HEPATOPROTECTIVE AND IMMUNOMODULATORY PROPERTIES OF Tinospora cordifolia IN CCl₄ INTOXICATED MATURE ALBINO RATS

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ABSTRACT — Effect of Tinospora cordifolia extract on modulation of hepatoprotective and immunostimulatory functions in carbon tetrachloride (CCl₄) intoxicated mature rats is reported here. Administration of CCl₄ (0.7 ml/kg body weight for 7 days) produces damage in the liver as evident by estimation of enzymes such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and alkaline phosphatase (ALP) as well as serum bilirubin level. CCl₄ administration also causes immunosuppressive effects as indicated by phagocytic capacity, chemotactic migration and cell adhesiveness of rat peritoneal macrophages. However, treatment with T. cordifolia extract (100 mg/kg body weight for 15 days) in CCl₄ intoxicated rats was found to protect the liver, as indicated by enzyme level in serum. A significant reduction in serum levels of SGOT, SGPT, ALP, bilirubin were observed following T. cordifolia treatment during CCl₄ intoxication. Treatment with T. cordifolia extract also deleted the immunosuppressive effect of CCl₄, since a significant increment in the functional capacities of rat peritoneal macrophages (PMφ) was observed following T. cordifolia treatment. The results of our experiment suggest that treatment by T. cordifolia extract may be the critical remedy for the adverse effect of CCl₄ in liver function as well as immune functions.

KEY WORDS: Tinospora cordifolia, CCl₄ intoxication, Immunomodulation, Hepatotoxicity

INTRODUCTION

Tinospora cordifolia is mentioned in Ayurvedic Indian literature as a constituent of several compound preparations used in general debility, dyspepsia, fever and urinary diseases (Chopra et al., 1956; Gupta et al., 1967). Previous studies have shown the pharmacological effect of T. cordifolia (Deshmukh, 1957). Antineoplastic activity of this plant has also been reported (Jagetia and Nayak, 1998). In experimental hepatic amoebiosis, a crude plant extract has been formulated (Sohni and Bhatt, 1996). Antiinflammatory, antidiabetic and hypoglycemic activities of various preparations from T. cordifolia have been observed (Dev, 1999; Gupta, 1964; Ainapure, 1985). An aqueous extract of the stem was found to antagonize the effects of various agonists such as histamine, 5HT, bradykinin, PGE₁ on the smooth muscles of rat and guinea pig (Patel, 1968). The active constituent in the extract was found to inhibit (in vitro) the growth of Mycobacterium tuberculosis (Singh, 1975). From the literature survey it has been revealed that there was lack of reporting related to the hepatoprotective and immuno-modulatory effects of T. cordifolia (in vivo) in wide areas of India and other countries where this plant is present maximally. Since CCl₄ causes liver damage, this model of hepatotoxicity (Recknagel and Glende, 1973) has been widely used to study the hepatoprotective effect of any exogenous drug (in our case T. cordifolia extract) in an experimental animal model. In our study for inducing hepatotoxicity, carbon tetrachloride was administered at 0.7 ml/kg body weight for 7 days (Recknagel and Glende, 1973). Animals were then fed an aqueous stem-extract of T. cordifolia.

In the present study, we have reported that administration of T. cordifolia extract in CCl₄ intoxicated hepatotoxic rat alters the serum enzyme levels, viz. SGOT, SGPT, alkaline phosphatase, and also modu-
lates the rat peritoneal macrophage responses in term of phagocytosis, chemotactic migration, etc. The hepatoprotective, immunomodulatory and antioxidant activity of T. cordifolia is also reported. Therefore, the results of this study will be very helpful to the community for general awareness of the beneficial effects of T. cordifolia on liver function as well in regulating immune functions.

**MATERIALS AND METHODS**

Adult male albino rats of the Wistar strain weighing 150-160 g were selected for this experiment. They were divided into four groups, each having 4 animals. Animals were maintained under standard laboratory conditions with free access to food and water. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed throughout the experimental schedule.

**Preparation of T. cordifolia extract**

T. cordifolia was used in the form of a polysaccharide preparation (Satwa). The preparation was defined in Ayurvedic literature as sedimented extracts of drugs predominantly starchy in nature. T. cordifolia Satwa obtained from stems of the plant is crushed in H2O, removing the fibrous material, followed by sun drying after the sediment has settled (Ramnarayan Baidya, 1985).

