

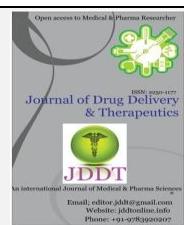


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Research Article

EVALUATION OF CYTOPROTECTIVE EFFICACY OF *ARISAEMA LESCHNAULTII BLUME* AGAINST CYCLOPHOSPHAMIDE INDUCED HEPATOTOXICITY IN TUMOR BEARING MICE

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ABSTRACT

Cyclophosphamide (CP) is a widely used antineoplastic drug, it is used for the treatment of several malignancies. However, upon treatment, it induces severe toxicity due to its oxidative stress capability. In this study we evaluate the cytoprotective efficacy of *Arisaema leschnaultii blume* (AL) against cyclophosphamide induced hepatotoxicity in tumor bearing mice due to its antioxidant property. Female Swiss albino mice were used. Group I-Naive control, Group II-DAL bearing, Group III-DAL + CP, Group IV-DAL + AL + CP, Group V- DAL + AL + CP, Group VI-DAL + Amifostine + CP. CP induced hepatic damage as indicated by significant elevation ($P < 0.05$) in aspartate aminotransferase, organ weight, and evidence by the histological study. CP also induced hepatic oxidative stress as indicated by significant elevation ($P < 0.05$) in malondialdehyde content, hydrogen peroxide (H_2O_2) generation, nitrite level, and the level of glutathione (GSH) peroxidase crashed in the CP treated group. AL enhanced the antioxidant defense system as indicated by elevation in GSH level, catalase activity, and GSH-S-transferase activity and by inhibiting MDA.

Keywords: hepatotoxicity, glutathione, antioxidants, cytoprotective etc.

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INTRODUCTION

Cyclophosphamide (CP) is a cytotoxic alkylating agent that has been in clinical use for over 50 years and it is effective in the treatment of neoplastic diseases such as solid tumors and lymphomas as well as nonneoplastic diseases such as rheumatoid arthritis and systemic lupus erythematosus.¹ However, the clinical use of CPA has been limited due to its ability to damage normal tissues which usually resulted in multiple organ toxicity mainly in the heart, testes, and urinary bladder.² Hepatotoxicity is a major side effect of CPA as it is metabolized principally within the hepatocytes by hepatic microsomal cytochrome p450 mixed function oxidase system to produce its two active metabolite phosphoramide mustard and acrolein.^{3,4} Phosphoramide is associated with its

immunosuppressive and antineoplastic effect, while acrolein is associated with its toxic effect.^{5,6} Studies have suggested that oxidative stress is associated with its hepatotoxic effect.⁷ CPA toxicity results from acrolein binding to cellular antioxidant nucleophiles such as glutathione (GSH) resulting in the depletion of the antioxidant defense system and it also initiates lipid peroxidation (LPO).⁸

Cytoprotective agents offer opportunities to reduce the treatment-related toxicity of anticancer therapy and perhaps to increase the dose and dose intensity of radiation and chemotherapy.⁹ Over 4000 cytoprotective agents were studied i.e. drde-07, herbal extract, Vit C, Vit E etc.¹⁰ The plant, *Arisaema leschenaultii* (Family Araceae) is a small tree distributed in the greater part of India on the hills of Assam, Karnataka, Kerala and

Tamilnadu has the antioxidant activity.¹¹ In this study we examine the cytoprotective efficacy of *Arisaema leschnaultii* blume against cyclophosphamide induced hepatotoxicity in tumor bearing mice.

MATERIALS AND METHODS

MATERIALS

Chemicals

Cyclophosphamide, amifostine, DTNB, alanine aminotransferas, alkaline phosphate, aspartase, bovine serum albumin, Bradford reagent, disodium hydrogen phosphate, disodium EDTA, EDTA, ethyl alcohol, formaldehyde, glacial meta phosphoric acid. Glutathione, lactate dehydrogenase, potassium chloride, ripa buffer, sodium chloride, tris buffer, sodium dihydrogen phosphate, thiobarbituric acid, tris carboxylic acid.

Animals

Female Swiss albino mice (25-30 g) were obtained from the central animal facility of Jiwaji University Gwalior and were maintained in polypropylene cages on rodent pellet condition of controlled temperature (22±2 °C) and acclimatized to 12/12 h light/dark cycle. Free access to food and water were allowed until 2h before the experiment. The care and maintenance of the animals were as per the approved guidelines of the "Committee for the purpose of control and supervision of experiments on animals (CPCSEA)". Food and water were provided 2h after the experiment. All experiments on animals were conducted according to the guidelines of establishment's ethical committee on animal experimentation.

