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Supporting the immune system through functional modulation of carbon tetrachloride intoxicated splenic macrophages by administering *Tinospora cordifolia*

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ABSTRACT

The suppression of immune system leads to the development of several chronic illnesses; in replying to this the available allopathic system of medicine is provided with limited tools for treatment and prevention. In this context, the present investigation focuses on immunostimulant properties of an aqueous extract of *Tinospora cordifolia* in splenic macrophages isolated from carbon tetrachloride induced immunosuppressive mice. Our results suggested that carbon tetra chloride intoxication (0.5ml/Kg body weight) significantly altered the functional status like morphology, cell adhesion, phagocytosis, myeloperoxidase (MPO) release, nitric oxide (NO) release, DNA fragmentation and killing capacity of splenic macrophages. However administration of aqueous fraction of *Tinospora cordifolia* stem parts at a dose of 40 mg/kg body weight (*in vivo*) in CCl_4 intoxicated male albino mice ameliorated the effect of CCl_4, as was evident from the scanning electron micrographs, percentage of morphologically altered macrophages, phagocytosis activity, cell adhesion, MPO release, NO release, DNA fragmentation and intracellular killing capacity. Thus, it has been coclude that bioactive compounds presents in polar fractions of *Tinospora cordifolia* were accountable for boosting the immune system.

Keywords: Splenic macrophages, *Tinospora cordifolia*, aqueous extract, CCl_4 intoxication, immunostimulant.

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INTRODUCTION

The abnormal functioning of the immune system leads to several chronic illnesses and protection of immune system using plant compounds or plant extracts is in recent times getting renewed interest because of their minimal side effects. Microbes that penetrate an epithelial barrier and enter a tissue site are encountered by the three types of sentinel immune cells in the tissues, viz., tissue macrophages, mast cells and immature dendritic cells. From phagocytosis to intracellular killing of ingested pathogens, macrophages cater to a host of immune functions as an important first line defense mechanism. Macrophage also plays a crucial role in antigen presentation in adaptive immunity (Kovalchin *et al*, 2011).

To mimic immunosuppressed condition in mice, carbon tetra chloride (CCl_4) was used because earlier it was correlated with not only in liver damage but also confers an immunocompromized state particularly concerning macrophage function (Bishayi *et al,* 2002). Alternative to allopathic medicine to support the immune system, we opted for *Tinospora cordifolia* (*T. cordifolia,* Menispermaceae) belonging from a group of medicinal plants that grows in the tropical and subtropical regions of India. This herb is extensively used in the Indian System of Medicine; the extract of different parts of the herb has found wide use in a variety of diseases (Aranha *et al,* 2012; Sharma *et al,* 2010). It is known for its antioxidant, antihyperglycemic activity and antibacterial properties (Shilpa *et al,* 2012). There are five glycosides (viz; clerodane furano diterpene glycoside, cordioside, syringin, cordifolioside B and cordiol) isolated from *Tinospora cordifolia* stem parts having anti-complement and immune-stimulating activities (Patel *et al,* 2011). In recent times, it has been established that *T. cordifolia* lays immunomodulatory effect on CCl_4 induced immunocompromised peritoneal macrophages (Sengupta *et al,* 2011). As macrophages have functional hetrogenicity (Gordon and Taylor 2005), it is a prerequisite to further validate the pharmacological effect exerted by *T. cordifolia* on all types of macrophages. In view of this, CCl_4-induced immunotoxicity and amelioration of such immunosuppressive effects by *T. cordifolia* in splenic macrophages have been further elucidated in the present study.

MATERIALS AND METHODS

Preparation of Extracts
Collection and grinding of the selected plant materials

T. cordifolia was collected from the neighboring geographical area of Assam University, Silchar, Assam, India and the plant was identified by a botanist of Life Science Department of Assam University (Voucher No: TC-11.21A) . After drying at 37^0C for 72 h the plant material (stem parts) was ground into powder. Exposure to sunlight was avoided to prevent the loss of active components.

Extraction of selected plant material powder by maceration method

One liter of double distilled water was mixed with 100 g of powdered *Tinospora cordifolia* stem, filtered twice with Whatman no.1 (twice) and then with nitrocellulose membrane. The extracted liquid was subjected to water bath evaporation to remove the water. For water bath evaporation, liquid extract material was placed into a beaker and subjected to water bath evaporation at 60^0C temperature for 7-10 hr daily for 2-3 days until a semisolid state of extracted liquid was obtained . The semisolid extract produced was kept in the deep freezer at -20^0C overnight and then subjected to freeze drying (Cos *et al,* 2006). Extract obtained by this method was then weighed and stored at -20^0C until further use. The mice were fed with powdered plant material with sterile tap water. Phytochemical screening of the aqueous extract of *T. cordifolia* was also carried out (Table1).

Table. 1: Phytochemical screening of aqueous extract of *Tinospora cordifolia* stem parts.

