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IMMUNOSTIMULATORY EFFECT OF METHANOLIC LEAVES EXTRACT OF *PSIDIUM GUAJAVA* (GUAVA) ON HUMORAL AND CELL-MEDIATED IMMUNITY IN MICE

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ABSTRACT

Psidium guajava (Guava) has been used in the traditional system of medicine to treat various inflammatory diseases. The present study investigates the immunomodulatory effect of methanolic extract of Guava leaves on humoral and cell-mediated immunity. Delayed type hypersensitivity (DTH) and cyclophosphamide-induced myelo-suppression assays were performed to evaluate the effect of Guava leaves extract on cell-mediated immunity. Haemagglutination assay and mice lethality test were conducted to evaluate the effects of Guava leaves extract on humoral immunity. A total of 36 mice were divided into six groups and each group comprised of six mice. Low, medium, and high dose groups received 100, 150, and 200 mg/kg b.w., intraperitoneal doses of Guava leaves extract, respectively.The treatment with Guava extract showed significant increase in the counts of WBC, RBC, platelet, monocyte, neutrophil, eosinophil, lymphocyte, and level of hemoglobin as compared with control group. Guava extract significantly prevented the cyclophosphamide-induced myelosuppression and increased the DTH after 24 h, 48 h and 72 h. The treatment with Guava extract caused significant increase in anti-SRBCs antibody titer and reduced the mice lethality ratio as compared with cyclophosphamide. In conclusion, current study suggests that Guava leaves extract can stimulate both humoral and cell-mediated immunity in mice.

Key words: Immunomodulation; *Psidium guajava*, Humoral immunity; Cell-mediated immunity.

INTRODUCTION

The immune system is an arrangement of naturally occurring molecules and methods inside living systemthat ensures protection against infections. The concept of immunomodulation includes non-specific activation of complement system, granulocytes, macrophages, lymphocytes, and natural killer cells. These activated cells, in turn, produce various effector molecules which also take part in modulation of immune system(Gummert *et al*., 1999; Vigila and Baskara, 2008). All these non-specific events generate an alternative to conventional chemotherapy and are expected to provide protection against different pathogens including fungi, viruses, and bacteria etc. (Sultana *et al*., 2011).

Cell mediated and humoral immunities are two important types of immune system that provide protection against diseases. The previous studies have shown that herbal drugs possess immunomodulatory properties and are known to activate both humoral and cell mediated immunity (Rinki and Mishra, 2011). Modulation of the immune system is emerging as a major area in pharmacology, especially in cases where undesired immunosuppression is the result of therapy **(**Thejass and Kuttan, 2007). Hematological parameters, like total leukocyte count, neutrophil count, lymphocyte count, monocyte count, RBC count, hemoglobin content, and platelet count are considered as key components of immune system. Health/immune system of the body is affected by the elevation or attenuation of these parameters as they recognize foreign antigens and build up an immune response (Sultana *et al*., 2011).

It has been noticed that the plants especially edible plants possess medicinal values along with their nutritional values (Shabbir, 2012). *Psidium gujava*, commonly called as guava in English, belongs to the family Myrtaceae (Torres-González *et al*., 2014).It is found in Asia, Africa, Europe, and South America, and is considered a native to Mexico. In traditional system of medicine, guava leaves have been used to cure cough, digestive sufferings, diarrhea, and dysentery(Heinrich *et al*., 1998; Leonti *et al*., 2001). Leaves have also been used in folkric medicine to manage hypertension and diabetes mellitus, and inflamed mucous membranes(Holetz *et al*., 2002; Ojewole, 2005; Oh *et al*., 2005). Leaves are chewed to alleviate toothache and are applied for rheumatic pain. Leaves are also applied on ulcers and wounds, and decoction is considered as febrifuge (Gutiérrez *et al*., 2008). Pharmacological evaluation showed that *Psidium guajava* possessed anti-oxidant (Thaipong *et al*., 2005), anti-microbial, wound healing (Chah *et al*., 2006), anti-tussive (Jaiarj *et al*., 1999), anti-allergic (Seo *et al*., 2005), anti-hypertensive (Ojewole, 2005), hypoglycemic (Mukhtar *et al*., 2004), anti-cancer (Chen *et al*., 2007; Bontempo *et al*., 2012;

Ryu *et al*., 2012), hepatoprotective (Roy *et al*., 2006), anti-inflammatory, and analgesic (Ojewole, 2006)properties. The present study investigates the immunomodulatory potential of methanolic leaves extract of *P. guajava*on humoral and cell mediated immunityin mice.