Plant Extraction

Ethanol extraction For preparation of ethanol extract 10g of root powder was dissolved in 100ml of ethanol and kept at 270C for two days and filtered through Whatmann no. 1 filter paper. The filtrate was then allowed to evaporate to get concentrated filtrate which was again reconstituted in small volumes of same solvent. The solvent was air-dried and the extract was weighed.

METHODS

Induction of Tumor

5×10⁶ cells of Dalton's lymphoma (DAL) were injected in the peritoneal cavity of each animal on day 0. Cyclophosphamide (CP) (200mg/kg) was administered intraperitoneally (i.p) to groups of mice on day 3 after inoculation of Dalton's Lymphoma (DAL) for 7 consecutive days. AL (100mg/kg, 200mg/kg) and Amifostine (300mg/kg) was given orally 30 min prior to CP administration for 12 days. . Group I-Naive control, Group II-DAL bearing, Group III-DAL + CP, Group IV- DAL + AL + CP, Group V- DAL + AL + CP, Group VI-DAL + Amifostine + CP. The body weights of the animals were recorded daily up to 12 days and after 12 days animals were anaesthetized with ether for collection of blood from orbital sinus, and then sacrificed by cervical dislocation for the removal

of vital organ. Various haematological and biochemical analysis were carried out.

Biochemical estimation

Blood biochemistry, hepatic glutathione and hepatic MDA were estimated after the exposure. Hepatic Glutathione concentration of tissue was assayed according to method. Blood GSH was estimated by the method ¹² **Beutler et al. (1963)**. In brief, 150 milligram of tissue was homogenized in 0.02 M EDTA buffer (pH 8.0) and 50% TCA was added to it. Supernatant was mixed with 0.4 M tris buffer (pH 8.9), and 0.01 M DTNB (5, 5'- Dithio- bis- (2-nitrobenzoic acid) and absorbance was read at 410 nm ¹³. Hepatic lipid peroxidation was determined by measuring the level of thiobarbituric acid substance (TBARS) formed, using thiobarbituric acid (TBA), according to the method ¹⁴. Blood Hb, RBC and WBC were analyzed by using Bechman Coulter Analyzer (USA). SGPT, SGOT, ALP and total protein concentrations were estimated by using kits (Ecoline, Merck).

Thiol assay

The reaction mixture containing 900 μL 2mM Na₂ EDTA in 0.2M Na₂HPO₄, 20 μL 10mM DTNB in 0.2M Na₂HPO₄ and 100 μL serum was incubated at room temperature for 5 min; the absorbance was read at 412 nm. Appropriate sample and reagent blanks were prepared simultaneously and the respective absorbance was noted. Corrected absorbance values were used to calculate serum protein thiol content using a molar extinction coefficient of 1600/M/cm and values were expressed as mM. The calibration curve was produced using GSH dissolved in phosphate-buffered saline (PBS) ¹⁵

GST assay

One mL of reaction mixture containing 850 μL of 0.1M phosphate buffer, pH 6.5, 50 μL 20mM CDNB (1-chloro 2,4-dinitrobenzene) and 50 μL 20mM GSH was pre-incubated at 37°C for 10min. Reaction was started by adding 50 μL serum and GST activity was assayed kinetically. Reaction was followed at one minute intervals for five minutes by measuring the absorbance at 340nm. GST was determined by using a molar extinction coefficient of 9.6/mM/cm and GST activity was expressed as IU/L. ¹⁶ and (Harvey, J.W. et al., 1982)

MDA assay

The reaction mixture containing 1mL 0.67% thiobarbituric acid (TBA), 1 mL 20% tricarboxylic acid (TCA), and 100 μL serum was incubated at 100 °C for 20 min and centrifuged at 12,000 rpm for 5 min. The absorbance of the supernatant was read at 532 nm and MDA concentration was determined by using a molar extinction coefficient of 1.56×10⁵ /M/cm and the values were expressed as mM. ¹⁷

Determination of SOD aaand cataase

The determination of hepatic superoxide dismutase (SOD) activity by measuring the inhibition of

autoxidation of epinephrine at pH 10.2 and 30 °C. SOD activity was expressed in U/mg protein. Hepatic catalase activity was determined according to the method of ¹⁸. by measuring the reduction of dichromate in acetic acid to chromic acetate at 570 nm. Catalase activity was expressed as $\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein.

Protein Expression (Western Blotting)

20% tissue was homogenated in RIPA lysis buffer (Sigma, U.S.A.). Equal amounts of proteins (quantified by Bradford assay) were mixed with Laemmli sample buffer, resolved on 12% SDS-poly-acrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with a buffer containing 5% non-fat skimed milk powder in PBST (1×PBS, 0.2% Tween 20) at 4°C for 1hour, and subsequently incubated in the same buffer containing primary caspase-3 antibodies in 1:2000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) for overnight at 4°C and then membrane was washed by PBS-T and incubated for secondary antibodies for 1 hour. The Proteins bands were identified by using an enhanced chemiluminescence detection system in accordance with the manufacturer's instructions (ECL or ECL, Amersham Biosciences).