Alkaloids	Glycosides	Reducing sugar	Saponins	Tannins	Poly phenols
Present	Present	Present	Present	Present	Absent

Animals

Twenty mice weighing approximately (20 ± 1.0 g) were taken and these mice were divided into four groups comprising of five mice each. The first group was kept as control. The second group was administered with CCl_4 at a dose of 0.5 ml/kg b.w. (i.p.) from 8th day to 15th day of the experiment. In the third group, the mice were fed with extract of *T. cordifolia* at a concentration of 40 mg/kg b.w. (orally) by feeding needle for 15 days. In the fourth group, the mice were given both *T. cordifolia* extract (for 15 days, orally) and CCl_4 (for last 7 days, i.p.). Animal experiments were in accordance with the instructions for the care and use provided by the institution at which the research was carried out.

Separation of splenic macrophages

Spleens were excised from sacrificed mice, immediately placed in Alsever's solution and then macerated using frosted glass slides to obtain a single cell suspension. Suspension was then transferred to sterile tubes and kept in ice for cell debris to settle. The supernatant was then layered over Histopaque 1077 (Sigma, USA) and subjected to density-gradient centrifugation. The band of leukocyte enriched fraction at the interface was collected, washed with DPBS and resuspended in RPMI-1640 containing HEPES (pH 7.2) and BSA (Merck, USA), and allowed to adhere on plastic surface. The adherent cells were collected and tested for viability as determined by Trypan Blue dye exclusion technique. Specificity of the cells was maintained by non-specific esterase staining. The cells were resuspended to a concentration of 10^6 cells/ml (Sikorski *et al,* 1991).

Morphological alteration assay by simple microscopy

A volume of 100µl macrophage cells in HBSS-BSA from all mice were taken separately and fixed in an equal volume of 2.5 % gluteraldehyde in HBSS. After 10 min, cells were centrifuged at 2000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in HBSS. Smears of cells were drawn on glass slides, air dried, fixed in methanol and stained with Giemsa. Cells were observed under an oil immersion microscope. Any cell extensively smoothened surface were scored as polarized and this was expressed as a percentage of the total no. of cells counted (Qu *et al,* 1995).

Scanning electron microscopy of splenic macrophages

All exposed and non exposed (control) mice were sacrificed and their spleens excised, and cut into small pieces. The pieces were fixed in 2.5% glutaraldehyde prepared in 0.1M sodium cacodylate buffer for 4 h at 4ºC. The samples were washed with the buffer and dried in tetra-methyl silane (TMS) following the method of (Dey, 1993) for critical point drying. The dry samples were secured horizontally to the specimen brass stub (10 mm diameter x 30 mm high) using double–coated adhesive tape, connected via a

patch of silver paint to ensure charge conduction. Care was taken to avoid any trapped air bubble. A thin conductive coating of gold was applied to the sample using a JFC-1100 (Jeol) ion sputter, at a relatively low vacuum of 10^{-3} torr in the sputtering chamber. The coated samples were examined in a JSM-6360 (Jeol) Scanning electron microscope operated at 20 KV. The tilt control was fixed at 0 degree for setting the specimen stage in a horizontal position; the working distance (WD) selector was used to set the WD to 8mm. A comparison was made on the morphological structure of the normal and CCl$_4$-treated spleen. In order to demonstrate the effect of CCl$_4$ and *T. cordifolia* on morphology of splenic macrophages, the scanning electron micrographs of spleen tissues from individual groups were developed in a random manner. The tissues were critical point dehydrated, metallised, and observed using a JSM-6360 (Jeol) SEM at the Sophisticated Analytical Instrument Facility (SAIF), North-Eastern Hill University (NEHU), Shillong, Meghalalya, India.

In vitro cell adhesion assay

Cell were seeded separately for different groups in 96-well microtitre plates and allowed to adhere for different times. Next, wells were washed with HBSS, and then 100µl of 0.5 % crystal violet in 12% neutral formaldehyde, and 10 % ethanol was added to each well and incubated for 4 h to fix and stain the cells. Wells were washed and air dried for 30 min. Crystal violet was extracted from the macrophage 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)

Phagocytosis assay

A volume of 100 µl of cells from exposed and non exposed mice were allowed to adhere separately on glass slides whereas non adherent cells were washed out with DPBS (1 X). To the glass slides containing adhered macrophages , 10 % heat killed *Staphylococcus aureus* was added and incubated for 3 h at 37°C which were then washed with DPBS (1 X) and dried. The cells were at last fixed in 50 % methanol, stained with Giemsa, observed under oil immersion microscope and the number of bacterial cells ingested were counted (Czuprynski *et al,* 1984).

