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ORTHOSIPHON ARISTATUS EXTRACT REDUCES CCl_4 INDUCED OXIDATIVE DAMAGE IN MICE

CHITHRA VIJAYAN, P. SHIKHA*, P.G. LATHA* and G.M. NAIR
Department of Botany, University of Kerala, Kariavattom, Kerala, India.
*Ethnomedicine and Ethnopharmocology Division, TBGRI, Palode, Kerala, India.

Free radical stress leads to tissue injury and progression of disease conditions such as arthritis, hemorrhagic shock, atherosclerosis, diabetes, hepatic injury, aging and ischemia, reperfusion injury of many tissues, gastritis, tumor promotion, neurodegenerative diseases and carcinogenesis. Safer antioxidants suitable for long term use are needed to prevent or stop the progression of free radical mediated disorders. The present investigation elucidated the role of free radicals in CCl_4 induced toxicity and protection by ethanolic extract of *Orthosiphon aristatus*. Male Wistar rats were administered with CCl_4 (2 ml /kg, i.p) for four days along with 0.5% Tween - 80 or EOA (250mg/kg, 500mg/kg, orally). At the end of the experiment, following decapitation, liver tissue samples were taken for histological examination or determination of malondialdehyde (MDA), Glutathione (GSH), Superoxide dismutase (SOD) , Catalase (CAT) and conjugated dienes. Silymarin was used as the standard drug. Studies on acute toxicity were also carried out and LD_{50} was determined. The results revealed that CCl_4 caused a significant decrease in GSH, CAT and SOD levels and increase in MDA and conjugated dienes level. EOA treatment (250mg/kg, 500mg/kg, orally) reversed all these biochemical indices towards normal values. The results demonstrate that EOA extract, by balancing the oxidant- antioxidant status, protects against CCl_4 induced oxidative organ injury.

Keywords: Catalase (CAT); Conjugated dienes; Malondialdehyde (MDA); *Orthosiphon aristatus;* Reduced glutathione (GSH); Superoxide dismutase (SOD).

Introduction

Due to pharmacological safety, there has been an increased interest in phytochemicals that may exhibit antioxidant activity which could be relevant in their nutritional incidence and their role in health and disease[1-3]. Antioxidants neutralize reactive oxygen species which stress, diseases our cells and inflict damage to biomolecules, resulting in aging and genetic changes that lead to cancer. Common sources of antioxidants are fruits, vegetables and medicinal plants. Therefore, a great number of different spices and aromatic herbs have been investigated for their antioxidant activity[4]. Some particularly belonging to Lamiaceae, have been found to be very effective with regard to natural antioxidants.

Orthosiphon aristatus, a member of the Lamiaceae family,is a popular medicinal herb in South-East Asia. *O. aristatus* has been proven to exert anti-diabetic and lipid lowering effect in diabetic rats[5]. In Malaysia, *O. aristatus* is traditionally used to promote urination and to alleviate bladder and kidney discomfort[6]. The therapeutic effects of *O. aristatus* have been ascribed mainly to its polyphenols, which have enzyme inhibition and antioxidant activity[7].

Animal grouping and extract administration- Twenty five male rats were randomized into five groups consisting of five each. Group 1 served as control and was given 0.5% Tween - 80 (0.5 ml) per day for four days (i.p). Group 2 animals served as antioxidant control, treated with CCl_4 in a single dose of 2 ml / kg administered orally for four days, while the animals in group 3 and 4 were treated like the control except that they received 0.5 ml of the extract corresponding to 250, and 500 mg/kg body weight, respectively. The animals in the group 5 received silymarin (100mg/kg) along with CCl_4. At the end of the experiment, following decapitation, liver tissue samples were taken for histological examination or determination of antioxidant marker enzyme status. The liver from each animal was excised, rinsed in ice cold 0.25 M sucrose solution and 10 %w/v homogenate was prepared in 0.05 M phosphate buffer (pH 7) and centrifuged at 12,000 × g for 60 min at 4°C. The supernatant obtained was used for the estimation of catalase (CAT), superoxide dismutase

Table 1. Effect of the drug on hepatic antioxidant enzyme related parameters. a-d test values carrying superscripts different from the control across each parameter are significantly different (P > 0.05). Each value is mean ± S.D (n = 5 rats). Results are expressed as percentage inhibition of the control.