• Histopathological Studies

Liver tissues of mice were removed and washed with normal saline. The cleared tissue was fixed in 10% natural buffered formalin solution (pH 7.0-7.2). After proper fixation tissue was processed for dehydration in ascending grade of ethanol, clearing with toluene, followed by impregnation in paraffin wax, then sections of 5 μ in thickness were cut with help of semi-automatic rotary microtome. Sections were stained with hematoxyline. Stained paraffin sections of liver were examined under phase contrast microscope (Lieca DMLB) and photomicrographs were taken. Representative area were captured and analyzed with the help of Lieca Qwin V3 digital image processing and analysis system.

• Hepatic Apoptosis Determination (TUNEL Assay)

Apoptosis was measured by the identification of apoptotic nuclei in sections of liver by fragment end labeling of DNA (Apoptosis detection kit, Chemicon, USA). In, brief, endogenous peroxidase activity was inactivated by 3% hydrogen peroxides. The DNA fragments were allowed to bind an antidigoxigenin antibody that was conjugated to a peroxidase. Diaminobenzidine (DAB) was applied to develop dark brown colour and then the slides were counterstained with haematoxylin. All fields in each sample were evaluated for positive stained liver cell. The results were expressed as the number of positive stained cells per high-power field.

• Organ to Body Weight Indices (OBWI)

After sacrificing the animals, Liver was removed and the washed free of extraneous materials and weighed.

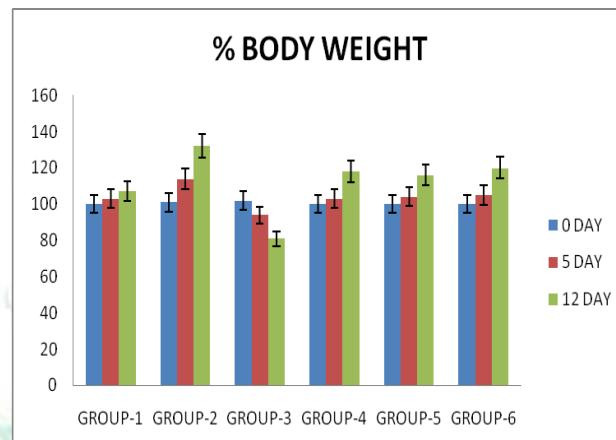
The organ to body weight indices (OBWI) were calculated as per the formula given below:

$$OBWI = \frac{Organ\ Weight}{Body\ Weight} \times 100$$

• Statistical Analysis

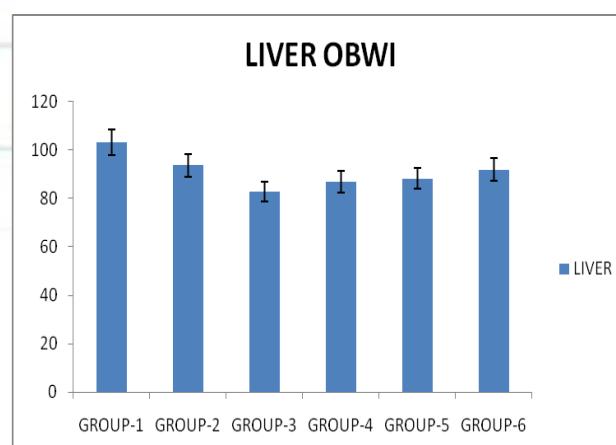
Statistical evaluations were made using one-way ANOVA followed by Dunnett's test. A probability of 0.05 and less was taken as statistically significant. The analyses were carried out using sigma stat for windows version 2.03 (SPSS Inc.USA).

RESULTS



$P<0.05$, as compared to control group (One Way ANOVA followed by Dunnett's t test).

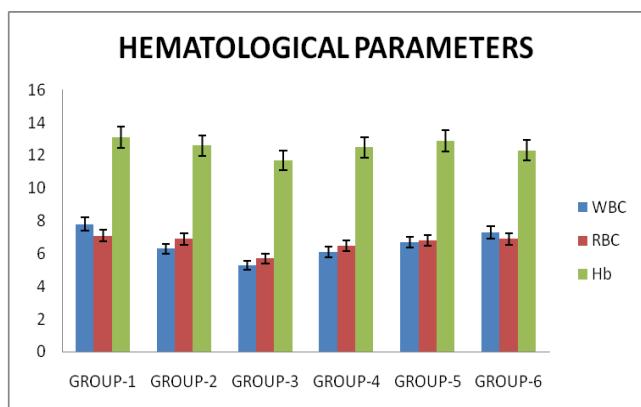
Figure 1: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on body weight of CP treated tumor bearing mice.