Myeloperoxidase release assay

A volume of 200 µl of cells from different groups were taken into micro centrifuge tubes and stimulated with LPS (100 ng/ml) for 1 h at 37°C and centrifuged at 13000 rpm for 10 min. The supernatant thus obtained from different sets was recovered separately and kept at -20°C until further use. The cell free supernatant was used for assay of the partial MPO release for different groups. The pellet that recovered from all the four groups were lysed in 0.01 % SDS and then centrifuged again; the supernatant was recovered as before for total MPO release assay. Subsequently 100µl of cell free supernatant as well as cell-lysate were allowed to react with 100µl Orthophenylenediamine substrate buffer and kept at 37°C for 20 min; then the reaction was stopped by adding 100µl of 2(N) H$_2$SO$_4$ and absorbance was measured at 492 nm (Bos *et al,* 1978).

Nitric oxide release assay

100µl of macrophage cells were isolated from respective groups and suspended in DPBS-BSA. The cells were than stimulated with LPS (100ng /ml) for 1 h at 37^{0}C and centrifuged at 13000 rpm for 10 min. The cell free supernatants were transferred to separate micro centrifuge tubes for nitric oxide release assay. Then 100 µl of Griess reagent was added and incubated at room temperature for 10 min. Reading were taken in a UV-spectrophotometer at 550nm and compared to a sodium nitrate standard curve (Sasaki *et al,* 2000).

DNA fragmentation assay

The DPA reaction was performed according to the method of (Perandones *et al,* 1993). Perchloric acid (0.5 M) was added to the pellets containing uncut DNA (resuspended in 200 µl of hypotonic lysis buffer) and to the other half of the supernatant containing DNA fragments. Then 2 volumes of a solution containing 0.088 M DPA, 98% (vol/vol) glacial acetic acid, 1.5% (vol/vol) sulfuric acid, and a 0.5% (vol/vol) concentration of 1.6% acetaldehyde solution were added. The samples were stored at 4°C for 48 h. The colorimetric reaction was quantified spectrophotometrically at 575 nm. The percent fragmentation was calculated as the ratio of DNA in the supernatants to the total DNA (Kurita *et al,* 2000).

Intracellular killing assay

In this procedure, 100µl of bacterial cells (10^7 cells/m) were incubated separately for all groups with 100µl macrophages in a total volume of 1 ml DPBS-BSA and kept in the shaker for 20 min at 37°C. Non ingested bacteria were then removed by differential centrifugation for 10 min at 10000 rpm at 4°C. Following that, two washes were given with ice-cold DPBS-BSA. The cells containing ingested bacteria were resuspended in DPBS-BSA containing 10 % normal serum and incubated at 37°C for 25 min. At 0 min and after 15, 30 and 45 min time intervals 0.1ml sample was removed each time and treated with gentamycin. The above content was then plated onto nutrient agar petriplate and the number of viable intracellular bacteria was determined by counting the individual colonies formed (Leigh *et al,* 1986).
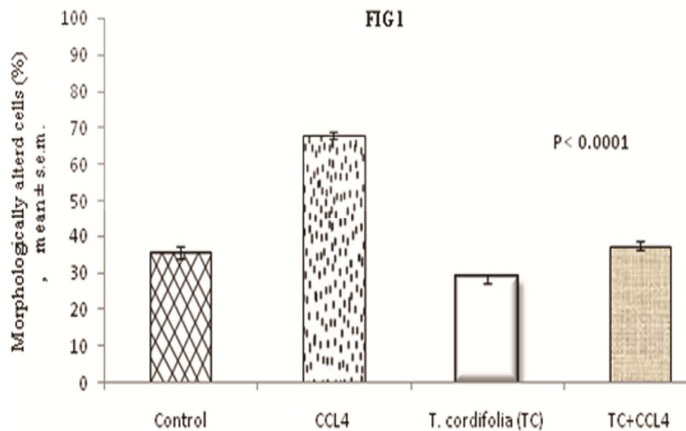
Statistical analysis

A one-tailed Student's t test as well as ANOVA was performed to compare the mean values between respective group. The results were expressed as mean ± standard error of the mean and all the experiment were done in triplicates.

RESULT

Effect of *Tinospora cordifolia* on morphology of splenic macrophages isolated from CCl$_4$ intoxicated male albino mice

