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ARTICLE

Comparative analysis of radical scavenging and immunomodulatory activities of *Tinospora cordifolia* growing with different supporting trees

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ABSTRACT According to Ayurveda, *Tinospora cordifolia* (Willd.) Miers (Menispermaceae) growing with physical support of *Azadirachta indica* Juss. (Meliaceae) (Neem) is medicinally more active than that growing on other supporting trees. In present study, comparative evaluation of antioxidant and immunomodulatory activities of ethanol extracts of T. *cordifolia* growing with the support of four different trees.viz *Azadirachta indica* (TCA), *Acacia leucophloea* (Roxb.) Willd. (Fabaceae) (TCB), *Butea monosperma* (Lamb.) Taub. (Fabaceae) (TCC) and *Prosopis juliflora* (Sw) DC. (Fabaceae) (TCD) was done. Evaluation of immunomodulatory potential of the extracts at the dose of 200 mg/kg body weight was done in rats, whereas phytochemical profile was established by HPLC. In *in vitro* antioxidant potential, TCA showed the highest i) total phenol content (21.5 mg/g dry weight), ii) total anti-oxidant activity (2144 µM Fe (II)/g dry mass), iii) DPPH free radical scavenging (86.36%), and iv) anti-lipid peroxidation potential (96.8%). It also showed the highest (11.7%) neutrophil adhesion and 275% more reduction in paw edema volume over control in TCA treated rats in vivo immunomodulatory activity. Only in humoral immune response, TCD (4.66 HA units) was found to be superior over the rest three extracts. In HPLC profiles of the extracts, TCA showed the presence of one extra peak at 9.88 minutes.
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KEY WORDS

medicinal plant
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humoral immune response

Tinospora cordifolia (Willd.) Miers (Menispermaceae) is an important medicinal plant (Kirtikar and Basu 1933). Ayurveda, the Indian medicinal system, strongly advocates use of *T. cordifolia* as a rejuvenator and it is also routinely prescribed to treat fevers, jaundice, diabetes, skin diseases, chronic diarrhea, urinary disorders, and dyspepsia (Singh et al. 2003a). It is used as Rasayana drug to improve the immunity and body resistance against a wide range of pathogenic attacks. The whole plant demonstrates an array of medicinal activities; however, the stem of this plant is widely used in medicines as described by the Ayurvedic Pharmacopoeia of India (Anonymous 2001).

Tinospora cordifolia has been extensively investigated for its immunomodulatory and antioxidant potential *in vitro* and *in vivo*. Immunomodulatory and hepatoprotective properties of aqueous extracts of *T. cordifolia* stem, in CCl_4 intoxicated Swiss albino mice, have been reported by (Bishayi et al. 2002). It was observed to modulate the immune system by activating the macrophages as evident by the enhanced secretion of lysozyme in treated cultured macrophage cell lines (More and Pai 2011). Its alcohol extract exhibited antiinflammatory action against acute and subacute inflammations (Wesley et al. 2008). Previously studied the effect of its aqueous extracts on cotton pellet granuloma and formalin induced arthritis model wherein, the effect of said extract was comparable to that of standard antiarthritic drug, indomethacine (Jana et al. 1999). Besides, *T. cordifolia* also has the memory enhancing action and it is regarded as 'Medhya Rasayana' (memory enhancing drug) in Ayurvedic texts. Agrawal et al. (2002) have proved the memory enhancing effects of aqueous as well as alcohol extracts of *T. cordifolia* by demonstrating the enhancement in learning and memory in normal and reverse cyclosporine induced memory deficit rats. Earlier reported anti stress activity of various extracts of *T. cordifolia* (Patil et al. 1997). A systematic reviewing of *T. cordifolia* with special reference to i) Ayurvedic pharmacology, ii) ethnobotanical, tribal and folk uses iii) phytochemical characterization and iv) biological actions has been done by (Upadhyay et al. 2010).