**Animal treatment and sample collection**

For inducing hepatotoxicity (*in vivo*), animals were injected (i.p) with carbon tetrachloride (CCl4) at the dose of 0.7 ml/kg body weight for 7 days (Recknagel and Glende, 1973). To study the effect of T. cordifolia, an aqueous stem extract of this plant was forcefully fed (by a feeding needle) at different concentrations (however, the 100 mg/kg body weight dose was optimized) during CCl4 intoxication and continued for 15 days. After completing the treatment, blood was collected from the retro-orbital plexus of respective animals, and serum was prepared by clot retraction and stored at –20°C for further use. Animals were then sacrificed for preparation of rat peritoneal macrophages from different sets of animals.

**Estimation of SGOT and SGPT level in serum**

First, 0.1 ml of non-hemolyzed serum was mixed with 0.5 ml of glutamic-oxaloacetic transminase substrate and incubated for one hr at 37°C. Then 0.5 ml of 2,4-dinitrophenyl hydrazine solution was added and stood for 15 min at room temperature. Then, 5 ml of 0.4(N) NaOH was added, mixed and kept at room temperature for 20 min. The intensity of the developed color was read at 540 nm after setting the instrument to zero density with water. The decrease in density represents the decrease in α-ketoglutarate from which the activity was calculated (Reitmen et al., 1957).

**Estimation of serum alkaline phosphatase**

Serum alkaline phosphatase activity was quantitatively determined using an ALP-Kit based on the modified method (Tietz, 1976) as described elsewhere according to the manufacturer’s instruction (Dr. Reddy’s Laboratories, Diagnostic Division, Survey No. 47, Bachupally, R.R., Dist. Hyderabad, 500123, A.P., India, Revised 10/97).

**Determination of serum bilirubin level**

The formation of pink-colored azo-bilirubin by the reaction between bilirubin and a diazo reagent was utilized. In practice, one estimation was done with addition of alcohol and another without alcohol. The former yields total bilirubin and the latter conjugated bilirubin, according to the method of Bergmeyer, 1985, as described elsewhere.

**Separation of rat peritoneal macrophages**

Five percent (w/v) bacto-peptone in saline (5 ml/100 gm body weight) was injected (i.p) into rats and peritoneal exudate cells were collected on Day 4 by washing the cavity with 20 ml ice-cold Ca2+ and Mg2+-free HBSS. Cells were washed twice and plated onto 60 mm plastic dishes in RPMI-1640 containing 1% (w/v) BSA. After 2 hr incubation at 37°C, nonadherent cells were removed by rinsing. The RPMI-1640 medium containing 1% BSA was added to the adherent cells used as macrophages. The viability of macrophages was >95% as assessed by Trypan blue dye exclusion (Matsumoto et al., 1997).

**Phagocytosis assay**

Cells in HBSS-BSA (100 µl from 10⁹/ml) were allowed to adhere on glass slides for 1 hr. Then 100 µl of 10% SRBC was added to the glass slides on which MØs had already adhered. The slides were incubated for 3 hr at 37°C. Then the cells were washed in HBSS, fixed in 50% methanol, and stained with Giemsa and observed under oil emersion microscope. Phagocytic index (PI) was calculated as described earlier (Czuprynski et al., 1984).
Immunobiological properties of *Tinospora cordifolia*.

Phagocytic index = Percentage of MΦ that contains SRBC × Average No. of SRBC per MΦ × 100

**In vitro** cell migration and determination of chemotactic index

Three small circular wells were punched out equidistantly in 0.8% agar gel. The central well was filled with peritoneal MΦ. One of the peripheral wells was filled with 0.9% saline and the other with chemotactic solution (immune serum). After 1 hr incubation at 37°C, cells from the saline well and from the serum well were aspirated and smeared on glass slides, air dried, fixed in methanol, stained with Giemsa and observed under oil emersion microscope. Chemotactic index was calculated as described elsewhere (Wilkinson, 1986).

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\text{Chemotactic Index} = \frac{\text{No. of cells migrating to serum well}}{\text{No. of cells migrating to saline well}}
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**In vitro** cell adhesion assay

Cells were seeded separately for different groups in 96-well microtitre plates and allowed to adhere for different times. In time, wells were washed with HBSS, and then 100 µl of 0.5% crystal violet in 12% neutral formaldehyde, and 10% ethanol was added to each well and incubated for 4 hr to fix and stain the cells. Wells were washed and air dried for 30 min. Crystal violet was extracted from the MΦ adhered in the wells by lysing with 0.1% SDS in HBSS. Absorbance was measured spectrophotometrically at 570 nm. Cell adhesion was expressed as increased absorbance at 570 nm (Lin *et al*., 1995).

