3: Table shows DPPH absorption inhibition (%) of aqueous crude extract.

Sr. no.	Conc. (µg/ml)	Abs. at 515nm	% Inhibition	IC ₅₀
1	10	0.267 ± 0.002	1.58 ± 0.25	
2	14	0.229 ± 0.003	15.84 ± 0.21	
3	16	0.203 ± 0.004	25.39 ± 0.71	
4	18	0.134 ± 0.002	50.01 ± 0.56	18 µg
5	20	0.091 ± 0.005	66.66 ± 1.35	

Pharmacological study

The hepatoprotective activity of fresh juice of young stem (tender) bark of *A. indica* was evaluated by measuring levels of serum marker enzymes like serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP). The serum levels of total proteins and bilirubin were also estimated. Adult rats of either sex weighing 150–200 g were divided into five groups each consisting of six animals. Group 1 received liquid paraffin only (3 ml/kg, s.c.) and served as control. Rats of remaining four groups received suspension of carbon

tetrachloride (CCl₄) in liquid paraffin (1:2, v/v, 1ml of CCl₄/kg, s.c.) to induce hepatic damage 24 h before start of treatment. Group 3 received in addition to CCl₄ suspension, silymarin (100 mg/kg, p.o.) daily. Groups 4 and 5 received juice extract of *A. indica* (200 and 500 mg/kg, p.o., respectively) orally every day in addition to CCl₄ suspension for 8 days. Blood withdrawn through retro orbital plexus of rats on 8th day. Serum was separated from blood of each rat by centrifugation for estimation of glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), alkaline phosphatase (ALP), bilirubin, and total protein.¹

Table 4: Table shows Experimental Design: Evaluation of Hepatoprotective activity by inducing CCl₄ induced hepatic damaged in rats.

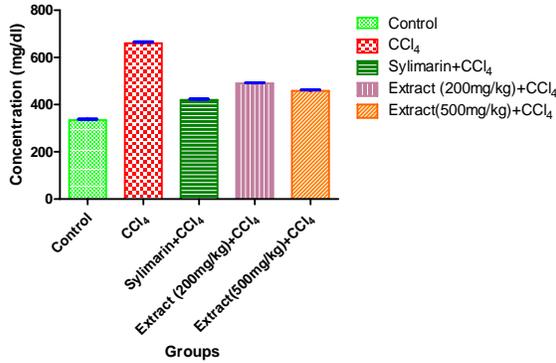
Group I	Control, received liquid paraffin (3ml/kg, s.c.)
Group II	Received suspension of CCl ₄ in liquid paraffin
Group V	(1:2, v/v, 1ml of CCl ₄ /kg, s.c.)
After 24 Hr.	
Group III	Received CCl ₄ suspension & Standard drug Silymarin (100 mg/kg, orally).
Group IV	Received CCl ₄ suspension & extract (200 mg/kg, orally).
Group V	Received CCl ₄ suspension & extract (500 mg/kg, orally).

Table 5: Table shows Effect of *A. indica* A. Juss treatment on different biochemical parameters in the serum of rats

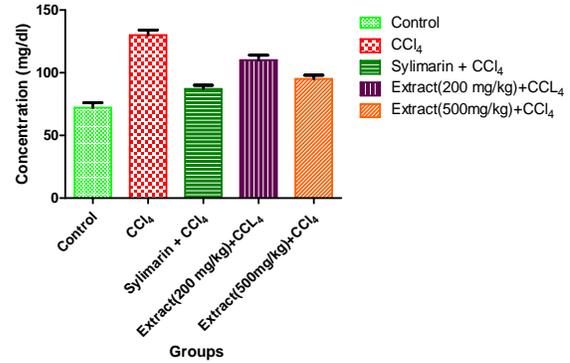
Parameter	Treatment				
	Control group	CCl ₄	Silymarin (100mg/kg, orally) + CCl ₄	Extract <i>A.indica</i> (200 mg/kg, orally) + CCl ₄	Extract <i>A.indica</i> (500 mg/kg, orally) + CCl ₄
SGPT	72 ± 4.0	130 ± 4.0 **	87 ± 3.0 **	110 ± 4.0	95 ± 3.0 **
SGOT	334.66 ± 4.5	660 ± 6.0 **	420 ± 3.9 **	490 ± 3.1	458 ± 4.0 **
ALP	221 ± 4.1	514 ± 4.0 **	280 ± 5.0 **	401 ± 3.0	370 ± 4.0 **
Bilirubin	0.68 ± 0.07	1.51 ± 0.11 **	0.7 ± 0.08 **	1.1 ± 0.13	0.9 ± 0.03 **
Total protein	6.8 ± 0.28	4.5 ± 0.19 **	5.6 ± 0.31 **	4.7 ± 0.21	5.2 ± 0.39 **

Values are expressed as mean ± S.D. of six animals in each group.