Groups	SOD Units/ mg protein	CAT Units/ mg protein	Conjugated Dienes mM/ 100g wet tissue	GSH mM/ 100g wet tissue	MDA nmol/ g wet tissue
Vehicle control	26.32 ± 0.4	28.92 ± 4.9	17.45 ±0.044	219.53 ±1.79	1.20 ± 0.30
CCl_4 control (ml/kg)	17.62 ±0.3[a]	18.72 ± 3.08[a]	26.10 ± 0.03[a]	141.63 ± 0.19[a]	2.86 ± 0.02[a]
CCl_4 + Drug(250mg/kg)	20.37 ± 0.6[b]	27.68 ± 1.76[b]	23.90 ± 0.09[b]	184.12 ± 1.71[b]	1.13 ± 0.15[b]
CCl_4 + Drug (500mg/kg)	20.83 ± 0.8[c]	34.42 ± 2.56[c]	16.93 ± 0.12[c]	214.80 ± 1.27[c]	1.06 ± 0.22[c]
CCl_4 + Silymarin (100mg/kg)	21.51 ±0.2[d]	39.52 ± 2.4[d]	15.91 ± 0.05[d]	233.69 ± 1.23[d]	1.00 ± 0.25[d]

(SOD), malondialdehyde (MDA), reduced glutathione (GSH) and conjugated dienes.

Acute toxicity - Acute oral toxicity study was performed using. Wistar rats of either sex selected by random sampling technique. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose level of 250mg/ kg, 500 mg/ kg, 1000mg/kg and 1500mg/kg by gastric intubations and observed for 24 hrs.

Assay of superoxide dismutase(SOD) : The activity of superoxide dismutase (SOD) was assayed[8]. Briefly, in a test tube, 0.5ml of supernatant of centrifuged tissue homogenate was taken. To this 1.5ml of carbonate buffer (pH10.2), 0.5ml of EDTA and 0.4µl of epinephrine was added and the OD was taken at 480nm. Epinephrine was added just before taking the OD.

Assay of malondialdehyde (MDA) : Lipid peroxidation was estimated in terms of Thiobarbituric Acid Reactive Species (TBARS)[9], using Malondialdehyde (MDA) as standard. The homogenized liver tissue (400µl) was mixed with 10% TCA and incubated for 15 min at 4°C and then centrifuged at 2, 200 g for 15 min at 4°C. To 1 ml of protein-free supernatant, 1 ml of fresh TBA reagent was added, mixed thoroughly and incubated at 60°C for 1 h in water bath. Then optical density was measured at 532 nm for the assay of MDA. Lipid peroxide is expressed in terms of nM of MDA mg^{-1} of liver tissue.

Determination of catalase activity -The activity of catalase was assayed following the method of Pari and Latha[10]. The percentage inhibition was evaluated following decrease in absorbance at 620 nm. The liver was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuge at 5000 rpm. The reaction mixture consisted of 0.4 ml of hydrogen peroxide (0.2 M), 1 ml of 0.01 M phosphate buffer (pH 7.0) and 0.1 ml of liver homogenate (10% w/v). The reaction of the mixture was stopped by adding 2 ml of dichromate-acetic acid reagent (5% $K_2Cr_2O_7$ prepared in glacial acetic acid). The changes in the absorbance was measured at 620 nm and recorded.

Determination of reduced glutathione activity: Reduced glutathione was determined using the modified method[11]. An aliquot of 1.0 ml of supernatant of liver homogenate was treated with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1 % sodiumnitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was measured at 412 nm.

Estimation of conjugated diene- Conjugated diene was estimated according to the method of Reckangel and Ghosal[12]. Tissue was extracted in tris HCl buffer (0.1M pH 7.5). 1ml of the homogenate wa mixed with 5ml of chloroform: methanol (2:1) and centrifuged at 2000rpm for five minutes. The upper layer was removed by aspiration. 2ml of the lower layer was taken in a test tube and evaporated to dryness in a water bath at 45°C. The residue was dissolved in 1.5ml of cyclohexane and absorbance was noted at 233nm against cyclohexane blank. The molar extinction coefficient of conjugated diene is 2.52 X $10^{-1}cm^{-1}$.

Histopathological Examination - Liver pieces were preserved in 10% formaldehyde solution for histopathological study. The pieces of liver were processed and embedded in paraffin wax. Slices were 4-6 mm thick, stained with hematoxylin and eosin and photographed.