Morphology of macrophage plays a prominent part of their function. To demonstrate the effect of both CCl$_4$ and *T.*
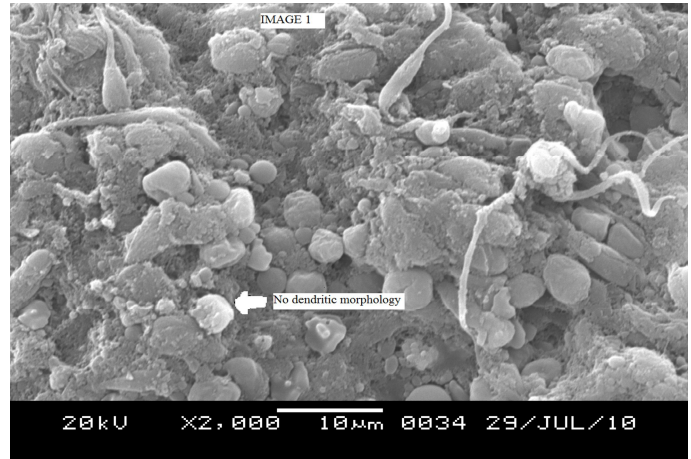
cordifolia on the number of differentiated macrophages, the macrophage cells of extensive rough surface or perfect spherical surface was measured and counted as morphologically altered macrophages. From this experiment, it was found that CCl_4 treatment increases the number of altered macrophages from 35.8 ± 1.69 % to 67.8 ± 1.02%. However the administration of *T. cordifolia* in CCl_4 intoxicated mice, the number of altered macrophages came to the level of 37.4 ± 1.17 %. Administration of *T. cordifolia* in control mice also showed some significant reduction of altered macrophages up to a level of 28.6 ± 1.21% (Fig 1; P <0.0001).
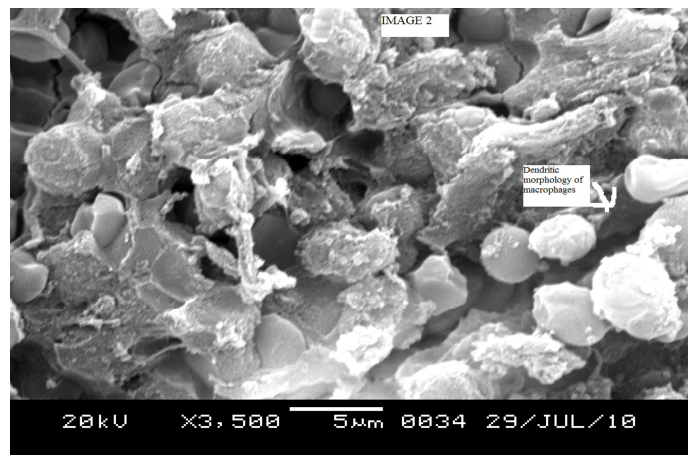

Fig. 1: Effect of *T. cordifolia* on morphology of splenic macrophages isolated from CCl_4 intoxicated male albino mice. The result was expressed as mean ± standard error mean. All the experiment were done in triplicate (n= 5).

Results of Scanning Electron Microscopy of splenic macrophages isolated from respective groups
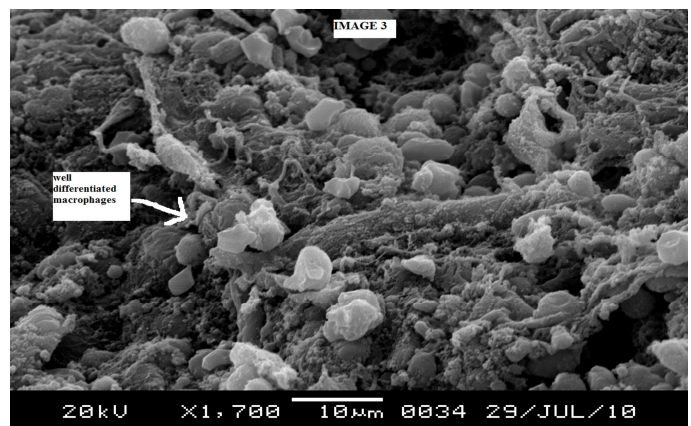
The killing of pathogen or foreign particles by macrophages must be preceded by the adherence of macrophages to that particular foreign body. The macrophages do this interaction with the foreign body, mainly because of their extended dendritic morphology. The pseudopods (dendritic extension) bind the foreign pathogen and phagocytose foreign body for killing. The scanning electron micrograph of splenic macrophages isolated from the respective groups showed that CCl_4 intoxication somehow render the differentiation of splenic macrophages and because of that CCl_4 intoxicated splenic macrophages were deficient of pseudopods (image 1) as compared to control groups (image 2). The CCl_4 intoxicated macrophages also found to be leaky in structure. Since simple microscope study showed, increase number of undifferentiated macrophages (without pseudopod) in CCl_4 intoxicated spleen compared to control group and this results also validated by electron microscope studies. However administration of aqueous extract of *T. cordifolia* retained the normal morphology as well as reduces the presence of undifferentiated macrophages in spleen isolated from CCl_4 intoxicated male albino mice (image 3). Administration of *T. cordifolia* in control mice did not show any change in morphology of splenic macrophages, as it was evident from scanning electron microscopy.

Image. 1: Effect of CCl_4 on the dendritic changes of mice splenic macrophages. The scanning electron photograph of CCl_4 intoxicated splenic macrophages at 2000 X and the bar is 10µm. This micrograph suggested that CCl_4 intoxication in control mice affect the differentiation of splenic macrophages and thereby less dendritic form of splenic macrophages.

