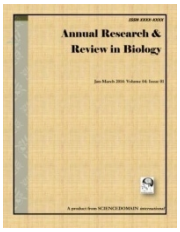

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Combinational Effect of *Cajanus cajan, Silybum marianum* and *Andrographis paniculata* on *In vivo* Antioxidant and Hepatoprotective Activities of Carbon Tetrachloride Intoxicated Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SS and AM designed the study. Most practical work was carried out by author SS. Statistical analysis was done by author Sapna S. Rest of the authors managed the preparation of the study and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To evaluate the combinational activity of *Cajanus cajan*, *Silybum marianum* and *Andrographis paniculata* for antioxidant and hepatoprotective potential.
Place and Duration: Department of Botany, Dr. H. S. Gour University (HSGVV), Sagar, Department of Zoology, HSGVV, Sagar, between September 2012 to April 2013.
Methodology: All three plants were subjected to Hydroalcoholic extraction. Adult albino

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rats were taken as experimental model for evaluation of hepatoprotective activity by measuring Aspartate amino transferase (AST), Alanine amino transferase (ALT) and total protein levels while liver was dissected out for measuring Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) level for antioxidant activity.

Results: *S. marianum* extract at 400 mg/kg.b.w showed better result among all three individual extracts by significantly decreasing the levels of AST and ALT (64.35 ± 8.17, 39.47 ± 5.61 U/L, respectively) and increasing the level of protein to 4.78 ± 0.41 mg/dl as compared to toxic control which is near to the value of standard drug. While the combination of the extracts showed enhanced activity as compared to that of *S. marianum* extract and standard drug (61.24 ± 3.7, 34.17 ± 3.21 U/L and 4.63 ± 0.22 mg/dl for AST, ALT and total protein respectively). For antioxidant activity, *S. marianum* increased the activity of SOD to 17.42 ± 0.63, CAT to 45.24 ± 1.84 and GPx to 21.96 ± 0.39 U/mg protein. Whereas, the combination of all three extracts in the concentration ratio of 1:1 increased the level of SOD, CAT and GPx to the near value of standard drug (18.12 ± 1.3, 44.24 ± 1.11 and 22.12 ± 0.46 U/mg protein).

Conclusion: All three plants showed potent hepatoprotective and antioxidant activity. Combinational study showed better results as compared to individual plant extracts and suggests that the polyherbal combinations may be used for enhanced activity.

Keywords: Hepatoprotective; antioxidants; aspartate amino transferase; alanine amino transferase; superoxide dismutase; catalase; glutathione peroxidase.

1. INTRODUCTION

The liver supports almost every organ in the body and is vital for survival. Due to its multi dimensional functions, the liver is prone to many diseases. Liver, the key organ for metabolism and excretion, is constantly endowed with the task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents [1]. Carbon tetrachloride (CCl4) is one of the most commonly used hepatotoxins in the experimental study of liver diseases. The hepatotoxic effects of CCl4 are largely due to its active metabolite, trichloromethyl radical. These activated radicals bind covalently to the macromolecules and induce peroxidative degradation of endoplasmic reticulum rich in polyunsaturated fatty acids. This leads to formation of lipid peroxidase, which in turn gives products like malondialdehyde (MDA) that causes damage to the membrane [2,3].

Free radicals are fundamental to any biochemical process; aerobic life and metabolism. They are continuously produced by the body's normal use of oxygen such as respiration. The Reactive Oxygen Species (ROS) inducing superoxide anionic radical ($O2^{\cdot-}$), peroxide ($O2^{\cdot-2}$) andhydroxyl radicals (·OH) are implemented in oxidative damage to various cellular macromolecules. Oxidative stress induced biochemical changes are crucial in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, inflammation and neurodegenerative disease [4]. Therefore, to overcome such problems, considerable attention has been directed towards identification of plants with antioxidant and hepatoprotective ability that may be used for human consumption [5].

Cajanus cajan, *Silybum marianum* and *Andrographis paniculata* belong to the families of leguminosae, compositeae and acanthaceae respectively. They share several medicinal properties including anthelmintic [6] and protection against alcohol induced liver damage [7,8]. "Thus, the present study investigated the combinational effect of *Cajanus cajan,*

Silybum marianum and *Andrographis paniculata* on the antioxidant and hepatoprotective activities in CCl4 intoxicated albino rats".