The different pharmacological actions of *T. cordifolia* like other medicinal plants can be attributed to the presence of array of secondary metabolites (alkaloids, flavonoids, phenolics, steroids, sapononins, tannins, glycosides etc.) in it (Singh

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et al. 2003b). Geo-climatic conditions are known to influence the secondary metabolites levels in medicinal plants which ultimately are reflected as a variation in the pharmacological actions. Being a climber, *T. cordifolia* grows with the physical support of a variety of the trees like, *Azadirachta indica* Juss. (Meliaceae), *Butea monosperma* (Lamb.) Taub. (Fabaceae), *Acacia leucophloea* (Roxb.) Willd. (Fabaceae), *Prosopis juliflora* (Sw) DC. (Fabaceae) etc. In India, Ayurvedic practitioners believe that different formulations prepared from the *T. cordifolia* growing with support of *Azadirachta indica* (Neem) is more potent by virtue of incorporation of medicinal values of latter (Sinha et al. 2004).

Though *T.cordifolia* has been investigated thoroughly for its number of medicinal properties, there are no studies reporting comparative medicinal activities of this celebrity plant with respect to its habitat, especially contribution of the supporting trees. In the present communication, we report the comparative radical scavenging and immunomodulatory potential of *T. cordifolia* growing with support of four different trees viz. A. *indica*, *A. leucophloea*, *B. monosperma* and *P. juliflora* growing in the identical geo- climatic conditions.

Materials and Methods

Plant material

The stem part of *T. cordifolia* growing with support of four different trees viz. *A. indica*, *B. monosperma, A. leucophloea* and *P. juliflora* was collected fresh from campus of North Maharashtra University, Jalgaon, India, during March-April, 2011. The plant stems were authenticated by Dr. P.G. Diwakar. Joint Director, Botanical Survey of India; Pune and voucher specimens (BMBTIC1, BMBTIC2, BMBTIC3 and BMBTIC4) were deposited in the Botanical survey of India, Pune.

Preparation of plant extracts

The collected stems of *T. cordifolia* were washed, dried in hot air oven at 50°C and then finely powdered. The powdered stem was extracted with ethanol (15 g per 100 ml ethanol) in Soxhlet apparatus for 8 h. The extracts were then concentrated to dryness under reduced pressure by using rotary vacuum evaporator at 45-50°C (Buchi, Switzerland) and the resultant residue was preserved in the dessicator for further use. Extracts of *T. cordifolia* growing with support of four different trees viz. A. *indica*, A. *leucophloea,* B. *monosperma* and P. *juliflora* were designated as TCA, TCB, TCC and TCD, respectively. For in vitro biochemical assays, each residue was dissolved in ethanol to attain a known concentration (10 mg/ml) and for animal experiments; the extracts were reconstituted in water to get the desired dose.

Determination of total phenolic content

The amount of total phenolics present in TCA, TCB, TCC and TCD was determined using Folin-Ciocalteu (FC) reagent by (Hazra et al. 2008). A gallic acid standard curve ($R^2 = 0.9$) was used to measure the phenolic content.

Total antioxidant activity (FRAP assay)

A slightly modified method of Benzie and Strain (1999) was adopted for the FRAP assay. The stock solutions included 300 mM acetate buffer (3.1 g CH_3COONa and 16 ml CH_3COOH, pH 3.6), 10 mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. This assay involved (i) preparation of fresh FRAP solution by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$, (ii) raising temperature of the solution to 37°C, (iii) allowing plant extracts (150 µl) to react with 2850 µl of the FRAP solution for 30 min in the dark and (iv) taking readings of the colored product (ferrous tripyridyl triazine complex) at 593 nm. The standard curve was linear between 200 and 1000 µM concentration of $FeSO_4$. Results are expressed in µM Fe (II)/g dry mass.

DPPH free radical scavenging assay :

The antioxidant capacities of TCA, TCB, TCC and TCD were confirmed by the DPPH scavenging assay according to (Brand-Williams et al. 1995) with slight modification. Different concentrations (0.1 to 1.0 mg/ml) of the extracts and ascorbic acid (standard) were thoroughly mixed with 5 ml of methanol DPPH solution (33 mg/L) in test-tubes and the resulting solution was kept standing for 10 min at 37°C before the optical density (OD) was measured at 517 nm. The measurement was repeated with three sets and average of the readings was considered. The percentage radical scavenging activity was calculated from the following formula:

$$\% \text{ scavenging [DPPH]} = [(A_0 - A_1)/A_0] \times 100$$

where, A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the extracts.

