



HEPATOPROTECTIVE EFFECT OF *CYNODON DACTYLON* ON CCl₄ INDUCED EXPERIMENTAL MICE

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Abstract

Context: Medicinal plants having diverse pharmacological properties including cytotoxic and cancer chemopreventive effects can be developed as novel drugs for cancer chemoprevention. One of the best approaches in search for anticancer agents from plant resources is the selection of plants based ethnomedical leads and testing the selected plants efficacy and safety in light of modern science.

Objectives: The present study aims in assessing the hepatoprotective activity of protein fraction of *Cynodon dactylon* on CCl₄ induced mice.

Materials and Methods: Fresh leaves were homogenized with phosphate buffered saline (PBS) at 4°C to obtain 20 % homogenate. The supernatant obtained was used for the ammonium sulphate fractionation. Hepatoprotective role was evaluated in the liver of mice administered with and without protein fraction. Paraffin oil and PBS serves as the vehicle control for Silymarin and protein fraction respectively. Silymarin was used as the standard antioxidant. CCl₄ acts as a hepatotoxin. The activity of enzymic antioxidants Catalase (CAT), Superoxide dismutase (SOD) and Peroxidase (Px) were determined in the liver homogenate of the control and experimental mice. The levels of these antioxidants were also assessed in the liver homogenate of the control and experimental mice.

Results: The protein fraction of *Cynodon dactylon* had significant hepatoprotective potential by enhancing the activities of enzymic antioxidants, increasing the levels of non enzymic antioxidants, marker enzymes and antilipid peroxidative role by decreasing the lipid peroxide levels. These effects were found to be more significant than that of silymarin, the standard antioxidant and the CCl₄ the hepatotoxin. Administration of *Cynodon dactylon* plus CCl₄ significantly decreases the levels of liver marker enzymes, hepatic enzymic and non enzymic antioxidants. The observed increased levels of lipid peroxides and decreased levels of enzymic and non enzymic antioxidants are the indications of liver damage due to high oxidative stress in CCl₄ induced mice when compared to the respective vehicle controls.

Conclusion: The findings of the present study revealed that the *Cynodon dactylon* had the potent hepatoprotective activity due to its antioxidant property against CCl₄ induced liver damage in mice.

Key words: Protein fraction, *Cynodon dactylon*, enzymic antioxidants, lipid peroxides, marker enzymes, CCl₄.

Introduction

Cynodon dactylon commonly referred to as Arugampul, is a valuable medicinal plant and is used as a curative for various ailments. This medicinal herb has a renowned position in Indian Systems of Medicine (www.herbalcure.com). The medicinal plant *Cynodon dactylon* is traditionally being used in diabetes (Kirtikar and Basu 1980), jaundice (Borah *et al.* 2006), kidney problems (Cheryl 2006), urinary diseases, GI disorders,

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constipation and abdominal pains (Paolo 2005). It has been reported to possess antimicrobial (Premkumar and Shyamsundar 2004), wound healing (Biswas and Mukherjee 2003), antioxidant (Auddy *et al.* 2003) and antimutagenic activities. The constituents reported in this plant are cynodin, hydrocyanic acid, triticin, proteins (Kao *et al.* 2005), carbohydrates, beta-carotene and minerals like calcium, phosphorus, iron and potassium (George *et al.* 1997). The present investigation explores the hepatoprotective effect of the protein fraction of the *Cynodon dactylon* on CCl₄, the hepatotoxin induced experimental animals.

Materials and Methods

Plant: The fresh plant material *Cynodon dactylon* (Arugampul) was collected from the pesticide free area, washed thoroughly to remove the dust particles, blotted dry between filter paper. The leaves were homogenized with phosphate buffered saline (PBS) at 4°C to obtain 20 % homogenate. The homogenate was strained through 8 layers of cotton gauze and centrifuged at 5000 rpm for 10 minutes at 4° C. The supernatant obtained was used for the ammonium sulphate fractionation.

Ammonium sulphate protein fractionation and estimation of protein content: Ten to hundred percentage ammonium sulphate fractionations of proteins was carried out and the precipitate obtained by this method was dissolved in 0.01 M PBS by the method of Jayaraman (1981). The protein content of all the fractions of the precipitate obtained after dialysis was estimated using the method of Shakir *et al.* (1994). DPPH assay was carried out by the method of Mensor *et al.* (2001) for finding ED₅₀ so that this dosage (40 µg) was used for administrating to experimental animals.