Statistical analysis: All data were analyzed with oneway ANOVA followed by Duncan's multiple comparison test. The intergroup difference was considered significant when P<0.05. The correlation between oxidative stress and immunological parameters was checked by Pearson

correlation analysis.

Results and Discussion

The results from examination of the effects of *Orthoiphon aristatus* ethanol extract on the rats with liver-injury induced by CCl_4 are summarized in Table 1. In this present study, the rats treated with single dose of CCl_4 developed a significant hepatic damage and oxidative stress, which was observed from a substantial increase in the lipid peroxidation. This indicates the inability of antioxidant defense mechanism in the system to prevent the formation of excessive free radicals. The treatment with ethanolic extract of *O.aristatus* was able to reduce the level of lipid peroxides in a dose dependent manner as compared with the CCl_4 induced group.

Superoxide dismutase has been reported as one of the most important enzymes in the enzymatic antioxidant defense system[13]. It removes superoxide anion by converting it to hydrogen peroxide, and thus diminishing the toxic effect caused by this radical. The observed decrease in percentage inhibition of superoxide dismutase may be due to the hepatocellular damage by CCl_4. However, an increase in the percentage inhibition of superoxide after plant extract administration implies an efficient protective mechanism of this plant.

Catalase is another antioxidant enzyme widely distributed in the animal tissues. It decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals[14].The reduction of activity of this enzyme may lead to deleterious effects as a result of superoxide and hydrogen peroxide assimilation. In the present study, the percentage inhibition of catalase was shown to increase after the administration of the extract in a dose related pattern. This indicates the hepatoprotective ability of this plant against liver damage.

MDA is the major oxidation product of peroxidized poly-unsaturated fatty acids and the increased MDA content is an important indicator of lipid peroxidation[15]. Liver is the main detoxifying organ in the body and as such it possesses a high metabolic rate and it is subjected to many insults potentially causing oxidative stress. Hence, a corrective measure to stabilize the hepatic antioxidant defense system is of paramount importance for the maintenance of health[16]. The present study was undertaken to assess the effect of administration of ethanolic extract of *O.aristatus,* on the *in vivo* antioxidant status through the estimation of MDA concentration in the liver of rats.

The hepatic MDA content animals subjected to CCl_4-induced oxidative stress was found to be significantly increased. This enhanced oxidative stress however was significantly ($p<0.05$) reduced in both the treatment groups. The plant extract showed dose dependent and statistically significant inhibition of lipid peroxidation as shown by the reduction in MDA level.

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C[17]. Administration of thiol compounds such as glutathione, cysteine and methionine have been shown to protect against oxidative stress in humans and animals. The liver tissue glutathione levels were significantly depleted in the CCl_4 treated control group after inducing the oxidative stress with CCl_4. The tissue glutathione levels, however, were significantly elevated in the groups supplemented with plant extract.

Treatment with *O.aristatus* resulted in significant ($p<0.05$) increase in hepatic glutathione levels compared to that of CCl_4-induced oxidative stress rats. Therefore, it clearly demonstrates that *Orthosiphon aristatus* have protective role against oxidative damage in the liver tissue. Hepatic glutathione status exhibited by *O.aristatus* was comparable to that obtained by silymarin treated group.

Histopatological Examination - This protective effect of ethanolic extract of *Orthosiphon aristatus* was confirmed by histological examination . Histopathological study of liver from control group (Fig.1) showed a normal hepatic architecture. Massive fatty changes, gross necrosis, broad infiltration of lymphocytes and of Kupffer cells around the central vein, and loss of cellular boundary (Fig.2) were observed in the livers of CCl_4-treated rats. In the groups pretreated with silymarin or *O. aristatus* extract (Fig. 3 and 4), the livers exhibited an almost normal architecture, with the presence of double nucleus hepatocytes, barring a little deformity of hepatocytes with pyknosis and clearing of cytoplasm.