$*P<0.05$, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control values for liver OBWI = 8 ± 0.06

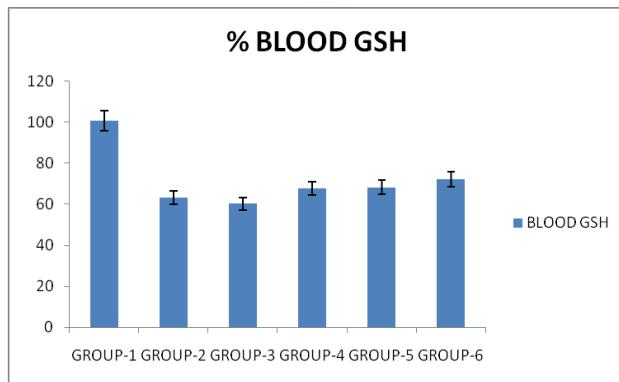
Figure 2: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on liver organ body weight indices (OBWI).



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control values for WBC= $7.4 \pm 0.7 \times 10^3$ cell/ μ l; RBC= 8.4×10^6 cells/ μ l; Hb= 13.2 ± 0.4 g/dl

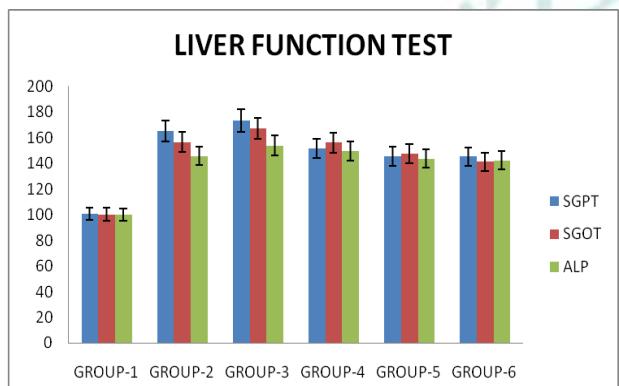
Figure 3: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on % WBC, RBC and Hb levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test)

Control values for Blood GSH= 0.075 ± 0.05 mg/dl.

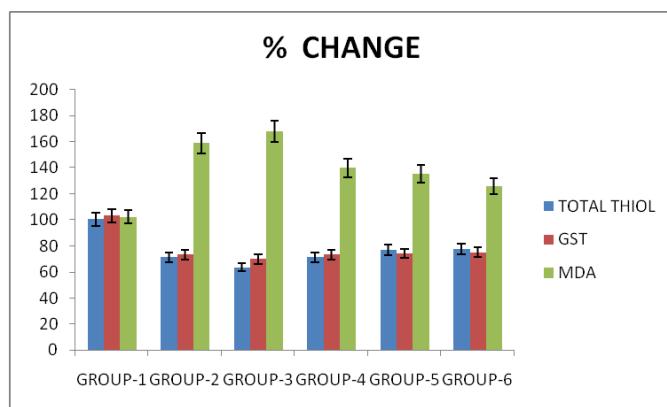
Figure 4: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on blood GSH (μ moles/mg) levels in SM treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control values for SGPT = 25 ± 1.01 IU/L; SGOT = 26.1 ± 1.02 IU/L; ALP = 29.2 ± 2.1 IU/L

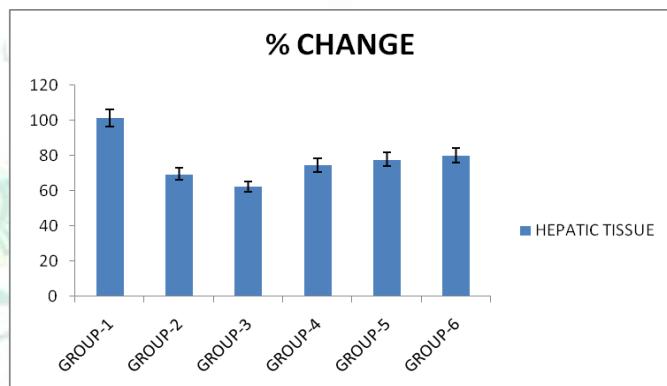
Figure 5: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on SGPT (IU/L), SGOT (IU/L) and ALP (IU/L) levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test)

Control values for GST= 0.248 ± 0.05 IU/L; Total thiol = 0.193 ± 0.07 mM; MDA = $0.22 \pm 0.08 \times 10^{-3}$ mM

