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Effect of methanolic extract of *Citrus limetta* peel on cellular and humoral immune response in mice

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Abstract: *Citrus limetta* is well known for its anti-inflammatory, antimicrobial, antifungal, antidiabetic and antioxidant properties. Methanolic extract of *Citrus limetta* (MECL) was used to assess cellular and humoral immune responses in mice by carrying out cyclophosphamide-induced neutropenia, delayed-type hypersensitivity (DTH), carbon clearance assay, haemagglutination assay (HA) and mice lethality assay. Methanolic extract of *Citrus limetta* peel was administered orally to mice in two doses 200mg/kg and 400mg/kg.The extract treated groups showed improvement in neutropenia induced by cyclophosphamide and improvement in the WBC profile. Skin thickness was significantly ($P<0.05$) higher in 200mg/kg and 400mg/kg groups in comparison to control in DTH. The phagocytic index was significantly ($P<0.05$) more in 400mg/kg group in carbon clearance assay. Mice were vaccinated with hemorrhagic septicemia vaccine before challenge with *Pasteurella multocida* for mice lethality test. Percentage mortality was decreased in 400mg/kg treated group in comparison to negative control Antibody titre response to sheep red blood cells was significantly ($P<0.05$) higher with dose 400mg/kg in HA. Results suggested the effectiveness of the methanolic extract of *Citrus limetta* as an immunostimulating agent.

Keywords: Methanolic extract, *Citrus limetta*, cellular, humoral, immune

INTRODUCTION

Medicinal plants contain constituents that are reported as potential candidates for their immune-boosting effect (Khodadadi, 2016). *Citrus limetta* peel is well known for its anti-inflammatory, antimicrobial, antifungal, antidiabetic and antioxidant properties (Perez *et al*., 2010). Pharmacological studies have shown that Citrus limetta possesses antithrombotic actions and anti-inflammatory activities due to inhibition of platelet cyclooxygenase and lipoxygenase (Morón *et al*., 2007; Nogata *et al*., 1996). Phytochemical study shows that saponin, flavonoids, tannins, and alkaloids are present in the methanolic extract of Citrus limetta peel. It contains limonoids and flavonoids (hesperidin, naringerin) which are considered to possess anticancer and anti-inflammatory activities (Middleton, 2000). Peels of Citrus limetta show marked antibacterial and antifungal activity against Salmonella typhimurium, Pseudomonas aurogenosa, Micrococcuss aureus, Microsporumcanis, Trychophyton mentagrophytes and Candida albicans using disc diffusion method. Methanol, ethanol, hot water, cold water and acetone extracts show antibacterial and antifungal activities (Khushwaha *et al*. 2012). It was hypothesized by taking in consideration the anti-inflammatory action that Citrus limetta peel extract may suppress or potentiate immune response. This project aimed to assess the effect of methanolic extract of *citrus limetta*peel on cellular and humoral immunity using an animal model and after treating with low and high doses of extract of *citrus limetta.*

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MATERIALS AND METHODS

Albino mice were purchased from the Department of Theriogenology UVAS, Lahore. 5 to 6 mice weighing 25 to 30g were used and were placed in the animal house of UVAS into stainless steel cages with all the suitable conditions maintained. All mice were provided with basal diet and water all over the experiment. All the ethical matters about handling and experiment use of animals were considered according to institutional guidelines of ethical committee of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan (Letter no. DR/734).

Collection of plant and preparation of extract

Fruit of *Citrus limetta* (sweet lemon) was bought from the local market of Lahore. Plant was identified from Botany Department GC University Lahore. Samples were stored in GC Botany Department Herbarium having voucher no. GC. Herb Bot. 3440. Fruit peel was separated from fruit after washing. Then the peel was dried under shade for 30 days until hard and brittle. Then, dried peels were ground mechanically using electric stainless steel blender. Peel powder weighing 100g was extracted with 900ml of methanol in a soxhlet extractor. The rotary evaporator was used under decreased pressure of 22-26mm Hg at 45°C for making extract more concentrated after complete evaporation. The extract was kept in the refrigerator at 4°C (Kundu *et al*., 2011).