Animal: Swiss albino mice of 5 to 7 weeks of (20 to 25 g) were obtained from animal breeding station, Kerala Agricultural University, Thrissur. The mice were acclimatized to laboratory conditions for 15 days before the commencement of experiments. All procedures described were reviewed and approved by the University Animals Ethical Committee (Reg. No.623/02/b/CPSCSEA).

Experimental Design: Hepatoprotective role was evaluated in the liver of mice administered with and without protein fraction. Paraffin oil and PBS serves as the vehicle control for Silymarin and protein fraction respectively. Silymarin was used as the standard antioxidant. CCl₄ acts as a hepatotoxin. The administrations were carried out for 21 days. The mice were divided into seven groups of six each.

Group I : PBS (intraperitoneally injected with 100µl).

Group II : Paraffin oil (intraperitoneally injected with 100µl)

Group III : Silymarin (intraperitoneally injected with 100µl paraffin oil containing 500µg Silymarin, i.e. 25mg / kg bwt)

Group IV : Protein fraction (intraperitoneally injected with 100µl PBS containing 40µg (ED₅₀) of protein)

Group V : CCl₄ (intraperitoneally injected 100µl with CCl₄)

Group VI : CCl₄ + Silymarin (intraperitoneally injected with 100µl of paraffin oil containing 500µg of silymarin and 100µl of CCl₄)

Group VII: CCl₄ + Protein fraction (intraperitoneally injected with 100µl of CCl₄ containing 40µg (ED₅₀) of protein).

Assessment of the enzymic activity and antioxidative role: The activity of enzymic antioxidants Catalase (CAT), Superoxide dismutase (SOD) and Peroxidase (Px) were determined in the liver homogenate of the control and experimental mice (Luck 1974). Apart from enzymic antioxidants, a spectrum of non-enzymic antioxidants namely retinol, ascorbic acid α-tocopherol and reduced glutathione, are important in cellular system in curtailing ROS. The levels of these antioxidants were also assessed in the liver homogenate of the

control and experimental mice. Liver marker enzymes Serum Glutamic Oxaloacetic Transaminase and Serum Glutamic Pyruvic Transaminase, were assayed in the serum of the control and experimental mice. Knowing the antioxidative role of protein fraction, the antilipid peroxidative effect also was evaluated by method of Bishayee and Balasubramaniam (1971):

Statistical analysis: The data presented here are means of 6 mice each group. The data were statistically analyzed by one-way ANOVA followed by Dunnet's method.

Results

The hepatoprotective potential of protein fraction of *Cynodon dactylon* leaves was evaluated by assessing the activity of enzymic antioxidants, the levels of non enzymic antioxidants, the levels of lipid peroxides and the liver marker enzymes in the experimental mice.

Activity of enzymic antioxidants: The activities of enzymic antioxidants like catalase, superoxide dismutase, and glutathione peroxidase were analyzed using the liver homogenate and the findings are depicted in Table 1. The catalase activity of CCl₄ group was found to be significantly lower than that found in control groups. Catalase activity in *Cynodon dactylon* treated group was found to be significantly increased when compared to vehicle control groups. In *Cynodon dactylon* plus CCl₄ administered group the enzyme activity was found to be more significant than that of silymarin plus CCl₄ treated group.

The SOD activity in CCl₄ group was found to be lower than that of other experimental groups. SOD activity of *Cynodon dactylon* plus CCl₄ group was found to be higher than that of silymarin plus CCl₄ treated group. Activity of GPx in liver was significantly decreased in CCl₄ treated group as compared to control mice. The intraperitoneal administration of *Cynodon dactylon* to CCl₄ induced mice afforded a significant increase in GPx activity in the liver when compared to the mice treated with PBS, paraffin oil and silymarin plus CCl₄ (Table 1).