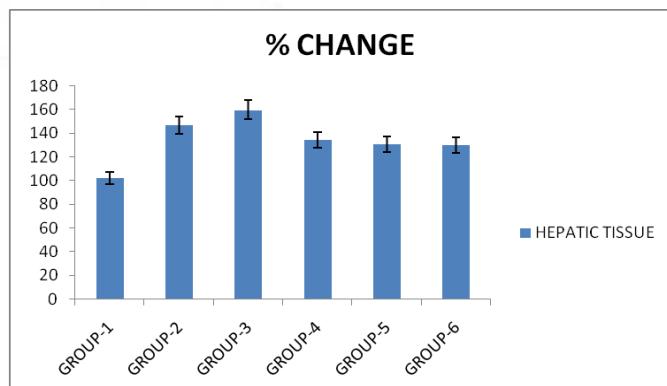
Figure 6: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on % plasma GST (IU/L), total thiol (mM) and MDA (mM) levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test)

Control values for GSH = 7.2 ± 0.4 μ moles/gm of tissue

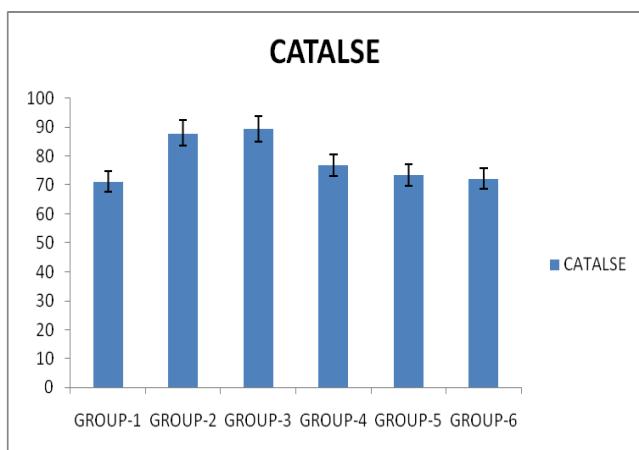
Figure 7: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on hepatic % GSH (μ moles/mg) levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test)

Control value for MDA = 4.43 ± 0.5 nmol/gm of tissue.

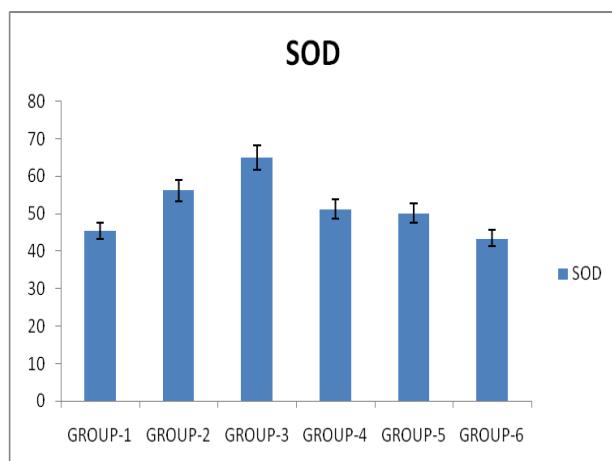
Figure 8: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on hepatic % MDA (nmol/mg protein) levels in SM treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control value for CATALASE = 72.12 ± 0.7 U/mg protein.

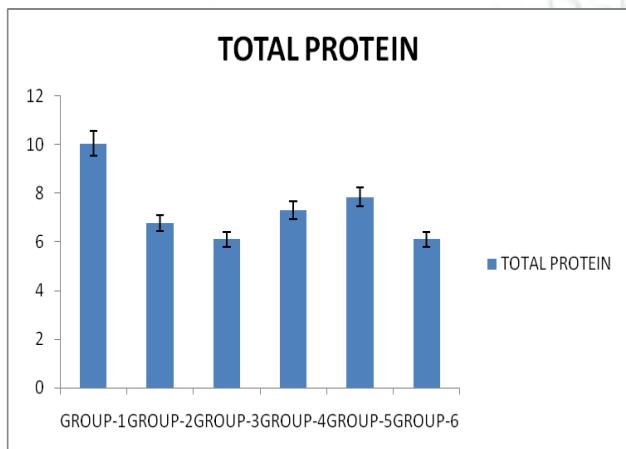
Figure 9: Effect of pretreatment of AL AL (100mg/kg), AL (200mg/kg) and Amifostine on catalase (U/mg protein) levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control value for SOD = 45.41 ± 0.67 U/mg protein.

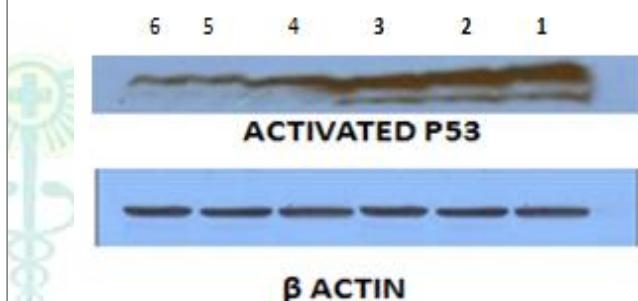
Figure 11: Effect of pretreatment of AL AL (100mg/kg), AL (200mg/kg) and Amifostine on SOD (U/mg protein) levels in CP treated tumor bearing mice.