Chemicals

Cyclophosphamide (Cyclomide) in powder form was purchased from Pharmedic laboratory Pvt Ltd. Phosphate buffer saline (PBS) from Bio plus Fine Research Chemicals Madison (USA). *Pasteurella multocida* strain was taken from the Department of Microbiology, UVAS, Lahore. Vaccine (Hemorrhagic septicemia vaccine) against *P. multocida* was taken from VRI (Veterinary Research Institute) Lahore, Pakistan**.**

Delayed type hypersensitivity assay (DTH)

A total of 20 mice were used for DTH assay. Four groups were made for DTH assay. Group A (negative control) received phosphate buffer saline (PBS) intraperitoneally, Groups B and C received 200mg/kg and 400mg/kg methanolic extract of *Citrus limetta* peel (MECl) respectively. Group D (positive control) was administered cyclophosphamide 150mg/kg. For this assay two areas right and left side of mouse skin were selected for sensitizing and challenging dose of antigen Dinitro chlorobenzene (DNCB) respectively. On 2nd day of the test, 2% DNCB in a dose of 0.1ml as sensitizing dose mixed in acetone was used on 4cm^2 on right side of the skin of all mice in treated and control groups. Afterwards, a challenging dose of 0.2ml of DNCB was used to left side as challenging dose on the skin of mice. Then, after a period of 24hr, 48hr and 72hr skin thickness was measured with Vernier calipers (Sajid *et al*. 2007).

Cyclophosphamide induced Neutropenia

After weighing, 15 mice were distributed into 3 groups. In Group A PBS was administered orally for 13 days. Group B and C were given a methanolic extract of *citrus limetta* peel 200mg/kg and 400mg/kg orally to each mouse for 13 days. On the 10th day, a blood sample was taken from heart, differential leukocyte count (DLC) and total leukocyte count (TLC) were done earlier to cyclophosphamide administration. Then the neutropenic dose 150mg/kg of cyclophosphamide was given to all groups. On 13th day i.e., three days after cyclophosphamide administration again TLC and DLC were performed. Percent reduction in DLC and TLC in treated groups (before and after) were compared with that of the control group (Asad and Srivathsa 2012).

Carbon clearance assay

A total 15 Mice were divided in to 3 groups, having 5 mice in each group. Group A (control) was given Sodium carboxy methyl cellulose 1.0% mixed with water and administered 0.3 ml/mouse for 5 days. Group B and C were administered a methanolic extract of *citrus limetta* peel 200mg/kg and 400mg/kg orally of *citrus limetta* orally for 5 days. After 48 hours, carbon ink suspension in dose 10µl/gm body weight was injected in the tail vein. Blood samples were collected from the retro-orbital vein at time 0 and 15min, 25-µl sample was dissolved with Na_2CO_3 solution 0.1% (2ml) and then its absorbance was evaluated at 660nm (Jayathirtha and Mishra 2004).

Mice lethality test

Preparation of Pasteurella multocida culture

Brain heart infusion (BHI) 37g was suspended in distilled water 1000ml. Heating was done to dissolve medium after soaking the mixture for 10 minutes. pasRevival of culture (*P. multocida*) pathogenicity was done.

PBS was used for the reconstitution of *P. multocida* culture. Two mice were taken and 0.2ml of this preparation was administered to each mouse subcutaneously. It resulted in death of both mice. Post mortem of mice was done and heart, kidney, liver and spleen were separated. These organs were preserved after cutting them into small pieces. Small piece of heart was taken and incubated for 24hrs on a petri dish having Brain heart infusion (BHI) at 37°C.

Procedure

Five groups A, B, C and D were designed having 5 mice in each. Group A was given PBS whereas Group B and C were administered methanolic extract of citrus limetta (MECl) 200mg/kg and 400mg/kg orally for 21 days. Group D was administered positive control (Cyclophosphamide). Control and treated groups were immunized with a 0.2 ml HS vaccine on 7th and 17th day of assay 0.2ml (25 × LD50) of *P. multocida* culture containing10^{7}cells/ml subcutaneously was used to challenge mice on the 21st day of the test. The mortality ratio was determined after keeping mice under observation for 72 hours (Sudha *et al*. 2010).

Haemagglutination Assay

Preparation of sheep red blood cells (RBCs) for immunization

Blood was taken from the jugular vein of sheep. Blood was transferred from syringe to the vacutainer tube and mixed to avoid clotting.0.5ml of blood was taken in falcon tube and was suspended in 2ml ice cold phosphate buffer saline. Then it was centrifuged at 1000rpm temperature 4°C for 7minutes. After centrifugation supernatant was discarded and then the RBCs were washed with ice cold PBS twice. After washing again the supernatant was discarded and cells were suspended in PBS and the bottom of tube was gently tapped. Then by using neubauer chamber number of cells per ml were adjusted by diluting with PBS.

