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Modulatory activity of a polyphenolic fraction of *Cinnamomum zeylanicum* L. bark on multiple arms of immunity in normal and immunocompromised mice

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ABSTRACT

To evaluate immunomodulatory activity of polyphenolic fraction of *Cinnamomum zeylanicum*bark (PP-CZ) against infection-related conditions using normal and immune-compromised mice. The normal and cyclophosphamide (CYP)-induced immune-compromised mice were sensitized with SRBCs and PP-CZ (10, 25, and 50 mg/kg, p.o.) was administered orally for 7 days. The haemagglutinin (HA) antibody titres (primary and secondary) and delayed type hypersensitivity (DTH) response was measured at 7- and 14-days post-immunization, respectively. In separate experiments, effects of PP-CZ on numbers of resident peritoneal macrophages in peripheral blood mononuclear cell (PBMC), against host resistance (*E coli*-induced abdominal sepsis) and phagocytic activity against *Candida albicans* were evaluated in mice. PP-CZ had shown a have beneficial effects on multiple arms of theimmune system in animal models and improves humoral (antibody production), cellular (DTH) and innate (PMN phagocytosis) responses of the immune system, as well as numbers of resident peritoneal macrophages. PP-CZ also showed protection to mice against lethal *E. coli* abdominal sepsis. PP-CZ demonstrated significant immunomodulatory activity through multiple arms of immunity in normal and infection-related immuno-compromised conditions.

INTRODUCTION

The immune system is a highly sophisticated defence mechanism against external biological invaders through the interconnected network between the brain, endocrine and immune system. It also serves to regulate the internal environment by eliminating aberrant cells or misplaced tissues within the body. Most of the immune disorders are a result of increased or decreased expression of the immune system (Geha *et al.*, 2007).

The immune system principally is responsible for the eradication of pathogens (Janeway Jr, 2001). Susceptibility to microbial, allergic and other disorders is higher in the presence of a compromised immune system resulted in state of immunodeficiency (or immune deficiency). In such conditions, the immune system's ability to fight infectious disease is compromised or entirely absent. Some people are born with defects in their immune system, or primary immunodeficiency but in most cases of immunodeficiency are acquired ("secondary") in disease conditions such as acquired immunodeficiency syndrome (AIDS) or many infections (e.g. Influenza)(Todoric *et al.*, 2013). The decreased ability of the immune system to clear infections in patients are responsible for causing autoimmunity through perpetual immune system activation(Grammatikos and Tsokos, 2012). The treatment of immune disorders mainly involves the use of immunomodulators, that respond in three ways namely immuno-suppression, tolerance, and immunostimulation (Mellman *et al.*, 2011). The concept of immunomodulation relates to nonspecific activation of the function and efficiency of macrophages, granulocytes, complement, natural killer cells and lymphocytes and also to the production of various effector molecules generated by activated cells. It is expected that theses nonspecific effects give protection against different pathogens including bacteria, viruses, fungi etc., and constitute an alternative to conventional chemotherapy.

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The category of immunostimulant comprises of drugs and nutrients that stimulate the immune system by inducing activation, or increasing activity of any of its components. Both immunostimulant and immunosuppressing agents have their own standing and search for better immunomodulatory agents is becoming the field of major interest all over the world since long (Patwardhan *et al*., 1990).

Competency of the immune system can be enhanced by the use of immunostimulant. Plant based natural medicines are believed to enhance the natural resistance of the body against infection and their immunomodulatory activities have been reported in numerous plants. In recent years, there has been growing interest in the field of herbal medicines research and search for cost effective and promising immunomodulatory compounds from natural products.

Consumption of dietary polyphenols leads to beneficial effects for human health as in the case of prevention and/or attenuation of cardiovascular, inflammatory, neurodegenerative and neoplastic diseases. Once ingested dietary polyphenols are able to interact and influence the function of many biological systems in the host, even including intestinal and systemic immunity (Magrone and Jirillo, 2010). Polyphenols from variety of natural sources are reported as stimulator of the immune response (Gonzalez-Gallego *et al.,* 2010, Hughes, 2005, Romeo *et al.,* 2010). Many *in vitro* and *in vivo* studies on Ayurvedic preparations and herbal extracts are reported for immune-stimulatory properties (Craig, 1999, Kumar *et al.,* 2011, Lee and Werth, 2004, Ravindran *et al.,* 2004). One of the most promising amongst them is Cinnamon (*Cinnamomum zeylanicum* Syn *C. verum*, family: Lauraceae) bark, a widely used food chain raw metrial, spice and flavouring agent(Kirtikar *et al.,* 1975, Warrier *et al.,* 1993).Moreover, cinnamon bark is a certified GRAS (generally recognised as safe) ingredient in USA.