MATERIALS AND METHODS

Plant Extract: *Psidium guajava* leaves (01 kg) were collected from local market of Lahore, Pakistan and identified by Prof. Dr. Rasool Bakhsh Tareen, a botanist at University of Balochistan, Quetta(Voucher #: UOL/DP/15-027).Leaves were then subjected to shade drying for 28 days and subsequently ground to powder form.Leaves powder (500 mg)was soaked in 1.5 liter pure methanol for 7 days and was subjected to occasional shaking on daily basis. Muslin cloth was used to separate the course particles, while Whatmann No. 1 filter paper was used for filtration.Filtrate was concentrated using vacuum rotary evaporator(IKA, Germany)under reduced pressure at 40°C.A thick semi solid paste was obtained and was kept at -20°C till further use for experimental purpose (Janbaz *et al*., 2014).

Experimental animal: Five to six weeks old albino mice of both sexes were purchased and kept in the animal house of Faculty of Pharmacy, The University of Lahore. During whole experimental period, all the mice wereprovided with standard diet and water *ad libitum*. Standard humidity (60-70%) and temperature conditions (28°C ± 2°C) were maintained and 24 h dark and light cycles were ensured (Shabbir *et al*., 2014).All experimental protocols were approved from Institutional Animal Ethics Committee, The University of Lahore.

Effect of Guava leaves extract on cellular immunity

Delayed type hypersensitivity test (DTH): DTH assay was conducted by following the protocol of Omer *et al*.(2012).A total of thirty six mice were divided into six groups and each group comprised of six mice. All the mice were shaved and sensitized using 0.1 ml of 1%dinitrochlorbenzene (DNCB) dissolved in acetone at day 2 and subsequently challenged at day 8 using 0.2 ml of 1% DNCB, except vehicle control group. DNCB was applied on the surface of the skin with the help of syringe in the form of circle. Group-2 served as a positive control group (DNCB only group) and received only normal saline intraperitoneally. Group-1 served as a vehicle control group. Only acetone was applied on the surface of skin of group-1 mice and intraperitoneally treated with normal saline only.Guava leaves extract was administered to group-3 (low dose; 100 mg/kg b.w.),group-4 (medium dose; 150 mg/kg b.w.), and group-5 (high dose; 200 mg/kg b.w.) by intraperitoneal route. Group-6 (immunomodulator) received 0.1 ml/kg intraperitoneal dose of immunomodulator. Skin thickness was measured with vernier caliper after 24 h, 48 h, and 72 h of challenging dose of DNCB.

Preparation of Immunomodulator: The method of Calin *et al* (2011) was used with slight modificationsto prepare the immunomodulator. For this purpose, 1 g sodium selenite, 15 g vitamin E, and 9 g sodium chloride were dissolved in distilled water q.s. to 1000 ml.

Cyclophosphamide-induced Myelosuppression: The assay was conducted by following the protocol of Sudha *et al*. (2010). The mice were weighed and divided into six groups. Low dose, medium dose, and high dose groups received 100 mg/kg b.w., 150 mg/kg b.w., and 200 mg/kg b.w., doses of Guava leaves extract, respectively by intraperitoneal route, starting from day 1 to day 10. Immunomodulator group received 0.1 ml/kg intraperitoneal dose of immunomodulator. Negative andpositive control groups received normal saline only. Onthe 10thday, neutropenic dose of cyclophosphamide (200 mg/kg) was administered subcutaneously to all the mice groups, except the negative control group. Blood samples were collected before the administration of cyclophosphamide and 3 days after the injection of cyclophosphamide. Total leukocyte count (TLC) and differential leukocyte count (DLC) were done using automated hemocytometer.