Table 1. Enzymic and non-enzymic antioxidants in liver of the experimental animals treated with protein fraction of *Cynodon dactylon*

Treatment	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Catalase ¹ (u/mg protein)	5.17	5.32	6.12	17.3	1.93 ^f	6.06 ^{abc}	7.9 ^{bcd}
SOD ² (u/mg protein)	3.06	2.12	3.68	4.49	5.46 ^f	5.56 ^{abc}	6.49 ^{bd}
Gpx ³ (u/mg protein)	2.02	2.09	3.02	3.30	1.07 ^f	3.49 ^{ac}	7.29 ^{bcd}
Vitamin A (µg / g tissue)	6.83	7.13	11.42	19.75 ^{abc}	3.6 ^f	23.96 ^{abc}	43.57 ^{bc}
Vitamin C (mg / g tissue)	1.55	1.65	2.13	3.08 ^{abc}	0.49 ^f	3.08 ^{abc}	4.6 ^{bc}
Vitamin E (µg / g tissue)	4.46	3.57	16.24	24.25 ^{abc}	2.08 ^f	6.93 ^{abc}	8.77 ^{bc}
Reduced Glutathione (nM / g tissue)	3.52	4.32	8.97	10.07 ^{abc}	4.66 ^f	14.1 ^{abc}	25 ^{bc}

Values are mean of six mice in each group (p< 0.001); Compared to paraffin oil group (a), PBS group (b), CCl₄ group (c), silymarin plus CCl₄ group (d), protein fraction plus CCl₄ (e), CCl₄ group compared to all other groups (f). 1- unit defined as the amount of enzyme required to decrease the absorbance by 0.05units at 240nm; 2- unit defined as the amount of enzyme that gives 50% inhibition of the extent of NBT reduction 1 min; 3- unit defined as the nano moles of GSH oxidized / min.

Activity of non-enzymic antioxidants: The activities of non enzymic antioxidants like vitamin A, vitamin C, vitamin E and reduced glutathione were analyzed using the liver homogenate and the findings are depicted in Table 1. The levels of vitamin A in the *Cynodon dactylon* plus CCl₄ treated group was found to be significantly increased when compared with the control and silymarin plus CCl₄ treated groups. The level of vitamin C in the liver of mice administered with *Cynodon dactylon* and CCl₄ showed significant increase when compared to the CCl₄, silymarin plus CCl₄ as well as control groups. The administration of *Cynodon dactylon* plus CCl₄ significantly increased the level of Vitamin E in the liver of mice when compared to the control groups and silymarin plus CCl₄ administered groups. The hepatic levels of reduced glutathione was found to be significantly increased in mice administered with *Cynodon dactylon* plus CCl₄ when compared to CCl₄ administered mice.

Liver Marker Enzymes: To evaluate the normal functioning of the liver, the hepatocellular marker enzymes such as serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were assessed in the serum of all the experimental mice. The activity of SGOT and SGPT was found to be significantly decreased in *Cynodon dactylon* administered mice when compared to PBS control. The activity of SGOT and SGPT was found to be significantly increased in CCl₄ the hepatotoxin induced animals (Table 3). In the present study, MDA content of the liver of *Cynodon dactylon* plus CCl₄ treated mice was found to be significantly ($p < 0.001$) decreased when compared to the control groups and silymarin plus CCl₄ treated groups (Table 2).

Table 2. Liver marker enzymes level of lipid peroxides in the experimental animals treated with protein fraction of *cynodon dactylon*

Treatment group	SGOT p(U/L)	SGPT q(U/L)	Lipid peroxides (nM MDA / mg protein)
Pbs	55.49	51.69	6.03
Paraffin oil	54.35	54.64	4.27
Silymarin	59.82	57.08	0.75
Protein fraction	35.59	36.12	0.74
CCl ₄	121.25	110.19	11.16 ^d
Silymarin + CCl ₄	48.49*	70.79*	5.04 ^{ac}
Protein fraction + CCl ₄	82.56*	77.72*	4.12 ^{ab}
CD($\alpha = 0.05$)	2.04	0.051	0.585

Values are mean of six mice in each group, $P > 0.001$ compared to CCl₄ group (a); silymarin plus CCl₄ group (b); protein fraction plus CCl₄ (c), CCl₄ group compared to all other groups (d); .micromole of pyruvate (p) and phenol (q) formed / minute.