An interesting factor about cinnamon is that it can act as both immunostimulant and suppressant depending on nature of constituents (Niphade *et al.,* 2009, Ravindran *et al.,* 2004). The cinnamon extract is reported to have immunostimulant effect on human lymphocytes proliferation, cytotoxic T-lymphocyte activity, immunoglobulin production by B-cells and interleukin (IL-1β) production by monocytes (Shan *et al.,* 1999). On the other hand, immunosuppressive potential of cinnamon cortex and oil (Ravindran *et al.,* 2004) and bark extract (Chang and But, 1986, Tang and Eisenbrand, 1992) has been demonstrated. However, exact proportions of each constituent(s) that are responsible for each of these contradictory immunomodulatory activities of cinnamon bark are not yet known.

The polyphenol fraction from *Cinnamomum zeylanicum* bark (PP-CZ) had shown to be responsible for its multifaceted pharmacological profile (Dudonné *et al.,* 2009, Mathew and Abraham, 2006). PP-CZ is reported to regulate immune function perhaps by regulating anti- and pro-inflammatory mediators as well as the gene expression of in macrophages(Cao *et al.,* 2008). Furthermore, polyphenols (mainly proanthocyanidins) was proposed to be a major contributor in antibacterial activity of ground cinnamon (*Cinnamomum burmannii*) against major pathogens (Shan *et al.,* 2007).

Recently, we have demonstrated ameliorative effects of the PPCZ in management of immune non-infectious disorders such as allergic rhinitis (Walanj *et al.,* 2014), asthma (26) and rheumatoid arthritis (Rathi *et al.,* 2013). However, immunomodulatory potential of PP-CZ on infection-related immunocompromised conditions is not yet explored. Therefore, we undertook the present work with an objective to evaluate immunomodulatory activity of PP-CZ on multiple arms of immunity in absence and presence of infections. The evaluation of PP-CZ in normal and cyclophosphamide (CYP) induced immunocompromised mice, on resident peritoneal macrophages peripheral blood peripheral blood mononuclear cell (PBMC), host resistance against E. coli (abdominal sepsis) and Candida albicans (phagocytosis) were investigated.

MATERIAL AND METHODS

Animals

Swiss albino mice (20-25 g) were obtained from National Toxicology Centre, Pune, India. The mice were housed in a group of 3 in polypropylene cages and separated gender-wise at a temperature of 24 ± 1 °C in 12 h: 12 h light: dark cycle, with free access to standard pellet feed (Chakan Oil Mill, India) and filtered water. Cages of mice were taken to the laboratory 1 h before on each day of actual experiment for acclimatization. All experiments were carried out between 08:00 h and 17:00 h in a quiet laboratory at an ambient temperature. The research protocol was approved by Institutional animal ethics committee (IAEC)as per Indian norms laid down by Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), New Delhi.

Chemicals

Fresh blood was collected from sheep's sacrificed in the local slaughter house. Sheep red blood cells (SRBCs) were washed three times in normal saline and adjusted to a concentration of 0.1 ml containing 1×10^8 cells for immunization and challenge. CYP was obtained as a gift sample from Khandelwal Laboratories, Mumbai, India and was used as a standard immunosuppressant agent. Carboxymethyl cellulose was purchased from Qualigens (Mumbai, India) and used as a suspending agent. All other chemicals used were of analytical grade.

The test compound, PP-CZ was prepared from Cinnamon bark as per reported procedure (Rathi *et al.,* 2013)and provided by Indus Biotech Private Limited, Pune, India(coded as IND02). The PP-CZ is a standardized polyphenolic fraction of *Cinnamomum zeylanicum* bark with total phenolic content of 860 mg gallic acid equivalent (GAE) per g and contains pentameric type A proanthocyanidin polyphenols (TAPP) as a marker compound with some trimer and tetramer content(Rathi *et al.,* 2013, Vetal *et al.,* 2013). The PP-CZ suspension was freshly prepared daily in distilled water with 0.2% carboxymethylcellulose (as suspending agent) to obtain concentration of 1 mg/ml. The doses of PP-CZ for biological evaluations were determined as 10, 25 and 50 mg/kg,

oral which was based on based on past reports of preclinical efficacy (Rathi *et al.,* 2013) and safety (Kandhare *et al.,* 2013) in animals.