Heamagglutination Assay: Mice of either sex weighing 30 to 35 g were randomly placed into six groups. All the groups were immunized with SRBCs (0.5 x 10^9 cells/0.1ml/animal) at day 0.This concentration of cells is frequently used to induce immunological response in mice(Ladics, 2007).Cyclophosphamide (a standard immunosuppressant) (25 mg/kg) and immunomodulatorwere used as reference controls. Low dose, medium dose, and high dose groups received 100, 150, and 200 mg/kg b.w., intraperitoneal doses of Guava leaves extract, respectively for seven consecutive days. Control group intraperitoneallyreceived normal saline only. Antibody titer was determined using HA titer method (Heden, 1946; Puri *et al*., 1993).Blood samples were drawn from tails of the mice and were subjected to centrifugation at 5000 rpm for 15 minutes for the collection of serum. 25µl of PBS was dispensed to the wells of all the rows, except the last row which was left as control.Then serum (25µl) was added in the wells of first column of 96 well microtitration plates and 2 fold serially diluted up to 8th row. Subsequently, 25µl of 10% (v/v) SRBCs in PBS were added in all the wells and microtitration plates were incubated at 37ºC for 2 h. The wells were microscopically evaluated for presence and degree of agglutination (Shivaprasad *et al*., 2006).

Mice lethality test

Preparation of *Pasturella multocida* culture: *Pasteurella multocida* was reconstituted in normal saline and subcutaneously administered into one rabbit at LD50 dose (10^5cells/0.5ml) (Virag*et al*., 2000). Blood was withdrawn after death of the rabbit and the animal was sacrificed for post-mortem. Specific organs, such as, liver, heart, spleen, and kidney were preserved after cutting into small pieces. Pyrogen free blood agar media was taken in a petri dish and small pieces of these organs were placed in it. The petri dish was incubated for 24 h.

Experimental design: Mice were divided into six groups having 6 mice in each group. Negative control group and hemorrhagic septicemia vaccine(HS) only treated group received vehicle only. Low dose, medium dose, and high dose groups were treated with 100, 150, and 200 mg/kg b.w. intraperitoneal dose of Guava leaves extract, respectively. *P. guajava* was administered for 21 days starting from day 1. Cyclophosphamide and immunomodulator were used as reference controls. All the mice were immunized with the hemorrhagic septicemia vaccine on 7th and 17th day, except negative control group. All the mice were then subcutaneously challenged with lethal dose of *P. multocida* on the 21st day of experiment. Mice examinations were conducted for about 72 h and following formula was used to calculate the mortality ratio (Sudha *et al*., 2010).

$$\text{Mortality ratio} = \frac{\text{Number of dead animals}}{\text{Total number of animals}} \times 100$$

Statistical analysis: Graph Pad prism v.6 software was used to analyse the data. We performed one way ANOVA followed by Tukey's test or student t-test, where applicable. The results were presented as mean ± S.E.M and $P < 0.05$ was considered as statistically significant.

RESULTS

Guava leaves extract significantly increased DTH at 24 h, 48 h, and 72 h: At 24 h (Fig. 1A), treatment with low (0.9500 ± 0.1169), medium (1.108 ± 0.1083), and high doses (1.350 ± 0.1033) significantly ($P < 0.001$) increased the skin thickness (a measure of DTH) as compared with vehicle control group (0.0100 ± 0.0044). The data also showed significant increase in DTH after treatment with immunomodulator (1.558 ± 0.0568; $P < 0.001$).

At 48 h (Fig. 1B), the positive control group (0.8333 ± 0.1537; $P < 0.01$) showed significant increase in DTH as compared with negative control (0.01833 ± 0.0040) along with the low dose (1.325 ± 0.1195), medium dose (1.378 ± 0.1063), high dose (1.642 ± 0.0943), and immunomodulator (1.758 ± 0.0789) treated groups ($P < 0.001$).

At 72 h (Fig. 1C), we found significant elevation in DTH in positive control group (0.8667 ± 0.1108; $P < 0.01$) as compared with negative control (0.0283 ± 0.0040) along with the low dose (1.392 ± 0.0830), medium dose (1.667 ± 0.1236), high dose (1.733 ± 0.0928), and immunomodulator (1.842 ± 0.0538) treated groups ($P < 0.001$).

We also compared the immunostimulatory effects of low dose (100 mg/kg b.w.), medium dose (150 mg/kg b.w.), high dose (200 mg/kg b.w.), and immunomodulator with positive control group and results showed dose dependent increase in all treated groups.

***P. guajava* leaves extract significantly increased antibody titer in Hemagglutination assay:** We found significantly decreased anti-SRBCs antibody titer in cyclophosphamide group (50.83 ± 0.7839; $P < 0.001$) as compared with the control group(118.7 ± 6.839)**.** Treatment with low (257 ± 4.583), medium (307.0 ± 4.583) and high (800 ± 51.41) doses of Guava leaves extract significantly ($P < 0.001$) increased the antibody titer as compared with the control group (118.7 ± 6.839). Similarly, immunomodulator (953.0 ± 23.59; $P < 0.001$) also significantly increased antibody titer as compared with the control group (Fig. 1D).