Discussion

Catalase (EC 1.11.1.6) is an enzymatic antioxidant widely distributed in all animal tissues and the highest activity is found in the red cells and liver. It decomposes H₂O₂ and protects the tissue from highly reactive hydroxyl and is thought to be the first line of defense against oxidative damage caused by H₂O₂ and other radicals induced by carcinogen. Inhibition of these protective mechanism results in enhanced sensitivity to free radical induced cellular damage (Alanivel *et al.* 2008). The present study showed the increased CAT activity in liver of the mice administered with the protein fraction of *Cynodon dactylon* to a statistically significant level when compared to control and silymarin groups. The catalase activity of CCl₄ group was found to be significantly lower than that found in control groups. A study by Ng *et al.* (2005) showed significant increase in CAT activity by administration of Rose (*Rose rugosa*) flower extract to 6-month old Swiss Albino mice. Another study by Manna *et al.* (2006) also showed increased level of CAT treated with aqueous extract of *Terminalia arjuna* to CCl₄ induced mice. Raja *et al.* (2007) observed that the administration of hydrochloric extract of *Cystisus scoparius* enhanced the activities of CAT and SOD in the liver of rats.

Super oxide dismutase (EC 1.15.1.1) is a ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress and provides the first line of defense against free radical damage. Superoxide radicals are one of the most important reactive O₂ free radicals constantly produced in living cells. Superoxide anion is the first reaction product of O₂ which is measured in terms of inhibition of generation of O₂. In our study the level was found to be increased to a more significant level when compared to control and silymarin groups. The SOD activity was significantly elevated in the liver of mice administered with protein fraction of *Cynodon dactylon* when compared with the control and Silymarin treated groups. The SOD dismutates superoxide radicals O₂⁻ into H₂O₂ plus O₂, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity (Moron *et al.* 1979). Koneri *et al.* (2008) have shown that the

ethanolic extract of roots of *Momordica cymbalaria* as well as silymarin increased the levels of antioxidant markers like GSH, SOD and CAT in CCl₄ induced hepatic damage in rats. The aqueous extract of *Eucalyptus globulus* were found to elevate the activities of SOD in rat liver (Arise *et al.* 2009) In line with these reports, a significant increase in SOD activity was caused by the administration of protein fraction of *Cynodon dactylon* and thus the protein fraction reduces superoxide radical induced oxidative damage to liver.

Glutathione per oxidase (EC 1.11.1.7) is also considered to be an important H₂O₂ removing enzyme in mammalian cells and is more important than catalase for removing H₂O₂. GPx is involved in the defense mechanism against oxidative damage. The induction of GPx, which is the central importance in the detoxification of peroxides and hydroperoxides, was measured in the hepatic cytosol where these processes have fundamental importance. In the present study, an increase in hepatic GPx activity was noticed in mice treated with the protein fraction of *Cynodon dactylon* when compared to that of control groups and silymarin administered groups. Administration of extracts of *P. aculeata* and silymarin were found to enhance the hepatic activities of GPx., GST, SOD and CAT in CCl₄ intoxicated rats. Alanivel *et al.* (2008), have shown increase of antioxidants GPx, SOD and GSH by extracts of *Sargassum polycystan* in liver of rats with D galactosamine induced hepatitis. Sudeesh and Vijayalakshmi (2005) have reported a significant increase in the levels of enzymic antioxidant in liver of male rats administrated with organic extract of *Punica granum*. Increased levels of catalase and superoxide dismutase was also reported by Natesan *et al.* (2007) in DLA mice by administration of *Careya arborea*. In accordance with these reports the enzymic antioxidants of the selected plant constitute the foremost defense system that limit the toxicity associated with free radicals and are intimately involved in the preventions of cellular damage.