Effect of PP-CZ on cellular immunity in normal mice

Effect of PP-CZ on HA titre and DTH response using SRBCs as an antigen in mice after 7-days of pre-treatment was tested as per reported method (Puri *et al.,* 1994). On day 0, twenty-four Swiss albino mice (18-25 g) were sensitized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0. The mice were divided into 4 groups, each group containing six mice and administrated with treatments as follows: Group I–Vehicle control (Vehicle, 10 ml/kg), and Group II–IV: PP-CZ (10, 25 and 50 mg/kg p.o. respectively). The treatments were administered orally for 7 days (day of challenge).

The primary (1°) and secondary (2°) antibody titres were measured on day 7 and 14 respectively by hemagglutination (HA) technique. Two individual serum samples of equal volumes from each group were pooled. Serial 2-fold dilutions of pooled serum samples was carried out in 25 µl volumes of normal saline in microtitration plates and 25 µl of 1% suspension of SRBCs in saline was added. After mixing, the plates were incubated at 37°C for 1 h and examined for HA titre under microscope. The reciprocal of the highest dilution of the test serum giving agglutination was considered as the antibody titre. On day 7, after sampling of blood, the thickness of the right hind foot-pad was measured using vernier calliper (Digimatic Series 500, Mitutoyo America Corporation, USA) and the mice were challenged by injection of 1×10^8 SRBCs in right hind foot pad. Foot thickness was measured again 24 h after SRBC challenge (Day 8) as per reported procedure (Doherty, 1981). The difference of foot thickness (between the pre- and post-challenge) was calculated and expressed in mm and taken as a measure of delayed type hypersensitivity (DTH). The body weights of mice were recorded.

Effect of PP-CZ on humoral immunity in immunocompromised mice

Effect of PP-CZ on HA titre and DTH response using SRBCs as an antigen in CYP induced immunocompromised mice after 7-days of pre-treatment was studied as per as per reported method (Puri *et al.,* 1994). Swiss albino mice (18-25 g) were divided into 5 groups, of 6 (3 male and 3 female) mice each and orally administered with treatments as follows: Group I–Vehicle control (Vehicle 10 ml/kg), Group II – CYP control (CYP 25 mg/kg + vehicle), and Group III-V –PP-CZ (10, 25 and 50 mg/kg) respectively. All the mice were immunized by injecting 0.1 ml of SRBCs suspension containing 1×10^8 cells intraperitoneally on day 0 of study. Immunosuppression was induced by CYP in mice from group II to IV with daily administration of CYP (25 mg/kg, oral) for 3 consecutive days. Mice from group III to V were administered with test compound, PP-CZ (10, 25 or 50 mg/kg p.o.) respectively from day-0 to Day-7 of the study. Antibody titres (1ºand 2º) and DTH response was measured by repeating the same procedure as that in normal mice. The body weights of mice were recorded.

Effect of PP-CZ numbers of resident peritoneal macrophages

The numbers of resident peritoneal macrophages were measured after subacute administration of PP-CZ in groups of Swiss albino mice as per reported method (Saxena *et al.,* 1991). The eighteen Swiss albino mice were randomised into group of 6 (3 male and 3 female) mice per group and were treated with vehicle or PP-CZ (25 or 50 mg/kg) once daily by gavages for 20 consecutive days. On day 21, the mice were injected i.p. with 5 ml cold phosphate buffered saline, then sacrificed by cervical dislocation. Peritoneal fluid was collected from the lower part of abdomen of each mouse and incubated at 37 °C for 1 h. The supernatant was then discarded and 2 % EDTA solution was added and maintained at 4 °C for 30 min. Macrophage cell suspensions were then centrifuged at 2000 rpm for 5 min and the pellet was suspended in 1 ml phosphate buffer saline. Number of peritoneal cells were counted by hemocyto-meter.

Effect of PP-CZ phagocytic activity against *Candida albicans*

The phagocytic activity of PP-CZ was assessed against *Candida albicans* as per reported method (Ponkshe and Indap, 2002). Eighteen Swiss albino mice (18-25 g) were randomly divided into 3 groups of 6 mice (3 male and 3 female) each and were treated with vehicle or PP-CZ (25, or 50 mg/kg) once daily by gavage for 20 consecutive days. On Day 21, blood samples were collected by retro orbital puncture and placed on a clean, dry glass slide and allowed to clot. The slide was incubated at 37 °C for 25 min to allow adherence of PMN cells. Slides were than rinsed and the PMN cells were incubated with $1x10^6$ cells of *Candida albicans* suspension for 1 h at 37 °C. Then, the slide was drained, fixed with methanol and stained with Giemsa stain. Slides were evaluated for PMN phagocytic activity by determining the phagocytosis (%) and phagocytic index (PI). The mean number of *Candida albicans* cells that are phagocytosed by PMNs on the slide was determined microscopically for 100 PMN cells using standard morphological criteria(Brune *et al.,* 1973)and considered as phagocytosis (%). The PI was calculated as Total no. of *Candida* in 100 PMN cells / Number of PMN cells.