Guava leaves extract significantly increased WBC, RBC, and platelet counts, and Hb content in healthy mice: Treatment with low dose (7.168 ± 0.1635; $P < 0.01$), medium dose (9.630 ± 0.239; $P < 0.001$), high dose (20.52 ± 1.546; $P < 0.001$), and immunomodulator (25.63 ± 0.9767; $P < 0.001$) significantly increased the total leukocyte count as compared with the control group (2.665 ± 0.08702) (Fig. 2A).

We found significant ($P < 0.001$) increase in RBC count after treatment with low dose (5.692 ± 0.3213), medium dose (6.223 ± 0.2355), high dose (6.217 ± 0.1794), and Immunomodulator (6.290 ± 0.3490) as compared with the control group (3.150 ± 0.2460) (Fig. 2B).

Low dose (432.3 ± 3.818), medium dose (470.8 ± 5.974), high dose (444.2 ± 3.818), andimmunomodulator (471.3 ± 3.180) showed significant ($P < 0.001$) elevation the platelet levels as compared with the control group (268.7 ± 6.004) (Fig. 2C).

Treatment with medium dose (12.28 ± 0.2626), high dose (12.62 ± 0.2455), and immunomodulator (12.87 ± 0.5814) significantly ($P < 0.01$) increased the Hb content as compared with the control group (8.183 ± 0.2482). However, treatment with low dose did not show significant difference (Fig. 2D).

Guava leaves extractalso significantly increased differential leukocyte count in healthy mice:

Treatment with low (68.17 ± 0.9458; $P < 0.01$),

medium (68.50 ± 1.478; P < 0.001) and high (69.00 ± 2.663; P < 0.001) doses of Guava leaves extract significantly increased the lymphocyte countsas compared with the control group (62.83 ± 0.6009). Treatment with immunomodulator (81.67 ± 2.011) also showed significant (P < 0.001) increase as compared with control group (Fig. 2E).

Treatment with low (17.33 ± 0.8028; P < 0.01), medium (19.50 ± 1.088; P < 0.01), and high doses (25.50 ± 0.9916; P < 0.001) of Guava leaves extract significantly elevated the neutrophil counts as compared with the control group (14.00± 0.5774). Similarly, immunomodulator (30.67 ± 1.282; P < 0.001) also showed significant increase in the neutrophil counts compared with the control group (Fig. 2F).

Treatment with medium (11.17 ± 0.6667; P < 0.001) and high (11.67 ± 0.6667; P < 0.0.1) doses of Guava leaves extract significantly enhanced the monocyte counts as compared with the control group (5.667 ± 0.4944). However, low dose (7.167 ± 0.4773) and immunomodulator (6.667 ± 0.8433) did not show the significant difference as compared with control group (Fig. 2G).

Treatment with medium (6.133 ± 0.2186; P < 0.01) and high (7.133 ± 0.3180; P < 0.001) doses of Guava leaves extract significantly increased the eosinophil counts as compared with the control group (3.833 ± 0.3073).However, treatment with low dose and immunomodulator showed non-significant difference as compared with control group (Fig. 2H).

Treatment with Guava leaves extract significantly prevented the effect on haematological parameters in cyclophosphamide-induced myelosuppressive mice: We found significantly (P < 0.001) decreased total leukocyte count in positive control group (0.6283 ± 0.03060) as compared with negative control group (2.183 ± 0.09247). Treatment with low dose (0.9483 ± 0.05522), medium dose (1.580 ± 0.1189),high dose (2.325 ± 0.03914), and immunomodulator (2.412 ± 0.1720) significantly (P < 0.001) prevented the reduction in WBC levels as compared with positive control group(Fig. 3A).

The data showed significantly (P < 0.001) suppressed RBCs count in positive control group (2.627 ± 0.1014) as compared with negative control group (4.187 ± 0.05823). Treatment with low dose (3.640 ± 0.09234), medium dose (3.672 ± 0.06940), high dose (3.995 ± 0.04965), and immunomodulator (3.800 ± 0.1442) significantly (P < 0.001) prevented the suppression of RBCs count as compared with positive control group (Fig. 3B).