Image. 2: Dendritic form of control splenic macrophages. The scanning electron photograph control splenic macrophages at 3500 X and the bar is 5 µm. The image showed dendritic morphology of macrophage
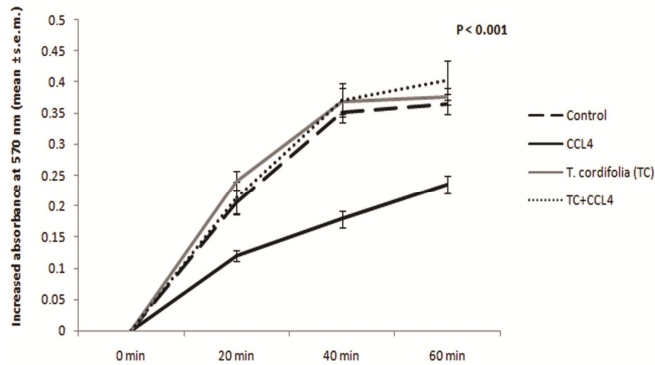

Image. 3: Effect of *T. cordifolia* aqueous extract on the dendritic changes in CCl_4 intoxicated splenic macrophages. The scanning electron photograph of CCl_4 + *T. cordifolia* splenic macrophages at 1700 X and the bar is 10µm. Administration of *T. cordifolia* stem aqueous extract at a dose of 40 mg/ kg b. wt. ameliorate the effect of CCl_4, as this micrograph showed that the restoration of dendritic form of CCl_4 intoxicated splenic macrophages.

Effect of *Tinospora cordifolia* extract on the adhesion property of CCl₄ intoxicated mice splenic macrophages
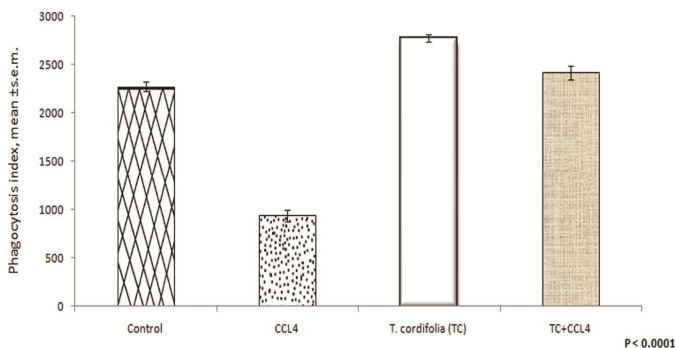
In vitro cell adherence assay may reflect the *in vivo* capacity for cellular adherence with treatment by *T. cordifolia* extract in hepatotoxic mice. In order to address this fundamental characteristic of macrophages, the cell adhesion property was assayed *in vitro*. Adhesion of splenic macropahges increased gradually after *T. cordifolia* administration in CCl₄ intoxicated mice (Fig 2; P <0.001).

Fig. 2: Effect of *T. cordifolia* on cell adhesion properties of splenic macrophages isolated from CCl₄ intoxicated male albino mice. The cell adherence property of CCl₄ increased after administration of *T. cordifolia*. The result was expressed as increased absorbance at 570 nm with mean ± standard error mean and. The experiment was carried out in triplicate. ANOVA analysis showed that the result was significant at P < 0.001.

Effect of *Tinospora cordifolia* on phagocytosis capacity of splenic macrophages isolated from CCl₄ intoxicated male albino mice
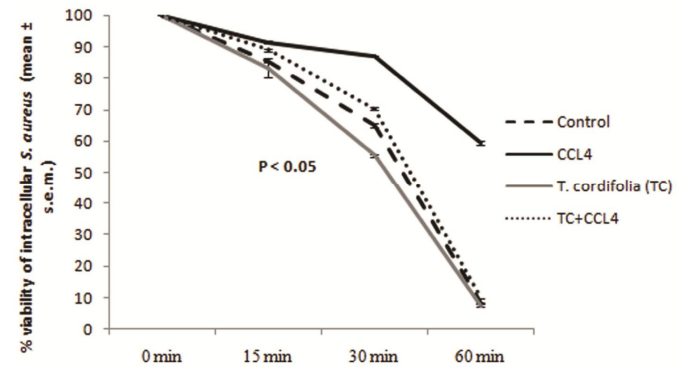
To appraise the effect of *T. cordifolia* on phagocytosis capacity of splenic macrophages, the phagocytosis index was measured. CCl₄ administration in control mice reduced the phagocytosis index from 2274 ± 48.23 to 937 ± 59.53. But after the administration of *T. cordifolia*, the phagocytosis index of CCl₄ intoxicated splenic macrophages increased up to 2418 ± 71.23. Apart from increasing the phagocytosis index of CCl₄ intoxicate splenic macrophages, *T. cordifolia* treatment also increased the phagocytosis index of control splenic macrophages to the level of 2773.8 ± 34.62 (Fig 3; P<0.0001).

Fig 3. Effect of *T. cordifolia* on phagocytosis of splenic macrophages isolated from CCl₄ intoxicated male albino mice. The result was expressed as mean ± standard error mean. All the experiment were done in triplicate (n= 5). One way ANOVA analysis showed the result was significant at P <0.0001.