Procedure

Mice were distributed into 4 groups. Treated groups (methanolic extract of *citrus limetta* 200mg/kg and 400mg/kg) were given extract orally for 28 days, while PBS was administered to the mice in the negative control. Cyclophosphamide was administered to the positive control. Groups treated with plant extract and control

groups were injected intraperitoneally with sheep's red blood cells (0.5×10^9) cells/ mouse mixed with PBS on the 14th and 21st days. Samples of blood were withdrawn from all mice treated and control groups on the last day of the test (i.e. 28th day). Antibody titre was calculated using a 96 well microtitre plate.

For HA titre 96 well microtitre plate was taken. PBS 50µl was added to each well. The 50µl of mice serum was added to the first well of column plate was tapped to mix the sample. From this well of mixture containing sample and PBS 50µl was taken with micropipette and added to the next well till the 10^{th} well. Last two wells served as control containing no serum. Then 1% sheep RBC solution was added to each well as antigen. Then incubate for two hours without disturbing the plate. Highest dilution showing haemagglutination was considered as haemagglutination titre. Values of HA titre of treated groups were compared with control groups (Bin *et al*., 2003).

STATISTICAL ANALYSIS

The results from the experiment were analyzed by on way ANOVA (Analysis of Variance) followed by multiple comparison LSD (Least significance difference). Software used was SPSS version 16, SPSS Inc and Chicago, IL, USA).values were significant at P<0.05.

RESULTS

Delayed type hypersensitivity

Thickness of skin was observed after application of DNCB compared in cyclophosphamide treated (positive control), negative control (PBS), 200mg/Kg and 400mg/Kg MECl treated group. Alteration in skin thickness of mice was observed on 24 hours, 48 hours and 72 hours after the application of DNCB. The significant difference (P<0.05) in an increase of skin thickness was seen in mice with MECl 200mg/Kg and 400mg/Kg in comparison to negative control respectively (fig. 1).

Cyclophosphamide induced neutropenia assay

After comparing DLC and TLC values it was concluded that the percentage decrease in DLC and TLC was higher in the control in comparison to the treated groups. A dose-dependent decrease in neutropenia was observed in treated groups in comparison to the control group. The maximum reduction in TLC and DLC was shown by the negative control. Improvement in the TLC and DLC profile were observed after the administration of MECl peel. There was a 12.8% reduction in neutropenia in the control group. The percentage reductions were 9.4% and 5.8% in 200mg/kg and 400mg/kg treated group respectively. There was more decrease of TLC 70% in the control group in comparison to MECl administered doses i.e. 200mg/kg and 400mg/kg 49.3% and 44.8% (table 1).

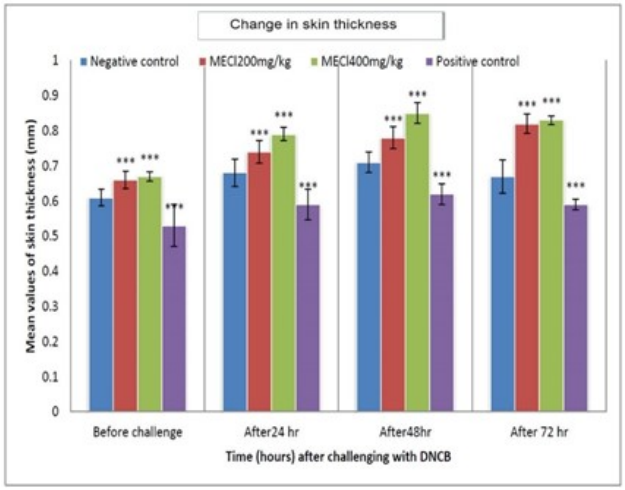

Fig. 1: Comparison between overall mean thickness values of skin thickness of mice in negative and treated groups. Difference was significant at P<0.05. Data were analyzed by ANOVA followed by multiple comparison, LSD

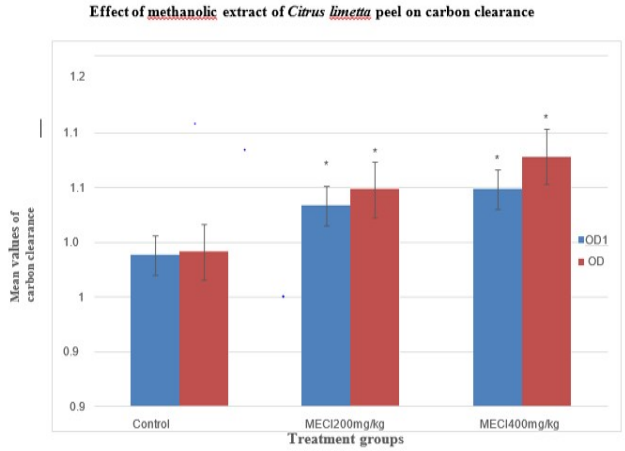

Fig. 2: Comparison of optical density values of control (CMC treated) and MECL (methanolic extract of *Citrus limetta* peel) 200mg/Kg and 400mg/Kg treated groups. Values were expressed as mean ± SE, n=5. P<0.05 as compared to negative control.