RBC counts were also found significantly (P < 0.001) alleviated in positive control group (54.83 ± 1.815) as compared with negative control group (139.7 ± 3.584). Treatment with low dose (72.00 ± 2.887), medium dose (85.67 ± 0.8819),high dose (97.50 ±0.6191), and immunomodulator (108.5 ± 2.045) significantly (P < 0.001) prevented the alleviation of platelet counts compared with positive control group (Fig. 3C).

We found significantly (P < 0.001) reduced Hb content in positive control group (5.053 ± 0.1917) as compared with negative control group (7.917 ± 0.1424). Treatment with low (6.217 ± 0.2600), medium (6.333 ± 0.04410), and high doses (7.083 ± 0.1302) of Guava leaves extract significantly (P < 0.001) increased the Hb content as compared with positive control group. Treatment with immunomodulator (7.378 ± 0.05913) also significantly (P < 0.001) prevented reduction in Hb content as compared with positive control group (Fig. 3D).

Lymphocyte counts were significantly (P < 0.001) reduced in positive control group (72.17 ± 1.778) as compared with negative control (90.67 ± 0.9545). Low dose (78.83 ± 2.104; P < 0.05) medium dose (86.17 ± 1.249; P < 0.01), high dose (91.00 ± 2.066; P < 0.001), and immunomodulator (96.00 ± 0.7303; P < 0.001) showed significant prevention in reduction of lymphocyte counts as compared with positive control group (Fig. 3E).

We found significantly (P < 0.05) suppressed neutrophil number in positive control group (5.000 ± 0.5774) as compared with negative control group (9.833 ± 0.4773). Treatment with low dose (16.83 ± 0.792), medium dose (19.33 ± 1.145), high dose (25.50 ± 1.176), and immunomodulator (20.50 ± 1.335) significantly (P < 0.001) prevented the suppression of neutrophil count as compared with positive control group (Fig. 3F).

The data showed significant (P < 0.01) reductionof monocyte counts in positive control group (14.50 ± 0.9220) as compared with negative control (20.00 ± 1.183). Treatment with medium dose (18.83 ± 1.400;P < 0.05), high dose (20.17 ± 1.470; P < 0.01), and immunomodulator(21.33 ± 1.892; P < 0.001)significantly prevented the decline in monocyte number as compared with positive control group (Fig. 3G).

We found a significant (P < 0.001) reduction in eosinophil count in positive control group (4.667 ± 0.4944) as compared with negative control group (9.333 ± 0.4944). Treatment with medium dose (7.333 ± 0.4944; P < 0.05), high dose (10.17 ± 0.6009; P < 0.001), and immunomodulator (9.833 ± 0.6009; P < 0.001) significantly prevented the reduction in eosinophil count as compared with control group (Fig. 3H).

Treatment with *P. guajava* extract reduced the mortality rate in mice: Negative control group and cyclophosphamide showed the 100% mortality rate. One mouse was found dead after 24 h, 2 died after 48 h, and 3 mice died after 72 h in both groups.Hemorrhagic septicemia vaccine only treated group decreased the mortality rate to 83.3% as compared with negative control. We found 3 mice dead after 48 h and

subsequently 2 more died after 72 h.Treatment with low dose further reduced the mortality rate to 50% as no mice was found dead after 24 h, however, one mouse died after 48 h and three more mice died after 72 h.Treatment with medium dose, high dose, and immunomodulator showed 0% mortality rate after 24, 48 and 72 h (Table. 1).