Effect of *Tinospora cordifolia* on intracellular killing capacity of splenic macrophages isolated from CCl₄ intoxicated male albino mice

Whether *T. cordifolia* administration in CCl₄ intoxicated mice has any effect in the killing capacity of splenic macrophages was the last and foremost important objective our study. Our results suggested that CCl₄ intoxication in control mice reduced the intra cellular killing capacity by seven folds but after administration of *T. cordifolia* aqueous extract in CCl₄ intoxicated mice restores the killing capacity of splenic macrophages (Fig 4; P < 0.05).
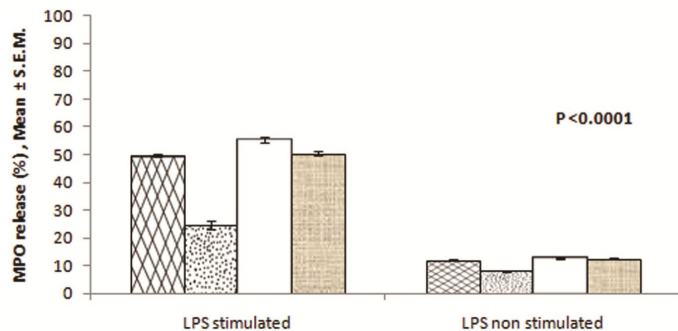

Fig. 4: Effect of *T. cordifolia* on killing capacity of splenic macrophages isolated from CCl₄ intoxicated male albino mice. After administration of *T. cordifolia*, the killing capacity of CCl₄ intoxicated splenic increased as viability of intracellular bacteria decreased steadily. The result was expressed as increased % viability of intracellular *S. aureus* at different time interval, with mean ± standard error mean. The experiment was carried out in triplicate. ANOVA analysis showed that the result was significant at P < 0.05.

Effect of *Tinospora cordifolia* on myeloperoxidase (MPO) enzyme release from splenic macrophages isolated from CCl₄ intoxicated male albino mice
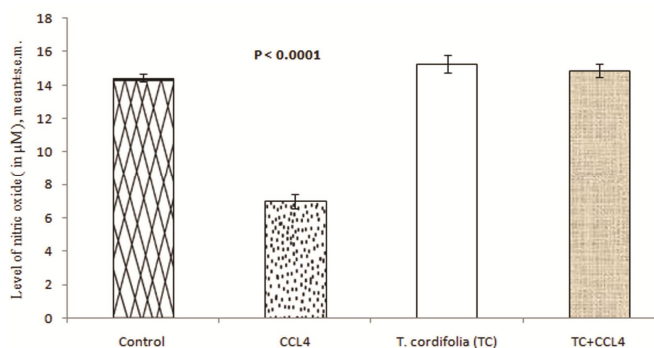
MPO decreases the free radical level in our system. CCl₄ causes an increase in the level of CCl₃ production, but whether *T. cordifolia* causes any alteration in the level of free radicals or not was the present objective of our study. CCl₄ intoxication reduces the enzyme release to 24.6 ± 1.33 from 49.8 ± 0.67 as in the case of a control group. However administration of *T. cordifolia* extract in CCl₄ intoxicated mice sufficed to protect the macrophage and induce 50.37 ± 0.73 %. Administration of *T. cordifolia* in control group also shows significant increase of MPO release from target cell up to 55.2 ± 1.05 % with LPS stimulation. The same trend was also observed in LPS non-stimulation (Fig 5; P <0.0001).

Effect of *Tinospora cordifolia* on nitric oxide (NO) release from splenic macrophages isolated from CCl₄ intoxicated male albino mice

Nitric oxide plays an important part to kill the microbial pathogen inside the macrophage by forming sodium hypochloride (NaOCl). CCl₄ intoxication significantly lowered the nitric oxide release to 7 ± 0.45 µM from 14.45 ± 0.23 µM as in the case of control group. However *T. cordifolia* administration ameliorated the affect of CCl₄ in CCl₄ intoxicated splenic macrophages (Fig 5; P =****).

Fig. 5: Effect of *T. cordifolia* on myeloperoxidase release of splenic macrophages isolated from CCl_4 intoxicated male albino mice. The result was expressed as mean ± standard error mean. All the experiment were done in triplicate (n= 5). One way ANOVA analysis showed the result was significant at $P < 0.0001$.