Anti-lipid peroxidation activity

Anti-lipid peroxidation potential of the extracts, TCA, TCB, TCC and TCD was determined by estimating the inhibition of thiobarbituric acid reactive species (TBARS) in goat colon tissue homogenate. Malonyldialdehyde (MDA) generated by the oxidation of polyunsaturated fatty acids upon reaction with two molecules of thiobarbituric acid (TBA) yielded a pink red complex, which was measured at 532 nm, from which % inhibition of lipid peroxidation by TCA, TCB, TCC and TCD was calculated. Reaction mixtures containing 0.5 ml of tissue homogenate, 1 ml 0.15 M KCl and 0.5 ml different concentrations of TCA, TCB, TCC and TCD (0.1 to 1.0 mg/ml) were prepared. Lipid peroxidation was initiated by adding 100 µl of 1 mM ferric chloride, Incubated at 37°C for 30 min. The reaction was stopped by adding 2 ml ice-cold 0.25 N HCl (containing 15% trichloroacetic acid (TCA), 0.38% TBA

and 0.2 ml 0.05% butylated hydroxyl toluene (BHT)). The reaction mixture was heated at 80°C for 60 min, cooled and centrifuged (5000 g, 15 min). Absorbance of the supernatant was measured at 532 nm against a blank, which contained all reagents except colon homogenate and plant extract under investigation. Identical experiments were performed to determine the normal (without drug and $FeCl_3$) and induced (with the drug) lipid peroxidation level in the tissue. The percentage of anti-lipid peroxidation effect (%ALP) was calculated by the following formula (Wade et al. 1985):

$$\%ALP = [A_{FeCl3} - A_{test} / A_{FeCl3} - A_{Normal}] \times 100$$

where A_{FeCl3}: Absorbance of $FeCl_3$, A_{Normal}: Absorbance of control reaction, A_{test}: Absorbance of test reaction.

Comparative evaluation of immunomodulatory potential of *T. cordifolia*

Animals

Wister rats (150-200 g) of either sex divided in 5 groups (n = 6), were used for the study. Animals were housed in animal house under standard conditions of temperature (25 ± 2°C), 12/12 h light/dark cycle and fed with standard pellet diet (Amrut, Sangali, MS, India) and tap water ad libitum. The Institutional Animal Ethical Committee reviewed the animal protocol prior to the experiment. All the animals were treated as per the guidelines for the Care and Use of Laboratory Animals (NIH Publication No.86-23, revised 1985) with the permission of Institute Animal Ethical Committee (Amrutvahini College of Pharmacy, Sangamner, 31/03/2010, protocol No.-7). Group I received only water and served as control. Group II, III, IV and V received 200 mg/kg body weight of TCA, TCB, TCC and TCD, respectively orally.

Preparation of antigen

Fresh sheep blood was collected in sterile Alsever's solution in 1:1 proportion and kept in refrigerator. Sheep red blood cells (SRBCs) for immunization were prepared by spinning sheep blood at 2000 rpm for 10 min, residue washed 4-5 times with physiological saline and then suspended in it with desired concentration (Patel et al. 2010).

Testing the neutrophil adhesion

Neutrophil adhesion test was performed according to method described by Fulzele et al. (2003). Rats divided in five groups were treated orally either with vehicle (water in present study) or drug (TCA, TCB, TCC and TCD) for seven days. On the seventh day of treatment, blood samples were collected from retro orbital plexus in to heparinized vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC). After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The TLC count and percentage of neutrophil gives the neutrophil index (NI) of blood sample. Percentage of neutrophil adherence was calculated by following formula:

percentage neutrophil adhesion = [NIu – NIt / NIu]

where, NIu is neutrophil index of untreated blood sample and NIt is neutrophil index of blood sample incubated with nylon fiber.

Evaluation of delayed type hypersensitivity (DTH) response

A method described by Chakraborthy (2009) and Gaur et al. (2009) was used with slight modifications to measure delayed type hypersensitivity response in rats as a function of treatment. Briefly, all rats were immunized on day zero by intraperitoneal administration of 0.4 ml of 5 x 10^9 SRBC/ml/rat. The treatment of TCA, TCB, TCC and TCD @ 200 mg/kg body weight was continued for next seven days and animals were challenged on seventh day with 0.1 ml of 1.25 x 10^9 SRBC/ml in right hind paw. The contra lateral paw received equal amount of saline and served as control. The paw thickness was measured at 24 h after challenge by using Vernier caliper. The difference in the thickness of right hind paw and the control was used as a measure of DTH response.