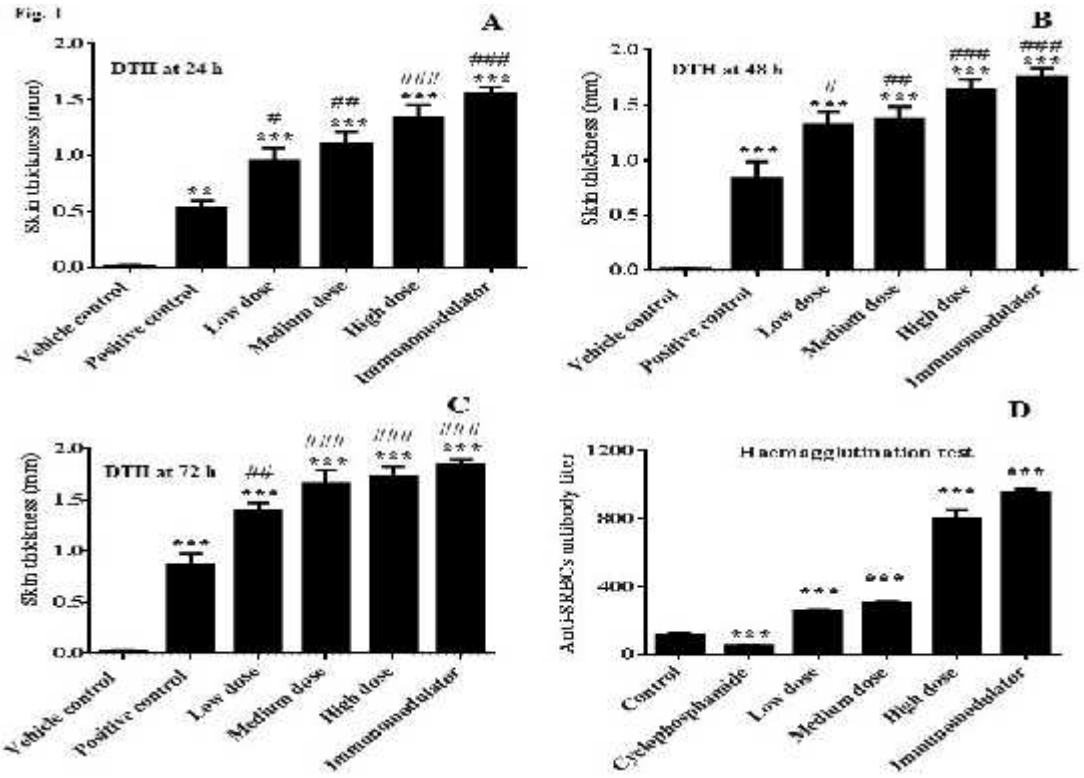

Fig. 1: Guava leaves extract significantly (P < 0.001) increased DTH as compared with both negative control and positive control groups at 24 h (A), 48 h (B), and 72 h (C).Mean ± SEM was given to represent the data where n = 6. Treatment with extract also significantly (P < 0.001) increased anti-SRBCs antibody titer as compared with control group in Haemagglutination test (D).

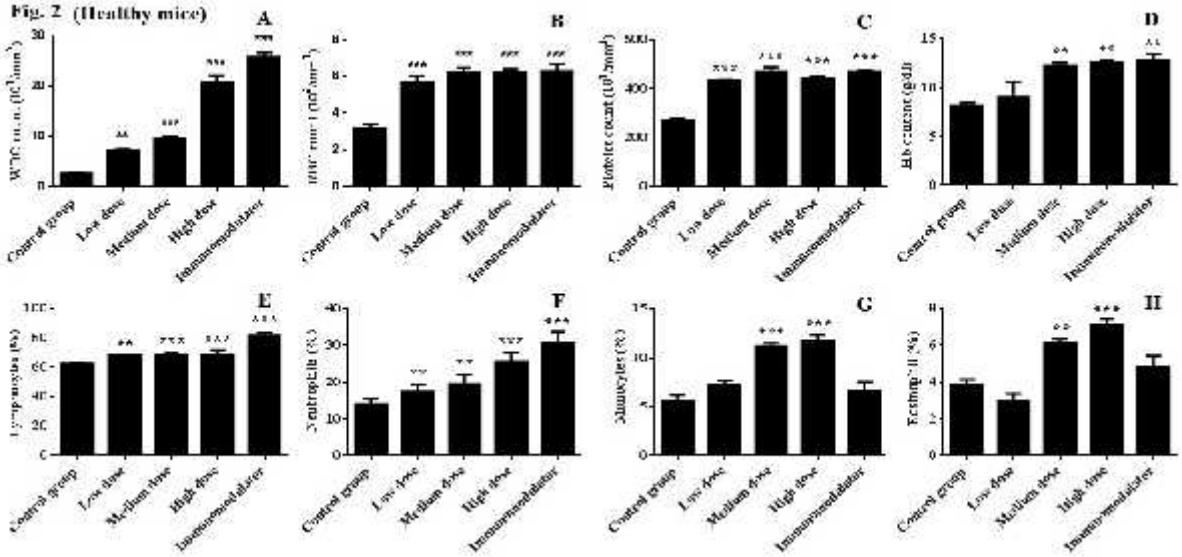

Fig. 2:Treatment with Guava leaves extract significantly increased WBC count (A), RBC count (B), platelet count (C), Hb content (D), and DLC (E-H) in healthy mice as compared with control group. Mean ± SEM was given to represent the data where n = 6.