**Myeloperoxidase (MPO) release assay**

Cells (200 µl) from different groups were taken into microcentrifuge tubes and stimulated with LPS (100 ng/ml) for 1 hr at 37°C. Cells were centrifuged at 13,000 rpm for 10 sec, and the supernatant from different sets was recovered separately and kept at −20°C until further use. This cell-free supernatant was used for assay of the partial MPO release from LPS as well as the LPS non-stimulated cells of different groups. The cell pellet was lysed in 0.01% SDS and then centrifuged, and the supernatant was recovered as before for total MPO release assay. A 100 µl cell-free supernatant was reacted with 100 µl substrate buffer and kept at 37°C for 20 min. Then the reaction was stopped by adding 100 µl of 2(N) H₂SO₄. Absorbance was measured at 492 nm. Total release (100%) was given by the supernatant which was taken after lysing the cells in SDS. Partial release was then calculated accordingly from different groups of both LPS-stimulated and LPS-non-stimulated cells (Bos *et al*., 1978; Philip, 1994).

**RESULTS**

**Effect of *T. cordifolia* administration on SGOT level in CCl₄ intoxicated rats**

Determination of SGOT level in the serum was a good indicator for jaundice manifestation (Reynolds and Moslen, 1980). In order to demonstrate this, SGOT level was determined following *T. cordifolia* extract treatment in CCl₄ intoxicated rats. The level of SGOT in control animal serum showed 47.93 ± 0.94 IU/litre, which was increased to 57.94 ± 2.38 IU/litre after CCl₄ intoxication. Administration of *T. cordifolia* extract in CCl₄ intoxicated rats caused reduction in SGOT level to 47.71±1.56. However, administration of *T. cordifolia* in control animals (p<0.05) also caused reduction of SGOT level to 25.76 ± 0.045 (Fig. 1).

![Fig. 1. Changes in serum levels of SGOT and SGPT in CCl₄ intoxicated rats treated with *T. cordifolia* extract (100 mg/kg body wt/day) for 15 days. Results are expressed as mean±S.E. (p < 0.001).](image-url)
Effect of *T. cordifolia* administration on SGPT level in CCl₄ intoxicated rats

Whether or not administration of *T. cordifolia* leads to any alteration in the serum SGPT level was a question. To demonstrate this, serum SGPT level following *T. cordifolia* treatment was measured. The result showed that CCl₄ caused a significant increase in SGPT level from control (37.15 ± 3.18 IU/litre) to 96.76 ± 4.43 IU/litre after intoxication. However, administration of *T. cordifolia* in CCl₄ intoxicated rats led to reduction of SGPT level to 45.83 ± 2.774 (p<0.001) (Fig. 1). *T. cordifolia* treatment in control animals showed no such significant alteration in SGPT level.

Effect of *T. cordifolia* extract administration on serum Alkaline Phosphatase level in CCl₄ intoxicated rats

Whether administration of *T. cordifolia* extract leads to any alteration in the serum Alkaline Phosphatase (SALP) level was an obvious question. In order to address this, SALP level was estimated after *T. cordifolia* treatment in CCl₄ intoxicated rats. SALP level in the control group increased from 1.24 ± 0.03 to 4.91 ± 0.27 in CCl₄ intoxicated hepatotoxic rat. Administration of *T. cordifolia* in CCl₄ intoxicated rats led to lowering of the SALP level to 1.52 ± 0.15 (p<0.001). However, *T. cordifolia* treatment in control animals also led to further reduction in SALP level to 0.90 ± 0.13 (Fig. 2).

Effect of *T. cordifolia* extract on serum bilirubin level in CCl₄ intoxicated rats

Destruction of hemoglobin yields bilirubin which is conjugated in the liver to diglucoroxide and excreted in the bile. Bilirubin accumulates in plasma when liver insufficiency exists, biliary obstruction is present or rate of hemolysis increases. Whether treatment with *T. cordifolia* during CCl₄ intoxication checks the serum bilirubin level was demonstrated by measuring serum bilirubin from different groups. The total bilirubin level (mg/100 ml serum) increased from 1.6 ± 0.20 in the control group to 8.3 ± 0.353 after CCl₄ intoxication. Administration of *T. cordifolia* extract in CCl₄ intoxicated rats also reduced the total bilirubin to 2.2 ± 0.2 mg/100 ml of serum (p<0.001). However, *T. cordifolia* treatment in control rats showed no such significant alteration in the serum bilirubin level (Fig.3).
Effect of *T. cordifolia* extract on the phagocytic capacity of CCl$_4$ intoxicated rat peritoneal macrophages

In order to determine whether there was any alteration in the phagocytic capacity of peritoneal macrophages due to *T. cordifolia* administration during CCl$_4$ treatment, the phagocytosis of SRBC by Mφs was assayed. Results showed that CCl$_4$ causes a marked decrease in the phagocytic index from control 27500 ± 6130 to 1800 ± 572 after CCl$_4$ treatment. Treatment with *T. cordifolia* in CCl$_4$ intoxicated rats also helped to regain capacity at 30800 ± 653 (p<0.01). However, *T. cordifolia* treatment in control animals caused no such alteration in the phagocytic capacity of peritoneal Mφs (Fig. 4).