*P<0.05, as compared to control group (One Way ANOVA followed by Dunnett's t test).

Control value for TOTAL PROTEIN = 10.02 ± 0.37 U/mg protein.

Figure 10: Effect of pretreatment of AL AL (100mg/kg), AL (200mg/kg) and Amifostine on total protein (U/mg protein) levels in CP treated tumor bearing mice.



GROUP-1	DAL
GROUP-2	DAL + CP
GROUP-3	DAL + CP + AL
GROUP-4	DAL + CP + AL
GROUP-5	DAL + CP + AMIFOSTINE
GROUP-6	CONTROL

Figure 12: Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on Caspase-3 protein expression in CP treated tumor bearing mice.

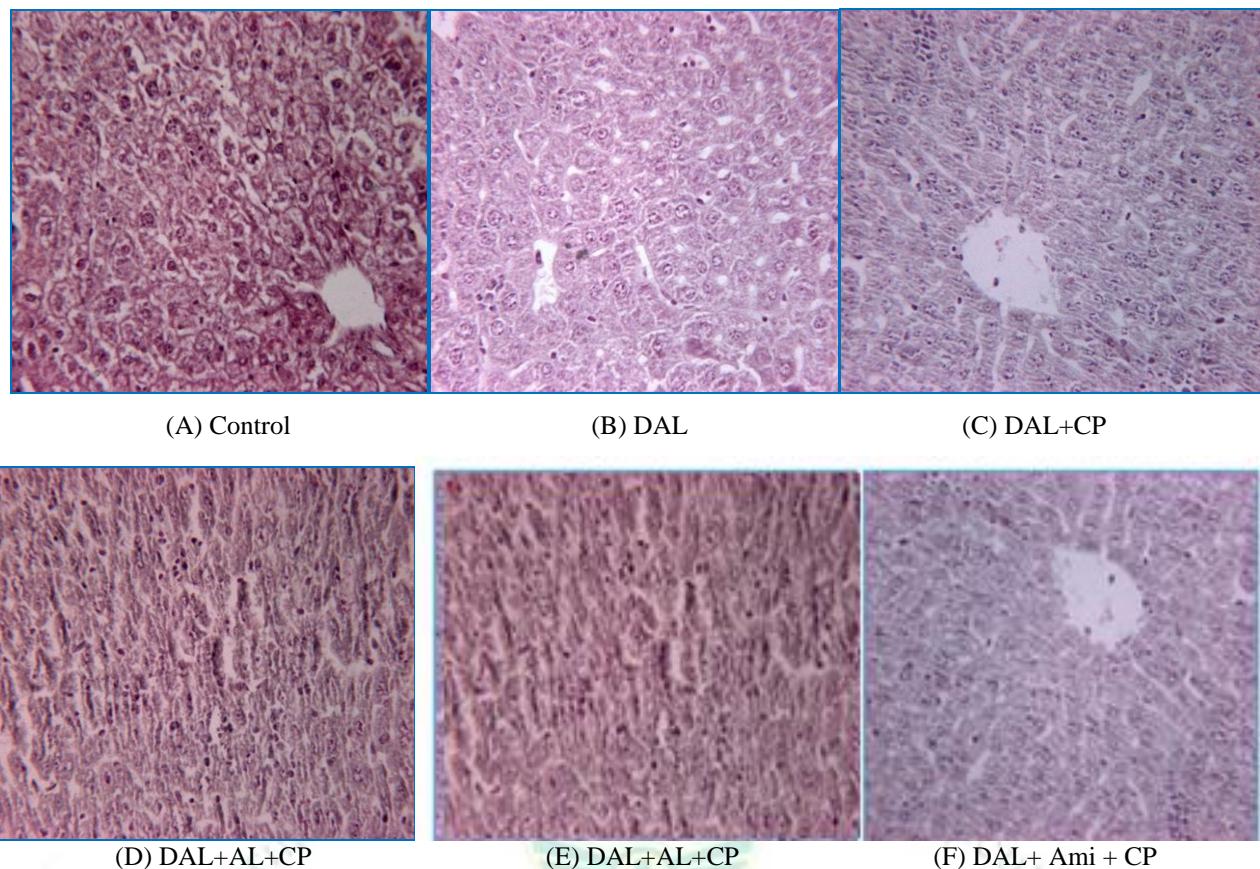


Figure 13 (A-F): Photomicrographs of CP administered (200 mg/kg, pc), AL (100mg/kg), AL (200mg/kg) on mice liver and other treatment