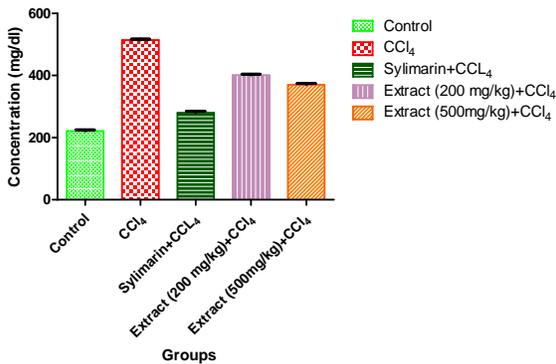
p < 0.01** as compared with group I, Experimental groups were compared with the CCl₄ group, p < 0.01 **



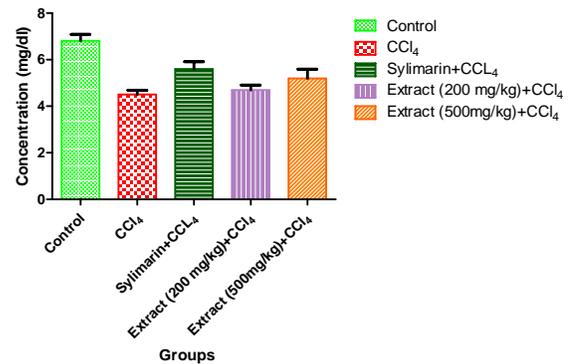
Graph 3: Shows Effect of extracts on SGOT level in hepatotoxic rats



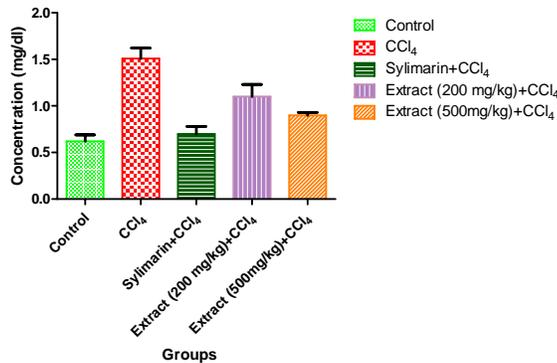
Graph 4: Shows Effect of extracts on serum SGPT level in hepatotoxic rats



Graph 5: Shows Effect of extracts on serum ALP level in hepatotoxic rats



Graph 6: Effect of extracts on serum Total Protein level in hepatotoxic rats



Graph 7: Shows Effect of extracts on serum Bilirubin level in hepatotoxic rats.

RESULTS AND DISCUSSION

Young (tender) stem barks of *A.indica* A. Juss, family *Meliaceae*, were collected and authenticated raw plant material used in the activity.

The authenticated and collected plant parts i.e. young (tender) stem bark of *Azadirachta indica* A. Juss were scrap by knives and added a measured quantity of water and juice was made in mixer. Juice was separated by squeezing and allowed to dry in Lyophilizer (CAT NO. MSW 137) at reduce pressure for freeze drying. So that it stop the degradation of sensitive constituents, that may be present in the juice, till all the water got evaporate and complete dry powder was formed. The dry juice was transferred to air tight glass or plastic container. This container was placed inside a vacuum container to avoid attack of moisture. All the extracts were subjected to chromatographic evaluation to check possible number of component in respective extracts using thin layer chromatography technique.

The plant *A. indica* belonging to family *Meliaceae*⁴ has been investigated in a systemic way to covering preliminary pharmacognostical evaluation. Fresh juice of young stem (tender) bark was collected and lyophilized immediately to obtained brown colour semisolid mass, so as to prevent degradation of thermo labile and hydrolysable compounds. The average % practical yield was found to be 1.86 %.

On preliminary Phytochemical screening, it was found that the dried fresh juice of young stem bark of *A. indica* A.Juss contains carbohydrates, proteins containing sulphur, Saponins, Flavonoids, terpenoides, tannins & Phenolics⁵. The plant extract shows the free radical scavenging and antioxidant activity. The fresh juice of young (tender) stem bark of *A. indica* extract exhibited significantly stronger free radical scavenging activities as determined by both assay using DPPH and total polyphenol, flavonoid contents.

For determination of dose, acute toxicity study was carried out in which no mortality was found, hence the doses of 200 mg/kg, 500mg/kg body weight were selected approximately for pharmacological studies. For assessment of hepatoprotective activity, the degree of hepatotoxicity developed was determined by withdrawing blood and evaluating different parameters on eighth day, the elevated levels of SGOT, SGPT, ALP, Bilirubin and decreased level of Total protein indicates the hepatotoxicity. Liver damage and recovery from damage was assessed on eighth day by measuring serum marker enzymes, biochemical changes in liver. It is well established that hepatotoxicity by CCl₄ is due to enzymatic activation to release radical in free state, which in turn disrupts the structure and function of lipid and protein macromolecule in the membrane of the cell organelles. The increased level of SGPT, SGOT, ALP, and bilirubin is conventional indicator of liver injury. In the present study, also it was seen that administration of CCl₄ elevates the levels of serum marker enzymes SGPT, SGOT, ALP, and serum bilirubin. Level of total protein is lowered. Fresh juice of *A. indica* and silymarin treated groups exhibited lower levels of SGPT, SGOT, ALP, and bilirubin as compared to CCl₄-treated group. The treatment with *A. indica* A. Juss also significantly elevated total protein levels significantly. The stabilization of serum bilirubin, SGPT, SGOT, and ALP levels by *A. indica* A. Juss is a clear indication of the improvement of the functional status of the liver cells. The characteristics feature of experimental hepatic damage observed is significant decrease in protein level. Decrease in the elevated level of the above enzymes would indicate reversal of the induced toxicity of the liver. The perusal pharmacological assay shows that fresh juice of young stem (tender) bark extract of *A. indica* A. Juss was good hepatotoxic agent at a dose level of 500mg/kg, the plant extract has decreased the enzyme level of SGOT, SGPT, ALP, Bilirubin and these result are statistically significant ($P < 0.01$) when compared with CCl₄ group While juice extract increases the proteins serum level significantly¹.

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