Effect of *T. cordifolia* extract on the chemotactic migration of CCl$_4$ intoxicated rat peritoneal Mφs

The chemotactic index was reduced to 7.52 ± 2.25 after CCl$_4$ treatment with respect to that of control 22.3 ± 6.3. Administration of *T. cordifolia* extract in CCl$_4$ intoxicated rats enhanced the chemotactic ability of cells to 30.5 ± 7.05 (p<0.05). Treatment with *T. cordifolia* in control animals led to no significant alteration (Fig. 5).

Effect of *T. cordifolia* extract on the adhesion property of CCl$_4$ intoxicated rat peritoneal macrophages

In *vitro* cell adherence assays may reflect the *in vivo* capacity for cellular adherence with treatment by *T. cordifolia* extract in hepatotoxic rats. In order to address this fundamental characteristic of Mφs, the cell adhesion property was assayed *in vitro*. Adhesion of cells increased gradually after *T. cordifolia* administration (p<0.001) (Fig. 6).

Effect of *T. cordifolia* extract on the myeloperoxidase (MPO) enzyme release from CCl$_4$ intoxicated rat peritoneal macrophages

MPO decreases the free radical level in our system. CCl$_4$ causes an increase in the level of CCl$_3$ production, but whether *T. cordifolia* causes any alteration in the level of free radicals or not was the present objective of our study. CCl$_4$ intoxication reduces the

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![Fig. 4](image_url) **Fig. 4.** Effect of *T. cordifolia* extract on the phagocytic capacity of CCl$_4$ intoxicated rat peritoneal macrophages.

The results in this figure represent the phagocytic index (mean ± S.E.) of rat peritoneal Mφs of three independent experiments (p<0.01).

![Fig. 5](image_url) **Fig. 5.** Effect of *T. cordifolia* extract on the chemotactic migration of CCl$_4$ intoxicated rat peritoneal Mφs.

The results in this figure represent the chemotactic index (Mean ± S.E.) of peritoneal Mφs from different groups of triplicate experiments (p<0.05).
enzyme release to 36.6±2.74% from 77±5.23% as in the case of a control group. However, administration of *T. cordifolia* extract in CCl₄ intoxicated rats sufficed to protect the macrophage and induce 90% enzyme release from the target cell with LPS stimulation (p<0.05). This trend was again observed in the case of LPS non-stimulation (Fig.7).

**DISCUSSION**

This study was undertaken to determine the effect of *Tinospora cordifolia* on liver functions as well as functional activities of peritoneal macrophages in CCl₄ intoxicated mature albino rats. Our results provide evidence for the adverse effect of carbon tetrachloride on the liver functions when enzymes such as SGOT, SGPT, ALP were found elevated in the serum. Since the estimation of serum enzymes such as SGOT, SGPT as well as bilirubin level was a good indicator for the pathological manifestation of jaundice, there was no doubt that CCl₄ induces liver damage in these experimental animals. However, *in vivo* administration of *T. cordifolia* extract causes hepatoprotective activities in CCl₄ toxicity. The enzyme level goes back to the normal level, as in the case of a control group. In our study, administration of *T. cordifolia* was found to provide significant protection against a CCl₄-induced increase in levels of SGOT, SGPT, SALP and serum bilirubin. CCl₄-induced elevated levels may indicate liver damage, since the major site of transamination reaction is in the liver. In transaminases, pyridoxal phosphate (PLP) the prosthetic group of the enzyme, forms a Schiff Base intermediate which remains tightly bound to the enzyme by multiple noncovalent interactions. *T. cordifolia* treatment during CCl₄ intoxication may enhance the above-mentioned molecular mechanism of enzyme action. The active principle or bioactive compound so far available may be used in some *in vitro* studies to elucidate the mechanism of enzyme action induced by *T. cordifolia*. Addition of a purified active compound from *T. cordifolia* in the assay buffer for SGOT enzyme activity (with or without PLP) may suggest the role of *T. cordifolia* in enzyme modulation at the molecular level. A CCl₄-induced rise in serum ALP...
level indicates the prevalence of jaundice, which is significantly protected by in vivo administration of an extract of *T. cordifolia*. Jaundice, with the appearance of yellow pigmentation in the skin and white of the eye, points to an elevated level of bilirubin in the blood. Jaundice can be caused by excessive breakdown of red cells, impaired liver function or mechanical obstruction of the bile duct. Measurement of the relative proportion of conjugated to unconjugated bilirubin is helpful in determining why a patient has jaundice.