Effect of PP-CZ on resistance to *E. coli* abdominal sepsis

The effect of pre-treatment with PP-CZ on host resistance was assessed in Swiss Albino mice using *E coli*-induced abdominal sepsis model in mice, (Subramoniam *et al.,* 1999). Twenty four mice weighing 20-25 g. were divided into 3 groups containing 8 (4 male and 4 female) mice each and orally treated as follows: Group I: vehicle control(vehicle, 10 ml/kg), Group II: PP-CZ (25 mg/kg) , Group III (50 mg/kg) once a day for 28 consecutive days. On day 29, lethal dose of *E. coli* suspension (2.5 x 10^9 cells) was injected intraperitoneally in all mice. Mice were then observed for percent mortality for 24 h post *E. coli* injection and during next seven days.

Table. 1: Effect of PP-CZ on cell mediated immunity (HA titre and DTH response after SRBCs challenge in normal mice).

Treatment (mg/kg, p.o, (14 days)	Body weight (g) on Day 14	HA Titre (Mean ± SEM)		DTH response (Mean ± SEM)
		1º Antibody Titer on Day 7 (count)	2º Antibody Titer on Day 14 (count)	Paw Edema Thickness (mm)
Vehicle Control	24.83 ± 0.87	5.33 ± 0.84	13.33 ± 1.69	1.50 ± 0.02
PP-CZ (10 mg/kg)	24.50 ± 1.03^{ns}	21.33 ± 3.37^{ns} (4 times)	32.00 ± 0.0^{ns} (2.4 times)	1.54 ± 0.09^{ns} (2.6%)
PP-CZ (25 mg/kg)	24.17 ± 0.87^{ns}	42.67 ± 6.75*** (8 times)	53.33 ± 6.74** (4 times)	1.85 ± 0.02*** (23%)
PP-CZ (50 mg/kg)	23.17 ± 0.79^{ns}	53.33 ± 6.75*** (10 times)	106.67 ± 13.49*** (8 times)	2.10 ± 0.03*** (40%)

n=6 (3 male and 3 female) per treatment group; Data represented as Mean ± SEM; Data was analysed by separate one-way ANOVA followed by Dunnett's test. * P < 0.05, ** P < 0.01 and *** P<0.001 as compared to Vehicle Control group. Increase and decrease is as compared with Normal mice.

Table. 2: Effect of PP-CZ on humoral immunity (HA titre and DTH response after SRBCs challenge in immunocompromised mice).

Treatment (mg/kg, p.o, (14 days)	Body weight (g) on day 14	HA Titre (Mean ± SEM)		DTH response (Mean ± SEM)
		1º Antibody Titer on Day 7 (count)	2º Antibody Titer on Day 14 (count)	Paw Edema Thickness (mm)
Vehicle Control	24.83 ± 0.87	5.33 ± 0.84	13.33 ± 1.69	1.50 ± 0.02
CYP Control	20.33 ± 0.56$^{###}$	0.33 ± 0.21$^{#}$ (93.8% decrease)	6.67 ± 0.84^{ns} (49.96% decrease)	2.48 ± 0.07$^{###}$ (65.33% increase)
CYP + PP-CZ (10 mg/kg)	22.50 ± 0.56^{ns1}	0.33 ± 0.20^{ns1} (No change)	12.33 ± 1.59^{ns1} (0.92 times decrease)	2.05 ± 0.07*** (36.66 % increase)
CYP + PP-CZ (25 mg/kg)	25.50 ± 0.43***	12.0 ± 1.79*** (2.2. times increase)	34.66 ± 6.42** (2.6 times increase)	2.08 ± 0.04*** (38.66% increase)
CYP + PP-CZ (50 mg/kg)	23.33 ± 0.61**	12.93 ± 1.69*** (2.33 times increase)	40.00 ± 8.10*** (3 times increase)	2.20 ± 0.04** (46.66% increase)

n=6 (3 male and 3 female) per treatment group;Data represented as mean ± SEM; Data was analyzed by separate one-way ANOVA followed by Dunnett's test.^{ns} - not significant, $^{#}$P<0.05 as compared to Vehicle Control group.^{ns1} - not significant,***P<0.0001 and **P<0.001 as compared to CYP Control group. The figures in parenthesis (Increase and decrease) are as compared with vehicle control mice.