In conclusion, by estimating the activities of blood marker enzymes (GPx and SOD) and other biochemical parameters (CD, GSH and MDA), an assessment of liver function can be made. The changed activities of these marker enzymes observed in CCl_4-treated rats in our study corresponds to the extent of liver damage induced by the toxin. The tendency of these enzymes to return towards a near normal level in groups treated with silymarin or *Orthosiphon aristatus* ethanolic extract is a clear manifestation of their anti-oxidant effect. Moreover, the ability of the plant to prevent the process of initiation and progression of free radicals may be

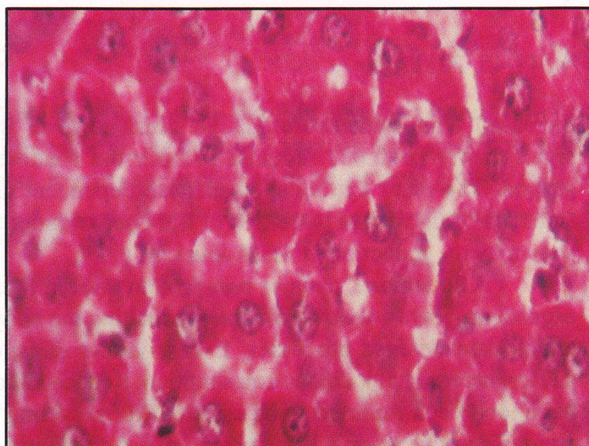

Fig.1. Section of liver of normal control rat showing hepatic cells with prominent nuclei and cytoplasm (x 350).

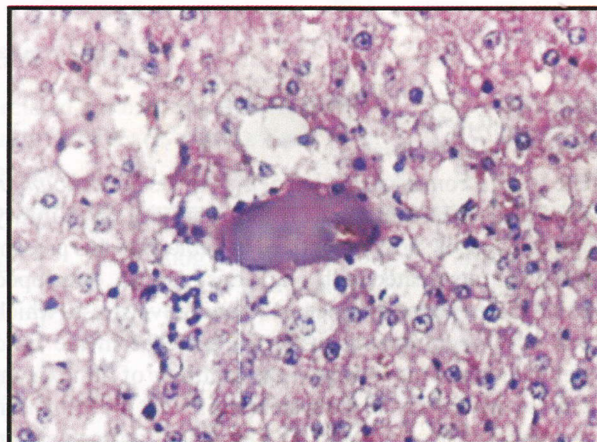

Fig.2. Section of the liver from Carbon tetrachloride - treated group, showing marked necrosis, severe congestion of blood vessels and inflammation with disappearance of nuclei (x 350).

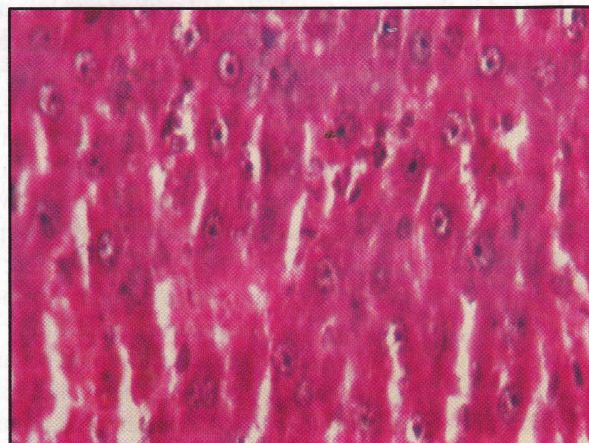

Fig.3. Section of the liver from OA (250 mg/kg) + CCl_4 - treated group showing marked improvement over CCl_4 control groups (x 350).

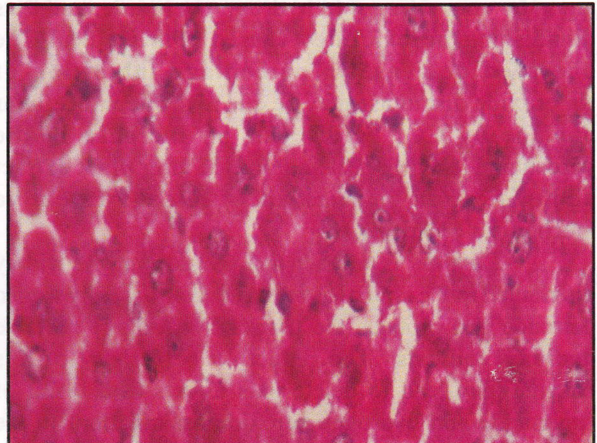

Fig.4. Section of the liver from Silymarin (100 mg/kg) + CCl_4 - treated group showing normal liver architecture (x 350).

attributed to the observed result.

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