Exposure to organisms or bacterial infection results in the activation of a variety of host defense mechanisms which include phagocytosis, degradative enzyme release and respiratory burst response. As it is evident from the phagocytic index after CCl₄ intoxication, it can be suggested that CCl₄ groups are more prone to infection, as they cannot phagocytose efficiently and, as a result, cannot clear out the invading microorganism, which may lead to a diseased state upon bacterial invasion. For the initiation of phagocytosis, contact between particulate antigen and phagocytic cells is important. That contact can be brought about by transport of particles via the blood or lymph to the sites of phagocytic cells. *T. cordifolia* extract level in the blood may increase the number of cells present in the peritoneum as well as alter the ability of those that remain to function normally. Treatment with *T. cordifolia* in CCl₄ intoxicated rats may increase myelopoiesis, monocyte to macrophage differentiation, Ag expression and release of cytokine, and also stimulate both tumoricidal and microbicidal activities. *T. cordifolia* thus interacts with the Mφ capability and amplifies an immune response.

Macrophages have the capacity to migrate specifically towards an inflammatory stimulus. Numerous substances generated during inflammation have the capacity to enhance macrophage speed and to orient movement in the direction of an increased concentration gradient of the agent. CCl₄ intoxication may lead to an alteration in the membrane level of leukocytes which alters the shape and orientation of Mφ, and cells migrate slowly. Also important in considering the attraction of macrophages to inflammation sites are substances that stimulate migration, such as chemotactic cytokines, and thus drive the cells toward inflammation sites. Continuous treatment with *T. cordifolia* extract during CCl₄ intoxication may lead to secretion of such chemokines in vivo, which somehow activate the cells for migration. Since the hallmark of inflammation is the infiltration of specific leukocyte subsets from the blood into the affected tissues, it can be suggested that *T. cordifolia*-induced activation of chemotactic activity may regulate inflammatory response.

Adhesion of macrophages also shows a marked decrease over time to vascular endothelium after CCl₄ intoxication. As an inflammatory response develops, a variety of cytokines and other immune mediators are released and act on local blood vessels, inducing increased expression of endothelial cell adhesion molecules (CAM). This requires that Mφs recognize it and adhere strongly enough so that the molecules are not swept away by blood flow. In vitro cell adherence assays may reflect the in vivo capacity for cellular adherence, and its increased value (after *T. cordifolia* treatment) reflects a greater recruitment of immunocompetent cells to the inflammation site. This increase in cell adherence might be primarily caused by an increased expression of cell adhesion molecules on the surface of Mφ.

Macrophages secrete lysosomal proteolytic enzymes active at tissue pH that may be important in their ability to kill tumor and bacterial cells. As macrophages mature, there is a progressive rise in lysosomes and their hydrolytic content. Continuous CCl₄ treatment may also lead to immaturation of macrophages in vivo, and it can be assumed that due to this exposure they have lesser lysosomal content and hence are less capable of secreting myeloperoxidase. Treatment by *T. cordifolia* extract during CCl₄ intoxication may somehow alter the maturation of macrophages so that they can regain their lysosomal hydrolytic content and release more MPO following activation. However, in our study, no focus was maintained to elucidate enzyme system interaction for microbial killing after *T. cordifolia* treatment during CCl₄ intoxication. Respiratory burst during phagocytosis results in activation of membrane-bound oxidase that catalyzes the reduction of oxygen to superoxide anion. As the lysosome fuses with the phagosome, myeloperoxidase, together with a halide ion, act on hydrogen peroxide to produce long-lived oxidants, including hypochloride, which are toxic. Hyperactivity of myeloperoxidase is thus equally as dangerous as hypoactivity, which may in turn cause immunosuppression. It may be predicted that *T. cordifolia*, by increasing the level of released MPO, might be serving a beneficial role as an antioxidant, and is capable of removing free radicals from a system either by prolonging the initiation phase or by inhibiting the propagation phase of autoxidation. From our results, it can be suggested that *T. cordifolia* would be a good alternative to costly allopathic therapy for jaundice with good efficacy and toleration. It will
also scavenge the free radicals in our bodies, preventing cardiovascular morbidity and mortality, and with its immunostimulatory effect it will prevent foreign pathogenic invasion.

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