Statistical analysis

All the responses were presented as mean ± standard error of mean (SEM). The data of antibody titres and DTH response (paw edema thickness) in normal and CYP mice were analyzed by one-way ANOVA followed by Dunnett's test. Data of number of peritoneal macrophages, % phagocytosis and PI was analyzed using Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test. The mortality data obtained during experiment in *E. coli*-induced abdominal sepsis was analyzed by Fischer's exact test for survival. The significance levels were considered at P < 0.05.

RESULTS

Effect of PP-CZ on body weights, HA titre and DTH responses in normal mice

The data of body weights, HA titre and DTH response after SRBCs challenge in normal a mice is shown in (Table 1). No significant changes in body weights were found in PP-CZ treated mice as compared with vehicle control group. On subacute treatment (7 days), PP-CZ (25 and 50 mg/kg, p.o.) showed dose-dependent increase in primary HA titre (8 and 10 times), secondary HA titre (4 and 8 times) and DTH response (23 and 40%) as compared with vehicle control group. PP-CZ at 10 mg/kg dose did not show significant change in HA titre or DTH response in normal mice.

Effect of PP-CZ on body weights, HA titre and DTH responses in CYP induced immunocompromised mice

The data of body weights, HA titre and DTH response after SRBCs challenge in CYP induced immunocompromised mice is shown in (Table 2). CYP (25 mg/kg, p.o.) caused drastic reduction in body weights (by 18.56%, P < 0.001), primary HA titre (by 93.8%, P < 0.05) and secondary HA titre (by 49.96%, not significant) respectively. On day 14, the body weights of CYP induced immunosuppressed mice with sub-acute treatment of PP-CZ (25 and 50 mg/kg) showed body weights of 25.50 g and 23.33 g respectively which was significantly (P < 0.001 and P < 0.01) more than body weight of CYP control mice (20.33 g). However, PP-CZ (10 mg/kg) treated group did not show significant difference as compared with CYP control mice.

Subacute treatment of PP-CZ (25 and 50 mg/kg, p.o.) for 7 days caused significant protection from CYP-induced reduction of body weights and HA titres. PP-CZ treatment at dose 25 and 50 mg/kg showed significant increase in primary (2.2 and 2.33 times) and secondary (2.6 and 3 times)HA titre as compared with HA tire of CYP control mice. However, PP-CZ (10 mg/kg, p.o.) did not show significant change in primary or secondary HA titre. CYP treatment showed significant increase in DTH response (paw thickness increase by 65.33%, P < 0.001). DTH response showed by PP-CZ (10, 25 and 50 mg/kg) treatment was 36.66, 38.66 and 46.66 % respectively, which was significantly (P < 0.01 to P < 0.001) less than CYP treated mice.

Effects of PP-CZ on resident peritoneal macrophages and phagocytic activity

The data of numbers of resident peritoneal macrophages and phagocytic activity (PI and % phagocytosis) is presented as Table. 3. The mean number of resident peritoneal macrophages was found to increase significantly ($P < 0.05$) in PP-CZ (50 mg/kg) treated group but not PP-CZ (25 mg/kg) treated group as compared to vehicle control group.

None of the tested doses of PP-CZ (25 or 50 mg/kg) could significantly enhance phagocytic index as compared to vehicle control group. The number of PMN with phagocytosis (% phagocytosis) in PP-CZ (25 mg/kg) treated groups were 95%, which was significantly ($P < 0.05$) more as compared to that of 81% phagocytosis in vehicle treated group. However, 92% phagocytosis recorded in PP-CZ (50 mg/kg) treated group was not statistically significant as compared to 81% phagocytoses found in vehicle control group.

Table. 3: Effect of PP-CZ on innate and adaptive immunity (phagocytic activity against *Candia albicans*).

Treatment (dose, p.o.)	Number of resident peritoneal macrophages	Phagocytic activity of cells	
		PI	% phagocytosis
Vehicle Control	3558.00 ± 1360.72	2.19 ± 0.34	81 ± 3.88
PP-CZ (25 mg/kg)	4300.00 ± 1240.82^{ns}	2.73 ± 0.37^{ns}	95 ± 2.73*
PP-CZ (50 mg/kg)	7758.50 ± 1512.16*	3.90 ± 0.48^{ns}	92 ±2.48

Data is presented as mean ± SEM, n =6 (3 male and 3 female) per treatment group, PI -Phagocytic Index. Data was analyzed using Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test. ns - not significant, * $P < 0.05$ as compared with vehicle control.