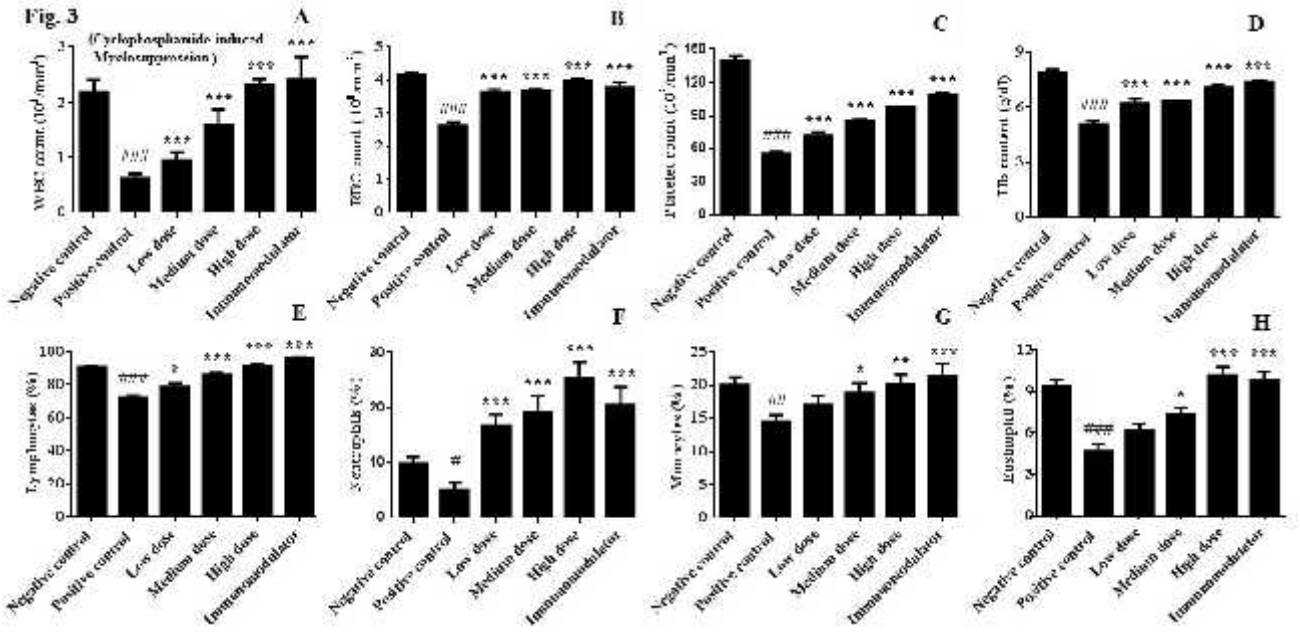

Fig. 3: Treatment with Guava leaves extract significantly prevented the reduction in WBC count (A), RBC count (B), platelet count (C), Hb content (D), and DLC (E-H) as compared with positive control groupin cyclophosphamide-induced myelosuppressive rats. Mean ± SEM was given to represent the data where n = 6.

Table 1.Treatment with *P. guajava* extract reduced the mortality rate in mice

Groups	24 h	48 h	72 h	Total mortality	Percentage Mortality
Negative control group	1	2	3	6	100%
HS only treated group	-	3	2	5	83.3%
Low dose (100 mg/kg b.w.)	-	1	2	3	50%
Medium dose (150 mg/kg b.w.)	-	-	-	-	0%
High dose (200 mg/kg b.w.)	-	-	-	-	0%
Cyclophosphamide	1	2	3	6	100%
Immunomodulator	-	-	-	-	0%

DISCUSSION

The current study evaluated the effect of Guava leaves extract on cellular immunity by performing delayed type hypersensitivity assay using DNCB. Evaluation of hypersensitivity by an antigen to which the individual has not been previously exposed is one of the ways to determine the cellular immunity (Friedmann and Pickard, 2014).The results showed that DNCB causes significant increase in DTH as compared with control group. DNCB forms a dinitrophenyl complex with skin proteins and acts as an antigen (Sajid *et al*., 2007). The sensitized T-lymphocytes when challenged with previously exposed antigen are converted into lymphoblasts and secrete lymphokines**.** These lymphokines, in turn, invite more scavenger cells to the site of reaction and cause the infiltration of inflammatory cells to exhibit the defensive response (Sharififar *et al*., 2009). In this study, treatment with Guava leaves extract significantly increased the DTH as compared with positive control group suggesting the boosted cell mediated immunity. The dose dependent increase in DTH response suggests the stimulatory effect of Guava leaves extract on accessory cell types and lymphocytes necessary for the expression of inflammation (Mitra *et al*., 1999; Fulzele, *et al*., 2003).