Fig. 6: Effect of *T. cordifolia* on nitric oxide release of splenic macrophages isolated from CCl_4 intoxicated male albino mice. The result was expressed as mean ± standard error mean. All the experiment were done in triplicate (n= 5). One way ANOVA analysis showed the result was significant at $P < 0.0001$.
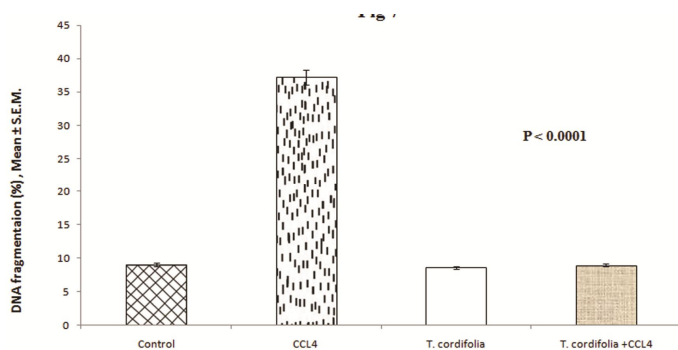
Effect of *Tinospora cordifolia* on induced DNA fragmentation of splenic macrophages by CCl_4

To assess the effect of *T. cordifolia* on apoptosis mechanism, the DNA fragmentation pattern of splenic macrophages isolated from the respective groups were studied. From our results, it was found that CCl_4 induced the DNA fragmentation of splenic macrophages by five folds. But administration of *T. cordifolia,* prevents the DNA fragmentation of CCl_4 intoxicated splenic macrophages (Fig 6; P=****).

No acute toxicity was observed on oral administration of the aqueous extract of *Tinospora cordifolia at* a dose of 150 mg/kg body wt.

Fig. 7: Effect of *T. cordifolia* on DNA fragmentation of splenic macrophages isolated from CCl_4 intoxicated male albino mice. The result was expressed as mean ± standard error mean. All the experiment were done in triplicate (n= 5). One way ANOVA analysis showed the result was significant at $P < 0.0001$.

DISCUSSION

The present study investigated the immunotherapeutic properties of aqueous extract of *T. cordifolia* stem parts through assessing some of the cellular activities of splenic macrophages, isolated from CCl_4 intoxicated male albino mice. CCl_4 intoxication altered the morphology of splenic macrophages by some means modulating the differentiation of macrophages, as it was evident from the scanning electron micrograph and light microscopy studies. However on administration of *T. cordifolia* in CCl_4 intoxicated male albino mice retained the dendritic morphology of macrophages. Apart from altering the morphology of macrophages, CCl_4 intoxication also reduced the cell adhesion, phagocytosis capacity of splenic macrophages but *T. cordifolia* intake ameliorated this effect of CCl_4. Myeloperoxidase and nitric oxide was found to be decreased after CCl_4 intoxication in control mice and this supports the CCl_4 reduced the functional killing status of macrophages because these two molecules play a major role in oxygen dependent killing mechanism of macrophages. Because of these functional abnormalities, the CCl_4 intoxicated splenic macrophages were found to be less effective in clearing intracellular *Staphylococcus aureus*. The DNA fragmentation pattern of CCl_4 intoxicated macrophages was also found higher as compared to the control group and this was probably an indication of cell senescence induction. Moreover, administration of *T. cordifolia* showed increased killing of intracellular *S. aureus* of CCl_4 intoxicated splenic macrophages through increased release of myeloperoxidase and nitric oxide. Along with that, *T. cordifolia* administration also lowered the DNA fragmentation pattern of CCl_4 intoxicated macrophages thus supporting recovery from premature senescence.

The first step in macrophage function is the activation or differentiation of macrophages. However intensive rough surface or almost spherical macrophages can be considered as altered macrophages because after encountering with the antigen, the macrophages must have dendritic surface morphology. The deviation in the normal shape of macrophages may be the cause of reduced functional status of splenic macrophages. Presence of spherical macrophage cells with smooth surface or highly polarized cells were an indication of morphologically altered or deformed cells. Deformity could indicate decreased functional potency of the macrophages. In our work, a significant deviation from normal shape of macrophages has been observed in CCl_4 intoxicated splenic macrophages as suggested from electron microscopy and light microscopic studies. We thus conclude that deformed cells in this case reflect inactive cells. However, after administration of *T. cordifolia* aqueous extract in that CCl_4 intoxicated macrophages retained the normal dendritic morphology. Previous work also showed that *T. cordifolia* ameliorated the CCl_4 induced immunosupression of peritoneal macrophages and the present study on splenic macrophages also validated that (Sengupta *et al,* 2011a). So, *T. cordifolia* has immunotherapeutic activity on resident and circulating macrophages thus supporting the hypothesis that this plant could be able to overcome the functional heterogeneity of macrophages. The mechanism involving this

protection by *T. cordifolia* extract is probably somehow related to the production of monocyte colony stimulating factor or granulocyte-monocyte stimulating factor (M-CSF/GM-CSF).

In order for resident macrophages to enter inflamed tissue or peripheral lymphoid organs, the cells must adhere to and pass between the endothelial cells lining the walls of blood vessels, a process called extravasation. Endothelial cells express leukocyte-specific cell adhesion molecules (CAMs). Some of these membrane proteins are expressed constitutively; others are expressed only in response to local concentrations of cytokines produced during an inflammatory response. Splenic macrophage bears receptors that bind to CAMs on the vascular endothelium, enabling these cells to extravasate into the tissues. The increase in cell adherence in CCl_4 intoxicated group after *T. cordifolia* extract treatment might be primarily caused by an increased expression of cell adhesion receptor molecules on the surface of macrophage cells.