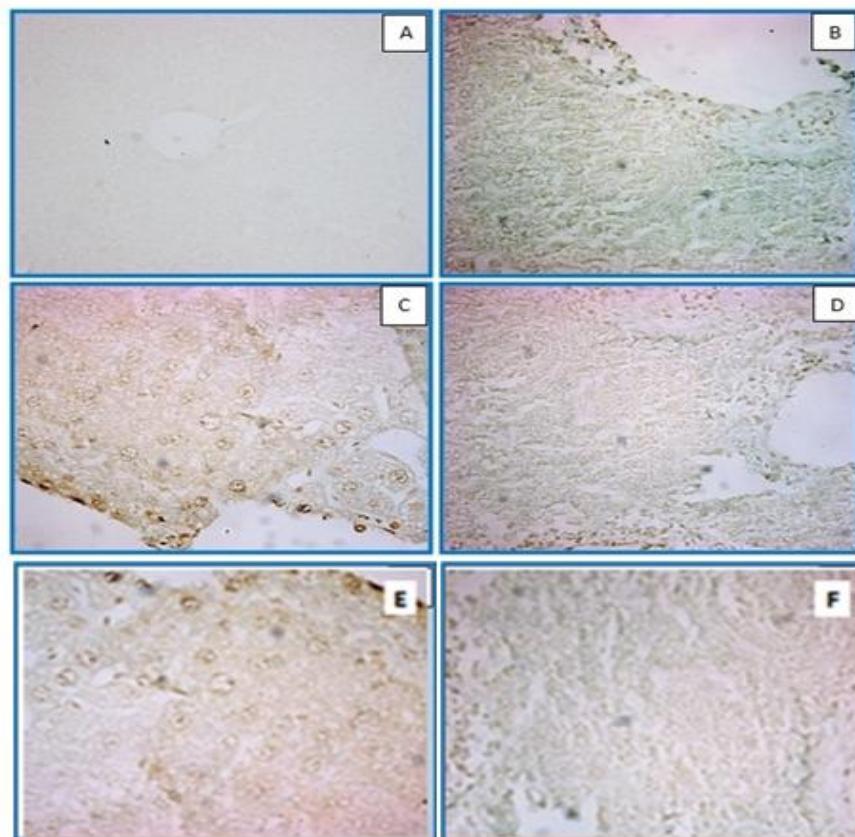


Figure 14 (A-F): Effect of pretreatment of AL (100mg/kg), AL (200mg/kg) and Amifostine on hepatocytes in CP treated tumor bearing mice (A) Control; (B) DAL (C) DAL+AL+CP; (D) DAL+ AL+ CP; (E) DAL+AL+CP ; (F) DAL+ Amifostine+CP

As per the literature the estimated cytotoxic dose of cyclophosphamide is 200 mg/kg. All the DAL bearing mice treated with 200mg/kg of CP to induce hepatotoxicity.

In **fig1** the percentage increase in body weight was significant ($p<0.05$) in treated groups compared to naive group. Body weight was increased significantly in DAL bearing mice ($p<0.05$) compared to naive group where as in CP treated mice body weight was significantly decreased compare to naïve control group. Body weight was not significantly changed by pretreatment of AL (100mg/kg, 200mg/kg) and Amifostine in CP treated tumor bearing mice compared to naïve control mice. No significant change was found in liver weight in any groups compared to control group (**Figure2**).

Figure 3 shows, WBC, RBC count and Hemoglobin level after 200 mg/kg of CP and other treatments in tumor bearing mice. There was significant ($p<0.05$) fall in the WBC, RBC and Hb count in DAL mice and CP treated mice compared to healthy control mice. A moderate improvement in WBC, RBC and Hb count was observed in AL (100mg/kg, 200mg/kg) and Amifostine treated mice but changes were not significant as compared to control group.

Figure 4 summaries, the changes in the reduced glutathione levels in blood. There was significant decrease ($p<0.05$) in blood glutathione level in tumor bearing mice and CP treated mice compared to naive group. In AL 100mg/kg, 200mg/kg and Amifostine pretreated groups, blood GSH level was also significantly increased ($p<0.05$) with respect to naive group. Blood GSH level was not significantly increased by pretreatment of CP treated tumor bearing mice compared to DAL bearing mice.

Figure 5 shows, the activities of serum SGPT, SGOT and ALP enzymes in all treatment groups. There was a significant increase ($p<0.05$) was observed in tumor bearing mice and CP treated tumor bearing mice compared to naive group. Amifostine and AL did show significant change in SGPT, SGOT and ALP activities compared to CP treated tumor bearing mice.