Carbon clearance assay

Phagocytic activity was assessed by performing a carbon clearance assay. Two doses of 200mg/Kg and 400mg/Kg were used and absorbance was noted at time 0 and 15 min after the injection of Indian ink suspension. A significant difference (P<0.05) was observed in enhancement in phagocytic index at 200mg/Kg MECl in comparison to the negative control. There was a more significant difference (P<0.05) in an increase in the phagocytic index at 400mg/Kg MECl compared to control (fig. 2).

Mice lethality test

This experiment was done to investigate role of MECL on the humoral immune response. Injection of *Pasteurella*

multocida to control group animals made 100% mortality within 72 hr of administration. No mortality was observed in the vaccinated control group. While all mice died in the non-vaccinated and positive control (Cyclophosphamide). In 200mg/kg MECl there was a 20% mortality ratio. On the other hand, 400mg/kg dose of MECl caused no mortality of mice (table 2).

Effect of methanolic extract of Citrus limetta Peel on Hemagglutination titre

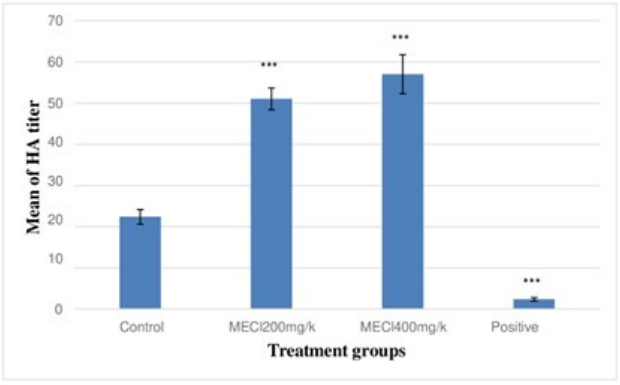

Fig. 3: Total antibody titre of negative control (normal saline). Positive control (cyclophosphamide) and treated (20mg/kg, 400mg/kg), Values were expressed as mean ± SE, n=5. Data were significant at P<0.05.

Haemagglutination Assay

Haemagglutination titre (HA) of negative control (PBS) was compared with treated groups (MECL 200mg/Kg and 400mg/Kg). A significant difference ($p<0.05$) was observed in an increase in titre of 200mg/kg dose of MECL in comparison to negative control. There was a more significant difference ($p<0.05$) in an increase in HA titre in 400mg/kg dose of MECL comparison to the negative control (fig. 3).

DISCUSSION

The objective of this project was to assess the role of methanolic extract of *Citrus limetta* peel on immune system of mice. DTH assay is used for the investigation of cellular immunity. Dinitrochlorobenzene (antigen) induces delayed-type hypersensitivity when applied to the skin and makes dinitrophenyl protein by reacting with skin (Sajid *et al*., 2007). This protein is involved in causing T cell activation which results in lymphokine formation. These lymphokines cause attraction for more scavenger cells toward the area of administration (Kuby 1997). T cells can maintain graft survival (Tan *et al*., 2013). A dose-dependent increase in skin thickness due to the administration of MECL this could be due to the increased activity of macrophages to the site of application of antigen. The results of present study correlate with the findings (Li *et al*., 2007).

Cyclophosphamide induced neutropenia is another assay used for determination of cellular immune responses in mice. Cyclophosphamide is an antineoplastic and immunosuppressive drug. It causes alkylation of DNA by producing breakage of strands and cross-linking. It affects the formation and function of DNA and triggering a decrease in neutrophils (Zuluaga *et al*., 2006). Neutropenia is a side effect of cancer therapy (Navarro *et al*., 2014). Organ infection like muscles, heart, CNS, and increases in susceptibility of the pancreas (Donadieu *et al*., 2011). Increase or decrease in WBS show vital role in immune system because they are responsible for recognition of pathogen and helpful for immune system. WBCs are involved in destruction of infectious microbes by phagocytosis (Ismail and Asad 2009). This study showed that gradually increasing the dose of methanolic extract of *Citrus limetta* peel resulted in a decrease in the effect of neutropenia and TLC caused by cyclophosphamide administration. This may be due to the stimulation of macrophages releasing colony-stimulating factor and interleukin 1.