Determination of humoral immune response

Indirect haemagglutination test with some modification was used to measure humoral immune respose (Ismail and Asad, 2009; Patel et al. 2010). Each pretreated rat including the control group was immunized with 0.4 ml SRBC (5 x 10^9 cells) by i.p. route at day zero. The treatment of TCA, TCB, TCC and TCD at 200 mg/kg body weight was continued for next seven days and blood samples were collected from individual rats from retro orbital plexuses on seventh day for the determination of Haemagglutinating Antibody (HA) titer. The titer value was determined by titrating serum dilution with SRBC (1.25 x 10^9 cells) in microtiter plate. The reaction mixture was incubated at room temperature for 2 h and examined visually for agglutination. The highest serum dilution showing haemaaglutination was noted and expressed as HA titer

Phytochemical profiling by HPLC

Shimadzu LC - 20AT Prominence binary HPLC system (Shimadzu, Japan) with C-18 reverse phase column was utilized for establishing phytochemical profile. 20 µl pre-filtered (0.2 µm) extracts (TCA, TCB, TCC and TCD) with fixed concentration (1 mg/ml) were injected in the column. The elution was done with acetonitrile: water (10:90), at a flow rate of 1 ml/min and peaks were detected using PDA detector at 266 nm (Srinivasan et al. 2008).

Statistical analyses

Data are expressed as mean ± S.E. Statistical analyses were

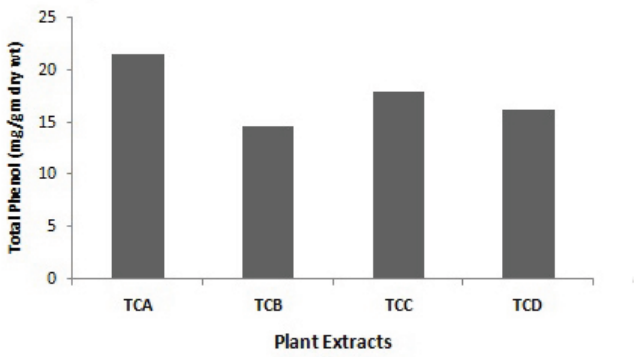

Figure 1. Total phenol content (gallic acid equivalent) of *Tinospora cordifolia* growing on A. indica (TCA), A. *leucophloea* (TCB), B. *monosperma* (TCC) and P.. *juliflora* (TCD).

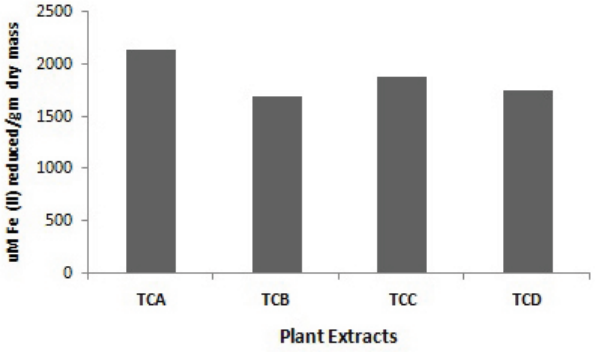

Figure 2. The antioxidant potential of *Tinospora cordifolia* growing on A. indica (TCA), A. *leucophloea* (TCB), B. *monosperma* (TCC) and P. *juliflora* (TCD).

carried out by one way ANOVA followed by Dunnette comparison test using graphpad prism 5.00 for Windows, Graphpad Software, San Diego, California, USA. P< 0.05 was considered as significant.