Effect of PP-CZ on resistance to *E. coli* induced abdominal sepsis

The mortality data of mice during *E. coli*-induced abdominal sepsis is presented in Table. 4. All the 8 mice in the vehicle control and PP-CZ (25 mg/kg) treated group showing 100 % mortality within 24 h of *E. coli* infection. On the other hand, only 3 out of 8 mice (37%) died in PP-CZ (50 mg/kg) treated group in 24 h with no mortality in remaining 7 days of observation period. The mortality rate in PP-CZ (50 mg/kg) treated group was significantly ($P < 0.05$) less as compared to mortality rate of vehicle control group (Fischer's exact test).

Table. 4: Effect of PP-CZ treatment on mortality of mice during *E. coli*-induced abdominal sepsis.

Treatment(dose, p.o.)	Number of dead mice	% Mortality
Vehicle Control	8/8	100
PP-CZ (25 mg/kg)	8/8^{ns}	100
PP-CZ (50 mg/kg)	3/8*	37.5

n = 8 in each group (4 male and 4 female), Data was analysed by Fischer's exact test for survival, *P< 0.05, ns- not significant as compared with vehicle control

DISCUSSION

Many immunostimulant activate different elements and mechanisms of the immune system of humans and animals, to reinforce the body's natural resistance to help in the treatment of infectious and non-infectious ailments and severe immune-suppression (Petrunov *et al.*, 2007). In the present study, immunostimulatory potential of PP-CZ (with TAPP as a marker compound) against immune responses of pathogenic infections were studied for the first time, on multiple arms of immunity involving pathogenic infections in the absence and in the presence of anti-infective (chemotherapeutic) agent using in vivo and in vitro experiments.

Control of disease by immunological means has two aspects, namely the development and improvement of protective immunity. Therefore, in the present study, we have evaluated immunomodulatory effects of PP-CZ against normal and CYP induced immunosuppression in mice. The response of PP-CZ on multiple types of immunity (cellular, humoral, innate and adaptive), responses (DTH, phagocytosis, host resistance, mortality) and type of pathogen (bacterial and fungal infection) was investigated. Based upon these studies, PP-CZ treatment was found to be immunostimulant on multiple arms of the immune system in dose dependent manner. PP-CZ treatment increased peripheral blood PMN phagocytosis activity in mice; treatment increased the number of resident peritoneal in mice. The results from this experiment suggest PP-CZ stimulates non-specific immunity by increasing the number of resident macrophages and the phagocytic activity in mice on subacute treatment. There was a dose -dependent trend for increased numbers of peritoneal macrophages and increased survival rate of mice.

Chemotherapy such as CYP acts at various levels on cells involved in defense against foreign invaders. CYP acts on both cyclic and intermitotic cells, resulting in general depletion of immunocompetent cells. CYP is an alkylating agent widely used in anti-neoplastic therapy. It is effective against a variety of cancers such as lymphoma, myeloma, and chronic lymphocytic leukemia (Baumann and Preiss, 2001). However, damage to the immune system is one of the major side effects of many chemotherapeutic agents including CYP. The immunosuppressant nature of these agents facilitates gradual deterioration of function of B cell mediated immunity and resulting in a decline in antibody titre.B-lymphocytes responsible for humoral immunity produce immunoglobulins which recognize and eliminate extra cellular antigens. Antigenic exposure could facilitate the proliferation and differentiation of B cells resulting in enhanced antibody titre. Challenge with SRBC produces rise in the hemagglutination antibody titre owing to sensitization of macrophages, T and B lymphocytes (Morris *et al.*, 2007). This reaction will act as a central role in humoral immune response against different antigens. In the present study, HA titre which is mediated by IgG and IgM type of immunoglobulins was shown significant dose dependent stimulation in normal mice (cellular immunity) and prevention of CYP-induced suppression of humoral immunity.