Figure 6 Summaries, the changes in the serum total thiol, lipid peroxidation and GST levels of animals of all groups. There was significant decrease in GST and total thiol level in CP treated tumor bearing mice and DAL bearing mice ($P<0.05$) compared to healthy control group. AL (100mg/kg, 200 mg/kg) and Amifostine treatment with CP in tumor bearing mice showed significant increase in serum GST and total thiol level compared to DAL bearing mice. Serum MDA level was significantly increased in DAL tumor bearing mice and CP treated mice with respect to healthy control group ($p<0.05$) and significant change has been observed by AL (100mg/kg, 200 mg/kg) and Amifostine in CP treated tumor bearing mice compared to DAL bearing mice.

Figure 7 summaries, the effect of various treatments on liver reduced glutathione levels in mice. There was significant decrease in reduced glutathione level in

tumor bearing mice and CP treated tumor bearing mice ($p<0.05$) compared to naive group. AL (100mg/kg, 200 mg/kg) and Amifostine showed any significant increase in hepatic tissue GSH level compared to DAL bearing mice.

In **Figure 8** the effect of various treatments on hepatic reduced MDA levels in mice. lipid peroxidation increased significantly in DAL bearing mice and CP treated tumor bearing mice compared to naïve group ($p<0.05$). MDA level was decreased significantly AL (100mg/kg, 200 mg/kg) and Amifostine pretreated tumor bearing groups compared to DAL bearing control mice.

Figure 9 shows the effect of pretreatment of AL and Amifostine on catalase levels in CP treated tumor bearing mice. The level of catalase significantly ($p<0.05$) increased in DAL bearing mice and CP treated group compared to naïve group. The level of catalase was decreased in AL and amifostine preteated tumor bearing mice compared to DAL bearing control mice.

Figure 10 shows effect of pretreatment of AL and Amifostine on total protein levels in CP treated tumor bearing mice. The level of total protein significantly ($p<0.05$) decreased in DAL bearing mice and CP treated group compared to naïve group. The level of total protein was decreased in AL and amifostine preteated tumor bearing mice compared to DAL bearing control mice.

Figure 11 shows the effect of pretreatment of AL Amifostine on SOD levels in CP treated tumor bearing mice. The level of SOD significantly ($p<0.05$) decreased in DAL bearing mice and CP treated group compared to naïve group. The level of SOD was decreased in AL and amifostine preteated tumor bearing mice compared to DAL bearing control mice.

Figure 12 shows, the expression of P⁵³ protein in liver of animals of various treatment groups. The western blot indicated that tumor results in the activation of P⁵³ protein in liver tissue. CP treated groups shows significantly reduction in caspase-3 protein band intensity compared to tumor bearing mice. In AL pretreated group P⁵³ protein also activates as compared to naive group. AL (100mg/kg, 200 mg/kg) and Amifostine treatment group does not show a significant reduction in band intensity (c P⁵³ expressions) compared to tumor bearing mice.

In Histological evaluation, liver section of control mice showed normal cord pattern, hepatic lobule, central canal and hepatocytes (**Figure13-A**). DAL bearing mice liver section showed mild vacuolar degeneration of hepatocytes and condensation of chromatin material indicative of cell death (**Figure 13-B**). In CP treated groups liver section showed abnormal cord and rearranged architecture of hepatocytes. Pretreatment with AL also showed the dead hepatocyte and feathery degeneration (**Figure 13 D, E**). The liver of CP administered mice pretreated Amifostine showed minimal to moderate lesions and some hepatocytes with granular degeneration (**Figure 13-F**).

Figure 14 indicates the number of apoptotic cells in liver section of animals of various groups. The number of apoptotic nuclei in the liver of control group was around 1 in a unit area (Figure 22-A). In the contrast, the numbers of apoptotic cells were observed frequently in centrilobular area in DAL group (Figure 14-B) and CP (Figure 14-C) and AL and amifostine treated tumor bearing mice (Figure 14-D,E,F) compared with control group.

CONCLUSION

Results concluded that *Arisaema leschenaultia blume* has accorded better protection in CP exposed tumor bearing mice. It not only protects the toxic effect of CP towards normal cells but also provides protection of tumor cells against cytotoxicity of CP. Thus *Arisaema*

leschenaultii blume neutralized the cytotoxic effect of CP towards normal cells as well as tumor cells.

FUTURE ASPECTS

As till date, there is no specific and effective cytoprotectants available against the adverse effects of chemotherapeutic agent. Therefore, there is need for the novel agent, which protects normal tissues from the deleterious effects of chemotherapy with the absence of tumor protection and tumor growth stimulation properties.

Studies are required on *Arisaema Leschnaultii Blume* and various other plants and synthetic compounds to come up as promising cytoprotective agent against chemotherapeutic agents.

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