Results and Discussion

Determination of total phenolic content and FRAP assay

The total phenol content (gallic acid equivalant) of TCA, TCB, TCC and TCD varied significantly as shown in Figure 1. TCA was on top in total phenol content with 21.5 mg/g dry weight followed by TCC (17.9 mg/g), TCD (16.3 mg/g) and TCB (14.7 mg/g) total phenolics on dry weight basis. It is known that total phenol content is responsible for the free radical scavenging activity in many plants (Pourmorad et al. 2006; Adedapo et al. 2008). The antioxidant potentials of TCA, TCB, TCC and TCD were estimated from their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II) (Figure 2). Reducing ability of TCA was 2144 ± 0.46 µM Fe(II)/g

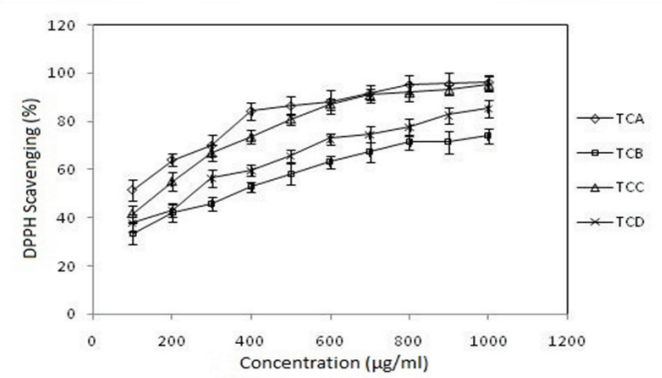

Figure 3. Comparative DPPH free radicals scavenging potential.

dry mass which was the highest amongst the four followed by TCC, TCD. and TCB in decreasing order. This activity is believed to be mainly due to the redox properties of phenolics which play an important role in (a) adsorbing and neutralizing free radicals, (b) quenching singlet and triplet oxygen and (c) decomposing peroxides. According to recent studies, a highly positive relationship between total phenols and antioxidant activity appears to be the trend in many plant species (Zheng and Wang 2001; Adedapo et al. 2008).

DPPH free radical scavenging potential

All the extracts of *T. cordifolia* growing on different support trees (TCA, TCB, TCC and TCD) were evaluated for DPPH free radical scavenging potential. A dose dependent radical scavenging was observed with all the extracts investigated (Fig. 3). However, a trend similar to that of FRAP was observed when the activities of different extracts were compared. TCA showed the maximum activity with 51.52 ± 4.28% DPPH radical scavenging at 100 µg/ml which increased to 86.36 ± 3.91 % at 500 µg/ml concentration. TCB, on the other hand, showed 33.21 ± 4.45% DPPH radical scavenging at 100 µg/ml which could increase up to 58.12 ± 4.36% at 500 µg/ml concentrations, the lowest among the four extracts studied. The other two extracts, TCC and TCD showed activities between the two. The DPPH free radical scavenging activity in present investigation indicated the presence of antioxidant molecule(s) capable of donating hydrogen to a free radical in order to remove the odd electron (Olayinka and Anthony 2010). The trend observed with all the extracts in radical scavenging study corroborates well with the respective total phenol content and total antioxidant activity of the different extracts.

Anti-lipid peroxidation activity

Depletion in enzymatic defense against free radical induced oxidative stress is known to promote generation of ROS thereby, affecting structural and functional integrity of cell mediated by peroxidation of membrane lipids (Biaglow et

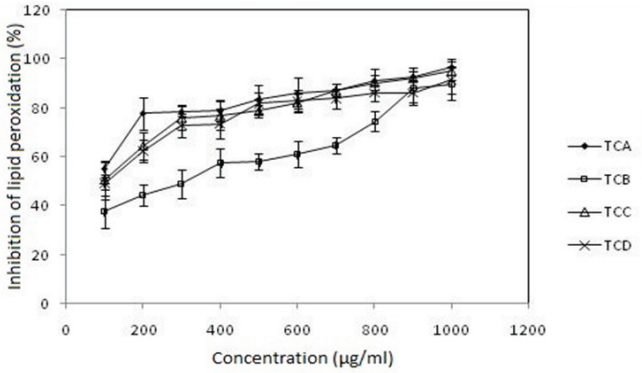

Figure 4. Comparative anti- lipidperoxidation (ALP) potential of T. *cordifolia* growing on A. indica (TCA), A. *leucophloea* (TCB), B. *monosperma* (TCC) and P.. *juliflora* (TCD).