Cyclophosphamide administration caused significant reduction in total WBC counts and differential leukocytes count in all the groups. Cyclophosphamide is an alkylating agent that belongs to nitrogen mustard subclass. It causes alkylation of DNA by interfering DNA synthesis and acts as an immunosuppressive agent (Zuluaga *et al*., 2006). We evaluated the immunomodulatory effect of Guava leaves extract in healthy animals as well as in cyclophosphamide-induced myelosuppressive mice. Guava leaves extract showed immunostimulatory effect by significantly increasing the TLC and DLC in healthy mice. It alsodose dependently prevented the cyclophosphamide-induced myelosuppression. Previously, different medicinal plants

have shown the ability to stimulate the haemopoetic system, similar to the results of current study. For example, *Acacia nilotica*, *Ficus glomerata*, and *Moringa oleifera* are known to ameliorate cyclophosphamide-induced myelosuppression (Gaikwad *et al*., 2011; Ahmad *et al*., 2012; Heroor *et al*., 2013).Neutrophils are the components of immune system and help in killing of microorganisms (McFarlane *et al*., 2008). Severe and prolonged neutropenia increases the susceptibility to fungal and bacterial infections (Boxer, 2012).Neutropenia is associated with different conditions, like cancer chemotherapy, rheumatoid arthritis, and allergic asthma, and the reversal of neutropenia is vital in these conditions (Vadhan-Raj, 2003). Monocytes and macrophages are important in commencement and resolution of inflammation through release of cytokines and activation of acquired immune system. They play vital role in cancer progression, wound healing, tissue homeostasis, arthritis, and atherosclerosis (Parihar *et al*., 2010). Lymphocytes are crucial player of immune response and are subdivided into T cells, B cells and NK cells. Among these cells, NK cells are responsible for mounting innate immune response, while T cells and B cells are involved in the adaptive immune response(Slifka *et al*., 2000). Platelets are also immune cells that have the potential to start and speed up many vascular inflammatory disorders like transplant rejection, atherosclerosis, rheumatoid arthritis, and malaria infection (Morrell *et al*., 2014).

Mice lethality test and haemagglutination assay were used to evaluate the effect of Guava leaves extract on humoral part of immune system. B-lymphocytes interact with antigen and differentiate into antibody secreting cells. Antibodies facilitate the phagocytosis of antigen or cause neutralization of antigen(Ismail and Asad, 2009). Results revealed that Guava leaves extract significantly increased the antibody titer which suggests the increase in production of IgG and IgM antibodies in the serum of mice against sheep's red blood cells (Manz *et al*., 2005).

Mice lethality test is one of the commonly used tests to analyze the immunological response in mice that are previously exposed to antigen. Mice lethality test determines the strength of mice to survive against the antigen (Rishi *et al*., 2002). *Pasturella multocida* is a gram-negative bacterium and is the causative agent of many important diseases, like avian fowl cholera, bovine hemorrhagic septicaemia, enzoonotic pneumonia, and swine atrophic rhinitis(Harper *et al*., 2006).Vaccination is responsible for the production of antibodies when antigen is administered. In current study, HS only treated group reduced mortality rate as compared with negative control group. This finding indicates the increased production of antibodies against *P. multocida* by B-lymphocytes of mice immunized with hemorrhagic septicemia vaccine (Yasin *et al*., 2011). Guava leaves extract showed the ability to increase the survival chances of mice against the antigen exposure which might be attributed to the increased production of antibodies against the antigen.

Conclusion: The data suggests that Guava leaves extract has immunostimulatory effects on both components of immune system i.e. cellular and humoral immunity. The immunostimulatory effects are characterized by increase in total and differential leukocyte counts, RBCs count, platelets count, and haemoglobin content in healthy mice. The Guava leaves extract also prevented the cyclophosphamide induced myelosuppression and increased delayed type hypersensitivity, which suggested the immunostimulatory effects on cell mediated immunity. The immunostimulatory effects on humoral immunity were evidenced by reduction in mice lethality ratio and increased antibody titer. Further studies are required to identify and isolate the active ingredients which are responsible for rendering the immunostimulatory effects.

Conflict of interest: The authors declare no conflict of interest.

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