Carbon clearance was used to evaluate the influence of MECl on reticuloendothelial (RES) cells. These reticuloendothelial systems consist of phagocytic cells. RES cells have a vital role in the removal of particles from the blood. When Indian ink is administered phagocytic cells of the reticuloendothelial system remove Indian ink from the blood. Macrophages engulf the colloidal Indian ink. The rate of carbon clearance from blood is called the phagocytic index (Gokhale *et al*., 2003). The phagocytic index was increased by the gradual enhancement of MECl treated groups. The augmented phagocytic index indicates the enhancement of the effect of MECl on the reticuloendothelial system.

In mice, lethality test humoral response is investigated before immunized with Haemorrhagic vaccine (HS) before administration of *Pasteurella multocida* culture and then the mortality ratio is calculated. *Pasteurella multocida* is infection producing mediator and it extends by airway. Vaccination is used to produce a humoral response (Finco *et al.,* 2001). This assessment is used to determine the production of antibodies under the influence of MECL. If a drug can produce enough antibodies to counter the pathogen then mice can survive. Otherwise, mice could not survive (Rishi *et al.,* 2002). Existence of mice was due to the capability of drug to form antibodies (Slifka *et el* 1998). In this study, effect of the methanolic effect of *Citrus limetta* peel (MECl) on mortality ratio was observed. In the Control group, there was a 40% mortality ratio. On the other hand positive control mortality ratio of 100%. In the lower dose of MECL, the mortality ratio was 20%. While there was no death of bacteria at a higher dose. The mortality ratio in treated groups has shown that antibody formation is increased causing less mortality. This could be due to the

Table 1: Effect of MECL on Cyclophosphamide-induced neutropenia

Treatment Groups	Total Leukocyte count			Neutrophils		
	Before	After	% Reduction	Before	After	% Reduction
Group A (Control)	2712±115.6	752±113.6	70	11.02 ±0.59	9.6±0.72	12.8
Group B (MECl 200mg/Kg)	4786±250***	2421±77	49.3	31.46±4.73*	34.42±4.23***	9.4
Group C (MECl 400mg/Kg)	5508±143.2***	3038±731	44.8	30.9±10.87*	32.7±3.30***	5.8

Values are represented as mean values ± standard deviation, n=5. ***P<0.001, *P<0.05 when compared to the control group, Data were analyzed by one way ANOVA following multiple comparison LS

Table 2: Effect OF MECL (Methanolic Extract of *Citrus Limetta* Peel) 200mg/Kg and 400mg/Kg treated groups on mice lethality

Groups	No. of Mice dead				% mortality ratio
	Between 0-24hr	Between 24-48hr	Between 48-72hr	No. of mice died after 72hr	
Non-vaccinated	5	0	0	0	100%
Vaccinated+ Normal saline	0	0	0	0	0
MECl 200mg/Kg	1	-	-	1/5	20%
MECl 400mg/Kg	-	-	-	0/5	0%
Cyclophosphamide	3	1	1	5/5	100%

increased production of antibodies against *Pasteurella multocida* after vaccination.

HA is an investigational test for a humoral immune response. It also proves the effect of mice lethality assay. Antibodies are secreted by B cells in response to antigen. These antibodies neutralize the effect of antigen. Differentiation of B cells into plasma cell secreting antibodies is a pathway of humoral response by which it works. These antibodies play a defensive role against the antigen. Antibody work as an effect or of humoral reaction by attaching to antigen and counteracting it or assisting its removal by forming latex through agglutination that is further readily ingested by phagocytic cells (Slifka *et al* 1998). In haemagglutination assay sheep RBCs were used as antigens. Haemagglutination titre was determined for control and MECl treated groups. Titre was gradually increased in both lower and higher doses of *Citrus limetta* extract treated groups. This higher titre could be due to the increased production of antibodies IgM and IgG from B cells (Seung, 2013).

After considering and analyzing our results, it is concluded that *Citrus limetta* peel extract along with its potential effects as an anti-inflammatory, analgesic, antibacterial, antiviral, antidiabetic and anthelmintic has also shown immunostimulatory effects on cellular and humoral immune response.

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