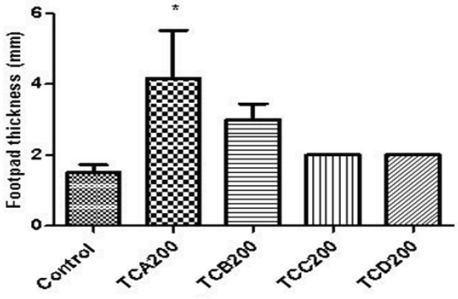

Figure 5. Effect of TCA, TCB, TCC and TCD (at 200 mg/kg body weight p. o.) on delayed type hypersensitivity (DTH) response in rats after 24 hours.

al. 1987). The estimation of lipid peroxidation (in present case the ability of extracts to inhibit lipid peroxidation) is therefore, important to monitor oxidative damage to cellular membranes (Tyagi et al. 2009). Comparative inhibitory effects of TCA, TCB, TCC and TCD in concentration range 100 to 1000 µg/ml, on TBARS formed in rat colon induced by $FeCl_3$ *in vitro* were studied. Among all extracts, TCA showed highest anti-lipid peroxidation activity (55.25% at 100 to 96.8% at 1000 µg/ml) followed by TCC (50.59% at 100 µg/ml to 95.24% at 1000 µg/ml), TCD (49.18% at 100 to 91.57% at 1000 µg/ml) and TCB (37.51% at 100 to 89.83% at 1000 µg/ml) (Fig. 4).

Consistency in the activity trend observed for all the parameters indicate the superiority of extract of *T. codifolia* growing with support of Neem tree over its counterparts growing on other support trees.

Comparative evaluation of immunomodulatory activity

Plants or plant products like its powder, extracts or fractions are well known for their ability to interact with the immune system of the mammals and affecting humoral and/or cellular immunity. Plants or their above said products alter the innate immunity or may show effect on specific cell types involved in acquired immunity (Borchers et al. 1997). In the light of these facts, a comparative immunomodulatory activity (effect on neutrophil adhesion and cell mediated and humoral immune responses) of ethanol extracts of stem of *T. cordifolia* growing with support of four different trees (TCA, TCB, TCC and TCD) was evaluated in anticipation of establishing the link between the activity variation as a function of supporting tree.

Table 1. Effects of ethanol stem extract of TCA, TCB, TCC and TCD on total WBC count and percent neutrophil adhesion.

Group	Treatment (mg/kg, p.o.)	WBC count cell/ mm^3	Percent neutrophil adhesion
I	Control	4683 ± 197.3	5.59 ± 2.5
II	TCA 200	5967 ± 302.9*	11.67 ± 2.6
IV	TCB 200	5533 ± 335.3	10.49 ± 0.007
VI	TCC 200	4933 ± 363.9	10.7 ± 0.31
VIII	TCD 200	5333 ± 336.3	11.6 ± 1.4

All values are mean ± SEM, n=6, * significantly different from control, $p < 0.05$.

Neutrophil adhesion test

Comparative effect of the extracts (TCA, TCB, TCC and TCD), on neutrophil adhesion, at 200 mg/kg body weight p.o. (per organism) are presented in Table 1. WBC count was observed to be increased in all the pretreated rats over control. The control group showed 4683 ± 197.3 WBCs/mm^3 which increased up to 5967 ± 302.9 WBCs/mm^3 in rats treated with TCA followed by TCB, TCD and TCC in decreasing order. Our observation with all the extracts of *T. cordifolia* (TCA, TCB, TCC and TCD) are in agreement with the previous studies (Mathew and Kuttan 1999; Manjrekar et al. 2000). Neutrophils represent a multifunctional cell type in innate immunity that contributes to pathogen clearance by recognition, phagocytosis and killing (Sharififar 2009). Activated neutrophils are the markers of boosted immune system and their activation can be studied by neutrophil adhesion test (Ghaisas et al. 2009). Among 4 extracts investigated, TCA treated rats showed maximum *i.e.* 11.67% neutrophil adhesion which is almost double to that of control. Other extracts were also equally effective in activating the neutrophils in our studies.