2. MATERIALS AND METHODS

2.1 Plant Material

The authenticated plant was collected from Natural Remedies Pvt. Ltd., Bangalore (sample invoice No. D119) and confirmed at Botany Department, Dr. H. S. Gour University, Sagar (M.P).

2.2 Chemicals and Drugs

The following drugs and chemicals were used: Ethanol (RANKEM), Aspartate amino transferase (AST), Alanine amino transferase (ALT), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) estimation kits (Merck), Carbon tetrachloride (RANKEM), total protein estimation kit (Commercial reagents kits from Span Diagnostics) and liquid paraffin (CDH). All chemicals used were of analytical grade.

2.3 Extract Preparation

Dried and powdered plant materials were extracted with 50% ethanol using soxhlet apparatus. The extracts were concentrated and dried at 68°C and kept at 4°C for further studies.

2.4 Phytochemical Test

Phytochemicals screening was performed to detect the presence or absences of various compounds such as tannins, flavonoids, alkaloids etc. as per standard methods [9].

2.5 Experimental Model

Adult albino rats (Wistar Strain) of either sex weighing between 150 – 200 g body weight were selected for the experimental study. The animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. They had free access to a commercial pellet diet and water *ad libitum*. The room temperature was maintained at 25±20C.

2.6 Experiment

A total of 78 animals were equally divided into 13 groups (n=6 in each group). The treatment period was for 6 days. Group I served as control and received vehicle (Normal saline) 10 ml/kg p.o. Group-II recieved CCl4 (2ml/kg) diluted with liquid paraffin (1:1) and given orally on third and sixth day, Group III recieved CCl4 and standard drug Liv 52 (150mg/kg p.o.). Group IV, V and VI received CCl4 and *C. cajan* extract, Group VII, VIII and IX received CCl4 and *S. marianum* extract, Group X, XI and XII received CCl4 and *A. paniculata* extract 100, 200 and 400 mg/kg p.o. respectively, once daily during the same period for 6 days. Group XIII received a combination of extracts of *C. cajan, S. marianum* and *A. paniculata* in the ratio 1:1:1 making their final concentration of 400 mg/kg p.o. (Table 1). Food was withdrawn

12hrs before CCl4 administration on the sixth day to enhance the acute liver damage in all the groups except group I animals. Rats were sacrificed on seventh day, 24 h after administration of the last dose. Blood samples were collected by abdominal aorta method and blood was collected in standard sampling tubes and serum was separated within 8 hours at room temperature for estimation of AST and ALT that determine the hepatoprotective activity of the drugs. The liver was excised and washed with 10 ml of 0.9% Sodium chloride solution to remove red blood cells. The tissue was then soaked in filter paper and blotted dry.

Table 1. Experimental setup and administration of various doses of drugs for the study of hepatoprotective and antioxidant activities of drugs individually and in combination in groups of rats (n=6)* for 6 days

Groups	Doses
Group I	Normal saline (10 ml/kg BW)
Group-II	CCl_4 (2ml/kgBW)
Group III	CCl_4 + Liv 52 (standard drug 150 mg/Kg BW)
Group IV	CCl_4 + CC extract (100 mg/kg BW)
Group V	CCl_4 + CC extract (200 mg/kg BW)
Group VI	CCl_4 + CC extract (400 mg/kg BW
Group VII	CCl_4 + SM extract (100 mg/kg BW)
Group VIII	CCl_4 + SM extract (200 mg/kg BW)
Group IX	CCl_4 + SM extract (400 mg/kg BW)
Group X	CCl_4 + AP (100 mg/kg BW)
Group XI	CCl_4 + AP (200 mg/kg BW)
Group XII	CCl_4 + AP (400 mg/kg BW)
Group XIII	CCl_4 + (CC + SM + AP in 1:1:1, making final concentration of 400 mg/kg BW)

AP= Andrographis paniculata
CC= Cajanus cajan
SM= Silybum marianum
Liv. 52= Standard drug
CCl_4= Carbon tetrachloride
** = 6 animals in each group*
1:1:1= 133.3:133.3:133.3 mg/kg BW

Then 200 mg of liver was homogenized with 200 µl buffer (0.05 M potassium phosphate and 0.1 mM EDTA, pH 7.8) using Teflon homogenizer and centrifuged at 10,000 RPM/30 min/4ºC for the estimation of antioxidant activity in liver tissue [10,11]. The study was approved by animal ethical committee (1030/9/07/CPCSEA).