Perturbations in immune milieu can arise due to cumulative pressure on the cellular and humoral types of immune system. Cell mediated immunity is modulated by thymus-derived lymphocytes (T lymphocytes) which are sensitized by the antigen and on subsequent contact they respond with a delayed-type

hypersensitivity reaction. DTH, a localized inflammatory reaction, is a part of the process of graft rejection, tumor immunity, and most importantly immunity to many intracellular infectious microorganisms especially those causing chronic diseases such as tuberculosis (Elgert, 2009). DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing of pathogen (Descotes, 1998). In the present study, DTH was measured by foot-pad edema thickness, after 48 h of antigenic in CP induced immunosuppressed rats. The DTH response was significantly increased by PP-CZ in normal and CYP, which indicated immunostimulatory effect of PP-CZ on lymphocytes and accessory cell types leading to enhanced production of antibodies and increasing cell mediated immunity. Our results are in line with past reports of immunostimulatory activity of cinnamon bark (Niphade *et al.,* 2009) and demonstrated the cinnamon polyphenols as a major constituent that is responsible for positive effects on cellular and humoral immunity.

Along with immunosuppressive effects, CYP, is known to generate of reactive oxygen species (ROS) and free radicals that is responsible for appearance of adverse effects, including cell death, and apoptosis (Mythili *et al.,* 2004). Many dietary compounds with antioxidant properties capable of various cells and tissues, are reported to offer protection against CYP induced ROS and free radicals (Bhattacharya *et al.,* 2003, Hamsa and Kuttan, 2011, Jnaneshwari *et al.,* 2013). Thus, administration of antioxidants during chemotherapy was found beneficial, and sometimes necessary, to reduce CYP-induced oxidative stress during chemotherapy regimen.

Cinnamon bark extract (Dudonné *et al.,* 2009, Mathew and Abraham, 2006)and its polyphenol content (Li *et al.,* 2013, Moselhy and Ali, 2009, Panickar *et al.,* 2012) are reported to have potent anti-oxidant activities. The procyanidine polyphenol from various natural sources reported to offer strong protection against oxidative stress toprimary glial cells (Roychowdhury *et al.,* 2001), human diploid fibroblast cells (Yokozawa *et al.,* 2013), heart (Bagchi *et al.,* 2003), neurons (Strathearn *et al.,* 2014), brain (Lu *et al.,* 2010), lungs (Yucel *et al.,* 2009) and blood (Morel *et al.,* 2014). Furthermore, proanthocynidine polyphenols from grapeseeds are reported to have protective effects against chemotherapy induced toxicity such as cisplatin (Sayed, 2009, Yousef *et al.,* 2009) and methotrexate (Gulgun *et al.,* 2010) in laboratory animals. Therefore, anti-oxidant potential of PP-CZ can be envisaged as one of the major mechanism observed protective effects against CYP-induced immunosuppression in the present study.

Recently, The role of inflammatory cytokines (IL-1β and TNF-α) signalling in the genesis of cytotoxic chemotherapeutic agents (CCA) related symptoms has been delineated (Smith *et al.,* 2014, Wood and Weymann, 2013). Inflammation and neural signalling are through to be important etiologic mechanisms of the clusters of cancer treatment-related symptoms (Wood and Weymann, 2013). CCAs shares a common ability to activate intracellular stress response pathways to trigger the synthesis, processing, and release of inflammatory cytokine such as IL-1β from immune cells (Wood and Weymann, 2013). Cinnamon extract and its polyphenols have been demonstrated potent protective effects against neuro-inflammation (Ho *et al.,* 2013) and inflammatory bowel disease (Ishimaru *et al.,* 2008, Kwon *et al.,* 2011) by virtue of its pro-inflammatory cytokines (especially IL-1β, IL-6, and TNFα) inhibition. Furthermore, presence of polyphenols, down-regulation of IFNγ expression in activated T cells without altering IL-2 production and inhibition of inflammatory markers (p38, JNK, ERK1/2, and STAT4) are found to mediate immunomodulatory action of cinnamon bark for the application of inflammatory disorders (Lee *et al.,* 2011). Recently, we have demonstrated the inhibitory effects of PP-CZ on pro-inflammatory cytokines (IL-2, IL-4, and IFNγ) release from Concanavalin (ConA)-stimulated lymphocytes *in vitro* (Rathi *et al.,* 2013). Furthermore, potent inhibitory effects by proinflammatory cytokines by proanthocyanidins (a marker component of PP-CZ), has been conclusively demonstrated by past reports (Ahmad *et al.,* 2013, Ahmad *et al.,* 2014, Kim *et al.,* 2011, Lee *et al.,* 2012, Sayed, 2012, Zhang *et al.,* 2005, Zhou *et al.,* 2011). Release of pro-inflammatory cytokines such as TNFα, IL-1, IL-6 are also known to play pivotal role in cancer chemotherapeutic agents induced weight loss (Haslett, 1998). During the present study, subacute co-administration of PP-CZ with CYP was found effective in terms of prevention of CYP-induced body weight loss. Taken together, inhibition of CYP induced pro-inflammatory cytokines can be envisaged as another possible mechanism behind observed effects PP-CZ in the present study.