In the present study the levels of vitamin A in the protein fraction treated group was found to be significantly increased in comparison with the control groups and silymarin treated group. Crespy and Williamson (2004) had showed a similar elevation in the level of Vitamin A on treatment of green tea in cardiovascular diseases containing animal models. Bhaya and Saini (2008) reported that the supplementation of Aloe to irradiated mice, lowered lipid peroxidation in liver, which was due to the enhancement of concentrations of antioxidants (Vit A, Vit C and Vit E) by the supplement. Skrzydlewska *et al.* (2002) also reported a significant reduction in the levels of vitamin C, E and A caused by the alcohol intoxication in the liver and blood serum of rats were reverted by the administration of the green tea. The levels of vitamin C in the liver of mice administered with protein fraction of *Cynodon dactylon* showed significant increase when compared to that of the standard antioxidant silymarin as well as the control groups. Narendhirakannan *et al.* (2005) reported that administration of *Cleome gynandra* L. leaf extract significantly increased the levels of vitamin C in arthritis induced rats. The administration of protein fraction of *Cynodon dactylon* afforded a significant increase in the levels of vitamin E in the liver of mice when compared to that of the control groups and silymarin administered groups. A flavonoid rich fraction from *Spermacoce hispida* significantly increased the hepatic levels of non enzymic antioxidants (Vit C, Vit E and GSH) in hyperlipidemic Rats (Kaviarasan *et al.* 2008) A similar kind of increase in hepatic antioxidative vitamins was shown by methanolic extract of *Careya arborea* Roxb and silymarin in rats with CCl₄ induced liver damage in a study by Sambathkumar *et al.* (2005).

The hepatic levels of GSH in mice administered with protein fraction of *Cynodon dactylon* plus CCl₄ showed a significant increase in comparison with the control groups and the silymarin group. The non enzymatic antioxidant Glutathione is one of the most abundant tripeptides present in the liver. Glutathione is the most important non-protein compound containing thiol group, which acts as a substrate for GST and GPx involved in preventing the deleterious effect of oxygen radicals. Das *et al.* (2003) reported a decreased activity of GPx in mice after ethanol exposure which might be due to the inactivation of enzyme and ROS. In consistence with this report, the protein fraction of *Cynodon dactylon* enhances the levels of GSH liver of mice.

When the liver cell membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream. In the present study, the activity of SGOT and SGPT was found to be significantly decreased in *Cynodon dactylon* administered mice when compared to PBS control. The activity of SGOT and SGPT was found to be significantly increased in CCl₄ the hepatotoxin induced animals. These findings are in accordance with the findings of Samudram *et al.* (2008) in ethanolic extract from the leaves of *Eclipta alba* and seeds of *Piper longum*, Ahamed *et al.* (2008) treatment with Methanolic extract of *Feronia limonia* (ME), Tenpe *et al.* (2009) in *Oroxylum indicum* extracts, Kalaivani *et al.* (2009) in aqueous and ethanolic extracts of *A. marmelos* and Thirupathi *et al.* (2009) in methanolic extract of leaves of *Balanites roxburghii* (BLR) showed significant decrease in the level of biochemical markers such as SGOT and SGPT.

Lipid peroxidation (LPO) refers to the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation involves the direct reaction of oxygen and lipid to form radical intermediates and to produce semistable peroxides which in turn damage the enzymes, nucleic acids membranes and proteins. The extent of lipid peroxidation is measured through malondialdehyde content (MDA), a pro-oxidant factor that determines the oxidative damage. In the present study, MDA content of the liver of protein fraction plus CCl₄ treated mice was found to be significantly ($p < 0.001$) decreased when compared to the control groups and silymarin plus CCl₄ treated groups. This decrease in MDA level indicated the inhibition of lipid peroxidation and enhancement of antioxidative defense mechanisms to prevent formation of excessive free radicals. The methanol extract of *Bauhinia racemosa* and silymarin were found to decrease LPO in rats in which liver damage was induced by paracetamol and CCl₄ Gupta *et al.* (2006). An increase in MDA level suggestive of enhanced LPO and failure of antioxidant defense mechanism leading to tissue damage in cancer bearing animals was also been reported by Sivalokanathan *et al.* (2006).

Conclusion

The results obtained clearly showed that the protein fraction of *Cynodon dactylon* had significant hepatoprotective potential by enhancing the activities of enzymic antioxidants, increasing the levels of non enzymic antioxidants, marker enzymes and antilipid peroxidative role by decreasing the lipid peroxide levels. These effects were found to be more significant than that of silymarin, the standard antioxidant and the CCl₄ the hepatotoxin. Administration of *Cynodon dactylon* plus CCl₄ significantly decreases the levels of liver marker enzymes, hepatic enzymic and non enzymic antioxidants. The results also imply that the hepatoprotective effects of *Cynodon dactylon* may be due to its antioxidant property.

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