2.7 Enzyme Assays

2.7.1 Hepatoprotective study

The estimation of hepatic marker enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was by standard colorimetric method [12,13,14] using standard kits. The results were expressed as units/litre (U/L).

2.7.2 Antioxidant study

The estimation of antioxidant enzymes Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx) was by standard methods [13,14,15] using standard kits.

2.8 Protein Estimation

The level of total protein was estimated in serum and liver tissues of experimental animals by Biuret method [16].

2.9 Statistical Analysis

The significance of difference among the groups was assessed using one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test between the data of control and treated groups. The values are expressed in mean±SEM.

3. RESULTS

3.1 Hepatoprotective Activity

The effect of hydroalcoholic aerial part extracts of *C. cajan, S. marianum* and *A. paniculata* on CCl4 induced liver damage in rats with reference to the variations in the levels of AST, ALT and total protein is shown in Table 2. CCl4 treated animals showed significant increases in the levels of AST (187.22 ± 12.50 U/L) and ALT (90.66 ± 8.60 U/L) while decrease in the level of total protein (1.57 ± 0.26) as compared to the normal control group (54.91 ± 6.30, 31.65 ± 4.30 U/L and 4.89 ± 0.41 mg/dL for AST, ALT and total protein respectively). All the extracts showed concentration dependent activity among which, *S. marianum* extract at 400 mg/kg.b.w showed better result by significantly decreasing the levels of AST and ALT (64.35 ± 8.17, 39.47 ± 5.61 U/L, respectively) and increasing the level of protein to 4.78 ± 0.41 mg/dl as compared to toxic control which is near to the value of standard drug (65.31 ± 7.30, 42.31 ± 4.50 U/L and 5.51 ± 0.47 mg/dL for AST, ALT and total protein respectively). While the combination of the extracts showed enhanced activity as compared to that of *S. marianum* extract and standard drug (61.24 ± 3.7, 34.17 ± 3.21 U/L and 4.63 ± 0.22 mg/dl for AST, ALT and total protein respectively).

Table 2. Hepatoprotective activity of *C. cajan, S. marianum* and *A. paniculata, values are for six animals in each group*

Groups*	Treatment (mg/kg BW)	Serum values of enzymes (U/L) AST	ALT	Total protein (mg/dl)
Group I	Normal Saline (Control)	54.91 ± 6.30	31.65 ± 4.30	4.89 ± 0.41
Group-II	CCl_4, 2ml (Toxic control)	187.22 ± 12.50***	90.66 ± 8.60***	1.57 ± 0.26***
Group III	CCl_4 + Liv 52 (St. drug, 150)	65.31 ± 7.30ns	42.31 ± 4.50ns	5.51 ± 0.47ns
Group IV	CCl_4 + CC (100)	112.36 ± 21.2	63.13 ± 4.46***	2.66 ± 0.31***
Group V	CCl_4 + CC (200)	97.41 ± 6.24***	46.43 ± 2.26**	3.11 ± 0.12**
Group VI	CCl_4 + CC (400)	67.32 ± 7.23ns	39.84 ± 5.44ns	4.76 ± 0.12ns
Group VII	CCl_4 + SM (100)	118.95 ± 14.43**	65.35 ± 6.21***	3.67 ± 0.47*
Group VIII	CCl_4 + SM (200)	90.16 ± 9.70**	51.16 ± 9.21*	4.27 ± 0.49ns
Group IX	CCl_4 + SM (400)	64.35 ± 8.17ns	39.47 ± 5.61ns	4.78 ± 0.41ns
Group X	CCl_4 + AP (100)	134.62 ± 12.20***	71.82 ± 6.70	3.36 ± 0.43**
Group XI	CCl_4 + AP (200)	107.21 ± 11.11**	59.88 ± 6.18***	3.98 ± 0.49ns
Group XII	CCl_4 + AP (400)	78.32 ± 9.18*	46.49 ± 7.81**	4.34 ± 0.48ns
Group XIII	CCl_4 + (CC + SM + AP in 1:1:1, making final concentration of 400 mg/kg BW)	61.24±3.7ns	34.17 ± 3.21ns	4.63 ± 0.22ns