Macrophages are actively phagocytic cells. Contact between pathogen and phagocytic cell is essential for initiation of phagocytosis. Our study reports a significant decrease in the phagocytic capacity of CCl_4 intoxicated splenic macrophages when allowed to ingest heat killed *S. aureus*. This can be explained in the light of morphologic alteration, reduction in membrane integrity and reduced adhering capacity of macrophages. As is evident from the phagocytic index after CCl_4 intoxication, it can also be suggested that CCl_4 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. However administration of *T. cordifolia* increases the phagocytosis capacity of splenic macrophages isolated from CCl_4 intoxicated mice.

In order to further clarify the role of *T. cordifolia* on immune modulation, we have studied the effect of *T. cordifolia* on the intracellular killing capacity of splenic macrophages. *S. aureus* is a virulent pathogen, has the ability to cause a variety of potentially life threatening infection varying from superficial soft abscesses to septic shock. Despite the availability of effective antimicrobial agents, *S. aureus* continues to cause life threatening infection including septic shock (Sheagren, 1984). Our findings suggest that CCl_4 intoxication contributes to the survival of *S. aureus* within splenic macrophages, since an increased colony of *S. aureus* was observed in CCl_4 intoxicated splenic macrophages. From the kinetics of *S. aureus* intracellular survival, it can be suggested that the bacteria persist and multiply within splenic macrophages. *In vivo* CCl_4 exposure may reduce the ingestion capacity of splenic macrophages, suggesting that the cells were not active enough or somehow less potent to kill efficiently but upon administration of *T. cordifolia* lowered the survival rate of intracellular *S. aureus*.

A number of antimicrobial and cytotoxic substances released by the activated macrophages are responsible for the intracellular destruction of phagocytized micro-organisms. Such mediators of cytotoxicity can be oxygen dependent (peroxidase, reactive oxygen species, reactive nitrogen species, peroxinitrate) or oxygen independent (lysozyme mediated killing) in their killing micro-organisms. Release of myeloperoxidase showed significant decrease in CCl_4 intoxicated groups compared to control groups. The neutrophil and macrophage posses primary (azurophilic) granules that contain acid hydrolase, myeloperoxidase and neuraminidases while the secondary granules contain lactoferin in addition to lysozymes. Ingested organisms are contained in vacuoles termed phagosomes, which fuse with the enzyme containing granules to form phagolysosomes. Myeloperoxidase enters the phagolysosomes following such macrophage-microbe encounter, stimulates a respiratory burst response and helps in the oxidative killing and scavenging of the microbe. On exposure, CCl_4, probably cause inhibition of myeloperoxidase release thus further decreasing the immune competence of splenic macrophages. However treatment of *T. cordifolia* in CCl_4 intoxicated mice increased the myeloperoxidase from splenic macrophages.

When macrophages are activated with bacterial cell wall lypopolysaccharide, they begin to express high level of nitric oxide synthase, which oxidizes L-arginine to yield citrulline and nitric oxide (NO). NO itself has potent antimicrobial effect. Besides its evident role in signal transduction, NO plays a significant role in killing of phagocytisized pathogens within macrophages. In the present study, it was found that, NO release significantly decreases in the CCl_4 challenged groups as compared to that of control. However treatment of *T. cordifolia* in CCl_4 intoxicated mice increased the nitric oxide release from splenic macrophages and this might have arisen from the increased expression of i-NOS gene (Sengupta *et al,* 2011b).

DNA fragmentation assay shows a greater degree of fragmentation in the splenic macrophages of CCl_4 treated mice. This may indicate an alteration in the integrity or morphology of the target cell. Apoptosis includes a series of changes in cell volume, disintegration of DNA and other morphological changes. That a greater percentage of DNA is susceptible to fragmentation on exposure to CCl_4 is only one of the indications that it may perhaps cause cell death in due course. This would suggest an absence of normally functioning of macrophage cells. In our study, a higher degree of DNA fragmentation of splenic macrophages on exposure to CCl_4, would indicate that a greater number of cells undergo senescence on CCl_4 exposure. This disturbed functional integrity would reduce the number of cells present in circulation, capable of normal function. However, treatment by *T. cordifolia* extract during CCl_4 intoxication may somehow alter the maturation of macrophages as well as prevent the DNA fragmentation, so that they regain their lysosomal hydrolytic content and release more MPO and NO following activation therefore, CCl_4 intoxicated splenic macrophages could be able to regain their intracellular killing capacity.

CONCLUSION

From our results it can be suggested that the aqueous fraction of *T. cordifolia* stem parts plays significant immunoprotective role in immunosuppressive condition through

not only modifying circulating macrophages but also by boosting the functional status of splenic macrophages. The detailed mechanism of this immunoprotective effect of *T. cordifolia* on macrophages needs to be further elucidated.

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