Delayed type hypersensitivity response (DTH response)

Sheep Red Blood cell (SRBC) induced delayed type hypersensitivity was used to assess the effect of extracts on cell mediated immunity. Figure 5 shows the comparative DTH response of TCA, TCB, TCC and TCD at 200 mg/kg body

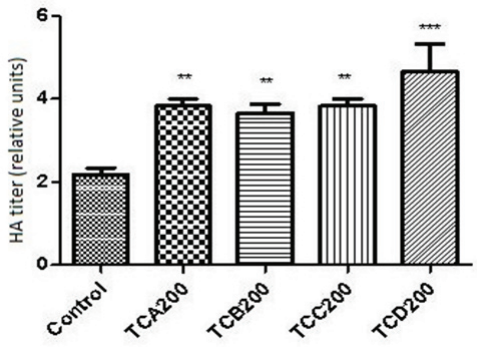

Figure 6. Effect of TCA, TCB, TCC and TCD (at 200 mg/kg body weight p. o.) on humoral immune response in rats after 7 days of treatment.

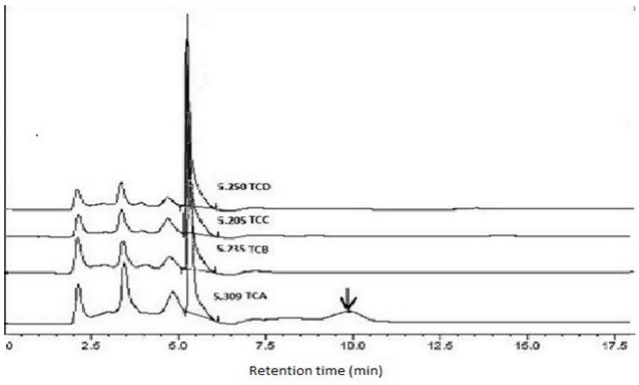

Figure 7. High performance liquid chromatogram of TCA, TCB, TCC and TCD stem extracts at 1 mg/ml. (↓) indicates an extra peak at RT 9.88 min in TCA extract.

weight p.o. (per organism), respectively. The DTH response in TCA treated rats was the highest and statistically significant.

In cell mediated immune response, sensitized T lymphocytes, when challenged by the antigen, are converted into lymphoblast and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus, immobilized to promote defensive inflammatory response reaction (Patwardhan et al. 1990). Earlier researchers reported the dose dependent stimulation of cell mediated immune response by *T. cordifolia*, which is in accordance with our investigation (Kapil and Sharma 1997; Aher and Wahi 2010).

Determination of humoral immune response

The haemagglutination antibody titer was used to assess humoral immune response. Figure 6 shows effects of TCA, TCB, TCC and TCD on haemagglutination antibody titer at 200 mg/kg body weight p.o. The results revealed that all the extracts (TCA, TCB, TCC and TCD) evaluated could significantly increase the humoral immune response, maximum being with TCD. *T. cordifolia*, earlier also proved to be efficient in stimulating the humoral immune response in a dose dependent manner (Kapil and Sharma 1997; Mathew and Kuttan 1999).

Comparative phytochemical profile

All the extracts were analyzed by HPLC for detecting the possible variation in secondary metabolite profile as a function of supporting tree. The method used for the separation could give a satisfactory chemical fingerprint. For this comparative analysis, only significant peaks were considered. The resultant chromatograms were almost super imposable albeit with varying peak area (Fig. 7). The chromatogram of TCA showed the presence of one extra peak (Rt 9.88) which is not present in other extracts (TCB, TCC and TCD). Three major groups of compounds, protoberberine alkaloids, terpenoids and polysaccharides have been reported to be responsible for the observed therapeutics activity of *Tinospora cordifolia* (Bisset and Nwaiwu, 1983; Chintalwar et al. 1999). However, a comparative evaluation of secondary metabolites of Tinospora cordifolia growing with the support of different trees has not been reported earlier.

Conclusion

T. *cordifolia* is an important medicinal plant and is being extensively used in Indian system of medicine, Ayurveda. A comparative analysis of antioxidative potential and immunomodulatory activity of its extracts with plants growing on four different support trees establish the superiority of the one which grows with the support of A. *indica* (Neem). Classically, it is neither a symbiotic relationship nor a case of parasitism and therefore the exact cause of this superiority is difficult to predict at this juncture and would need further detailed investigations. Presence of an extra peak at retention time (Rt) 9.88 min in HPLC with extract of the plant growing with the support of Neem is indicative of possible change in secondary metabolite profile as a function of support tree, which need to be investigated further. Nevertheless, the present study does validate Ayurveda literature and belief of its practioners that *Tinospora cordifolia* growing with the support of A. *indica* (Neem) has superior medicinal properties and value.

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