*Results are expressed as Mean±SEM. P value; ***p<0.001, **p<0.02, *p<0.05.*
ns= non significant
CC= C. cajan
SM= S. marianum
AP = A. paniculata
CCl_4 = Carbon tetrachloride
Liv 52 = St. drug
AST= Aspartate amino transferase
ALT= Alanine amino transferase
** = 6 animals in each group*

3.2 Antioxidant Activity

In the present study, the hydroalcoholic extract of *C. cajan, A. paniculata* and *S. marianum* at different concentrations (100, 200 and 400 mg/ml) and their combination (1:1:1) to make a final concentration of 400 mg/ml) were assayed for antioxidant activity, analyzing SOD (Superoxide dismutase), CAT (Catalase) and GPx (Glutathione peroxidase). On assessment of the antioxidant enzymes, CCl4 treated animals showed significant decrease in the levels of SOD, CAT and GPx (11.21 for SOD, 27.57 for CAT and 17.14 for GPx U/mg of protein) as compared to the normal control group (19.30 for SOD, 52.43 for CAT and 25.34 for GPx U/mg of protein). *S. marianum* increased the activity of SOD to 17.42 ± 0.63, CAT to 45.24 ± 1.84 and GPx to 21.96 ± 0.39 U/mg protein. Whereas, the combination of all three extracts in the concentration ratio of 1:1:1 increased the level of SOD, CAT and GPx to the near value of standard drug (18.12 ± 1.3, 44.24 ± 1.11 and 22.12 ± 0.46 U/mg protein). (Table 3).

Table 3. Antioxidant activity of *C. cajan, S. marianum* and *A. paniculata Liver tissue. Values of anti oxidant enzymes, for six animals in each group*

Groups*	Treatment (mg/kg BW)	(U/mg protein) SOD	CAT	GPx
Group I	Normal Saline (Control)	19.30 ± 1.30	52.43 ± 4.38	25.34 ± 0.48
Group-II	CCl_4, 2ml (Toxic control)	11.21 ± 0.94***	27.57 ± 1.43***	17.14 ± 0.26***
Group III	CCl_4 + Liv 52 (St. drug, 150)	18.71 ± 1.12***	48.19 ± 3.87ns	23.14 ± 0.46**
Group IV	CCl_4 + CC (100)	13.46 ± 1.2**	21.18 ± 3.78***	9.26 ± 0.28***
Group V	CCl_4 + CC (200)	14.12 ± 3.6ns	27.33 ± 1.24***	14.14 ± 0.24***
Group VI	CCl_4 + CC (400)	16.11 ± 5.12ns	36.12 ± 2.34**	19.33 ± 0.36***
Group VII	CCl_4 + SM (100)	12.10 ± 0.79**	28.11 ± 1.59***	16.32 ± 0.38***
Group VIII	CCl_4 + SM (200)	16.35 ± 0.71*	38.58 ± 1.64**	20.10 ± 0.41***
Group IX	CCl_4 + SM (400)	17.42 ± 0.63ns	45.24 ± 1.84ns	21.96 ± 0.39***
Group X	CCl_4 + AP (100)	11.34 ± 0.90***	25.12 ± 1.33***	12.98 ± 0.38***
Group XI	CCl_4 + AP (200)	15.67 ± 0.78*	35.01 ± 1.25**	18.23 ± 0.38***
Group XII	CCl_4 + AP (400)	16.64 ± 0.65ns	40.19 ± 2.87*	20.34 ± 0.39***
Group XIII	CCl_4 + (CC + SM + AP in 1:1:1, making final concentration of 400 mg/kg BW)	18.12 ± 1.3ns	44.24 ± 1.11ns	22.12 ± 0.46***