Sustained proinflammatory response (Murphey *et al.,* 2004) due to overexpression of pro-inflammatory mediators, such as TNF-α and IL-1β (Liaudet *et al.,* 2001) is associated to diminished bacterial clearance (Liaudet *et al.,* 2001), increases severity of infection and initiates multi-system organ (Weber and Swirski, 2014) and shows immense mortality in sepsis (Nameda *et al.,* 2005). The virustatic potential of cinnamon bark against pathogenic infections has been reported in the past. Kaishi-ni-eppi-ichi-to (TJS-664), a Chinese herbal preparation containing cinnamon as its main constituent, has been shown to exhibit antiviral action with 100% survival rate in influenza A2 virus infected mice with no effects in vitro (Ball *et al.,* 1994). Therefore, we have evaluated potential of PP-CZ against different types of infections and attempted to delineate possible mechanisms.

Phagocytosis represents an important innate defense mechanism against ingested foreign materials. Macrophages play a pivotal role in humoral and cellular immunity as they orchestrate both cytotoxic and phagocytic response. The release of macrophage inflammatory proteins and subsequent recruitment of leukocytes and natural killer cells plays vital role in antigen-stimulated immune responses. Macrophages function in both non-specific defence (innate immunity) as well as help initiate specific

defence mechanisms (adaptive immunity) of vertebrate animals. Their role is to phagocytose, or engulf and then digest, cellular debris and pathogens, either as stationary or as mobile cells. They also stimulate lymphocytes and other immune cells to respond to pathogens. They are specialized phagocytic cells that attack foreign substances, infectious microbes and cancer cells through destruction and ingestion.(Ovchinnikov, 2008) . In the present study, subacute treatment of PP-CZ showed increase in peritoneal macrophages numbers and PI. Therefore, increased capabilities of peritoneal macrophages through improvement of innate (non-specific) immunity can be envisaged as a underlying mechanism of PP-CZ in demonstrating protection against bacterial infections. Furthermore, PMN serve as modulators of immune function resulting in the elevation in neutrophil count (Soehnlein *et al.*, 2008). As PMN are considered as frontline cells in the immune system, and capable of recognizing and destroying foreign agents such as bacteria, increased PMN by PP-CZ treatment observed in the present study, indicate potential of PP-CZ in stimulating adaptive immunity against infectious pathogens.

The innate immunity response is the important line of defence against bacterial infection. However, innate immune function is impaired by progressive immunosuppression at late stage of sepsis (Weber and Swirski, 2014). While the initial immune response is crucial for effective clearance of invading pathogens, an overly exuberant host response to infection can cause septic shock, tissue damage, and death. Profuse inflammation in sepsis is frequently followed by global immunosuppression that increases susceptibility to viral and bacterial infections (Murphey *et al.*, 2004, Weber and Swirski, 2014). In the present study, PP-CZ(50 mg/kg) treated mice showed significant decrease in mortality as compared to 100% mortality seen in vehicle control group during *E. coli*-induced abdominal sepsis.

The promising effects of the test compound, PP-CZ, during the present study can be attributed to its high content of proanthocyanidins. Proanthocyanidins are reported to have diverse biological effects even though they are not absorbed into the systemic circulation (Donovan *et al.*, 2002). The procyanidines are reported to be degraded by intestinal microflora and forms metabolites such as phenolic acids (Deprez *et al.*, 2000) which are then absorbed through the intestinal or colonic barrier to demonstrate biological effects (Donovan *et al.*, 2002).Our results are in support of potential of proanthocyanidins from grape seeds shown in potentiating anti-tumor activity of doxorubicin via immunomodulatory mechanism (Zhang *et al.*, 2005) and protection against cisplatin-induced nephrotoxicity (Sayed, 2009). Suppression of bone marrow and immunity are the major drawbacks of many chemotherapeutic agents. Furthermore, potent immune-suppression is reported to prompt various types of infection (Fleming, 1997). Therefore, modulation of the immune system with improved effectiveness against pathogenic infections is highly desirable clinical need. The results from present study showed promise towards multi-faceted protective effects against CYP (a chemotherapeutic agent) and infections (bacterial and fungal).However, detailed studies with this regard will be required.

CONCLUSIONS

In conclusion, the present study demonstrated promising immunomodulatory activity of PP-CZ on multiple arms of immunity and can be explored as an adjuvant to chemotherapy in management of malignant and infectious diseases.

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