*Results are expressed as Mean±SEM. P value; ***p<0.001, **p<0.02, *p<0.05.*
ns= non significant
CC= C. cajan
SM= S. marianum
AP = A. paniculata
CCl_4 = Carbon tetrachloride
Liv 52 = St. drug
SOD = Superoxide dismutase
CAT = Catalase
GPx = Glutathione peroxidase
** = 6 animals in each group*

4. DISCUSSION

One of the most commonly used chemical agents for liver damage in hepatoprotective study is CCl4 [17]. The active radical of this compound is CCl3 which binds to the macromolecules and induce peroxidative degradation of membrane lipids of endoplasmic reticulum. This results in the formation of lipid peroxides whose product malondialdehyde (MDA) causes severe membrane damage [18,19]. The extent of hepatic damage is assessed by the elevated levels of serum marker enzymes AST and ALT which were significantly lowered by the administration of all three extracts at a concentration of 400mg/kg BW and their combination (in the ratio 1:1:1) in the tested groups suggesting their hepatoprotective potential. The total protein estimation is useful in hepatoprotective study as its decreased level indicates severe non viral liver cell damage [20]. After CCl4 administration, the total protein level was lowered which was significantly elevated on treatment with all the four extracts, indicating their possible protective role against liver cell damage. The hepatoprotective potential of a drug depends upon its ability in reducing the harmful effects caused by a hepatotoxin [21]. The medicinal property of a plant is due to the presence of its chemical constituents. In hepatoprotective study, these phytoconstituents play a vital role in

inducing microsomal enzymes thereby accelerating the excretion of CCl4, or inhibiting the lipid peoxidation induced by CCl4 [22]. Phytoconstituents such as alkaloids [23] and flavonoid [24] have been found effective in the hepatoprotection against CCl4 induced liver damage. The phytochemical analysis of the hydroalcoholic extract of all three extracts showed the presence of such phytochemicals (alkaloid and flavonoid, Table 4) which may be responsible for the hepatoprotective efficiency of the plants against CCl4 induced liver damage [25].

Antioxidants are intimately involved in the prevention of cellular damage which is the common pathway for cancer, aging, and a variety of diseases [26]. Free radicals are atoms or groups of atoms with an odd (unpaired) number of electrons which can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction. Their chief danger comes from the damage they can do when they react with important cellular components such as DNA, cell receptors or the cell membrane. The body has a defense system of antioxidants to prevent free radical damage. They are the molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Catalase (CAT) and other antioxidant enzymes like Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) are produced naturally within the body. They help the body to convert superoxide to hydrogen peroxide and that into water and oxygen. GPx uses hydrogen peroxide to break down potentially harmful toxins in the body, including alcohol, phenol, and formaldehyde. When our body uses oxygen it produces free radicals that damage cell membranes, proteins and DNA. Catalase works closely with superoxide dismutase to prevent free radical damage to the body. SOD converts the dangerous superoxide radical to hydrogen peroxide which is converted to harmless water and oxygen by CAT and GPx. When the level of these enzymes decreases in the body, the antioxidant system cannot function properly [27].

The results obtained in the present study showed that all the extracts and their combination were found to be effective in increasing SOD, CAT and GPx activity. It also indicates that effects of these extracts may be associated with decreased oxidative stress, free radical-mediated tissue damage and that they prevent the accumulation of excessive free radicals and protect the liver from CCl4 induced liver damage in rats. Flavonoids, other phenolic compounds and poly sulfides of garlic oil of plant origin have been reported as scavengers of free radicals [28,29,30]. The results obtained in the present investigation of phytochemical studies show that all three plants are rich in flavonoids and phenolic compounds (Table 4).

Table 4. Phytochemical analysis of *C. cajan*, *A. paniculata* and *S. marianum*

Phytochemical/Test	*A. paniculata*	*S. marianum*	*C. cajan*
Alkaloids	+	+	+
Flavonoids	+	+	+
Tannins	+	+	+
Terpenoids	+	+	+
Steroids	+	+	+
Glycosides	+	+	-
Carbohydrates	+	+	+
Anthraquinones	-	-	-
Saponins	-	+	-

+ : Present
— : Absent.

5. CONCLUSION

All three plants showed potent hepatoprotective and antioxidant activity. Combinational study showed better results as compared to individual plant extracts and suggests that the polyherbal combinations may be used for enhanced activity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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