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Research Article

In vitro and *in vivo* evaluation of immunomodulatory activity of methanol extract of *Momordica charantia* fruits

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ABSTRACT

The fruits of *Momordica charantia* have been traditionally used in India and other Asian countries. The methanol extract of *Momordica charantia* fruits (MCM) was evaluated for immunomodulatory activity using *in vitro* and *in vivo* methodologies. Effect of extract was evaluated at various concentrations (832 – 6.5 µg/ml) for secretion of mediators like nitric oxide, superoxide, lysosomal enzyme and myeloperoxidase activity of isolated murine peritoneal macrophages. The extract showed stimulation of nitric oxide, lysosomal enzyme and myeloperoxidase activity. The extract was also evaluated for *in vivo* phagocytic activity by carbon clearance assay in mice and it showed significant increase in the phagocytic index at 100, 200 and 400mg/kg dose. The effect of the extract on delayed type hypersensitivity (DTH) and antibody titre assay were evaluated in ovalbumin immunized mice. MCM showed no significant effect on DTH response but significant stimulation of antibody titer at 200 and 400mg/kg dose. The effect of the extract in cyclophosphamide induced myelosuppressed mice was not significant. The results suggest that the extract stimulated non-specific murine immune system, both *in vitro* and *in vivo*.

Keywords: Macrophages; nitric oxide; phagocytosis; ovalbumin; antibody

INTRODUCTION

The immune system evolved to protect the host from potentially pathogenic agents including microorganisms (viruses and bacteria), parasites, and fungi; to eliminate neoplastic cells; and to reject non-self components. The structural and functional alterations of the immune system may lead to immunosuppression, which may modify the host defense mechanisms against infection, cancer, and induction of abnormal immune responses resulting in allergy and autoimmunity[1]. Immunostimulant drugs are required to overcome the immunosuppression induced by drugs or environmental factors and immunosuppressants are required where there is undesired immunopotentiation. There is strong requirement of the drugs which can boost immune system to combat the immunosuppressive consequences caused by stress, chronic diseases like tuberculosis, conditions of impaired immune responsiveness (e.g. AIDS) etc[2].

Momordica charantia, a member of the Cucurbitaceae family, is known as bitter melon, bitter gourd, balsam pear, karela, and pare. It grows in tropical areas of the Amazon, East Africa, Asia, India, South America, and the Caribbean and is used traditionally as both food and medicine. The plant is a climbing perennial with elongated fruit that resembles a warty gourd or cucumber. The seeds, fruit, leaves, and root of the plant have been used in traditional medicine for microbial infections, sluggish digestion and intestinal gas, menstrual stimulation, wound healing, inflammation, fever reduction, hypertension, and as a laxative and emetic[3]. Clinical conditions for which *M. charantia* extracts (primarily from the fruit) are currently being used include diabetes, dyslipidemia, microbial infections, and potentially as a cytotoxic agent for certain types of cancer[4].

Although constituents of Momordica have not been definitively determined, research indicates the primary constituents responsible for the hypoglycemic properties are charantin, insulin-like peptide (plant-insulin), cucurbutanoids, momordicin, and oleanolic acids[5]. P-insulin is structurally and pharmacologically similar to bovine insulin and is composed of two polypeptide chains held together by disulfide bonds[6]. *Momordica charantia* also has numerous other constituents including proteins (momordin, which may have anticancer properties), glycosides, saponins, and minerals[3]. It is also rich in vitamins A and C and beta-carotene, as well as the minerals iron, phosphorus, and potassium[7]. The objective of the present study was to prepare and evaluate the methanol extract of *Momordica charantia* fruits (MCM) using *in vitro* and *in vivo* immunomodulatory

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activity in murine immunopharmacological system.

MATERIALS AND METHODS

Plant material and Preparation of extract

Fruits of *Momordica charantia* were purchased from local supplier in Jan. 2008 and authenticated by botanist of the Institute. The voucher specimen (No. 2008/01/01) was deposited in the herbarium of the Institute. The fruits were cut into small pieces and dried at controlled temperature 45^{0}C and powdered. The powder was defatted with petroleum ether (60-80^{0}C) and then extracted with boiling methanol under soxhlation to give MCM. The extract was filtered and vacuum dried.The MCM extract was tested for presence of phytochemicals [8].

Experimental animals

Swiss albino mice were obtained from Haffkine Bio-pharmaceuticals Ltd., Mumbai. The animals were acclimatized for 10 days before being used for the experiments. They were housed in a room with controlled temperature (23±2^{0}C) and a 12-h light/ 12-h dark cycle. The animals were fed with standard pellet diet and water *ad libitum.* The experimental protocols were approved by the Institutional Animal Ethics Committee of institute and conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

Chemicals

Ovalbumin, Freund's complete adjuvant (FCA), bovine serum albumin (BSA), nitroblue tetrazolium (NBT) and tetramethyl benzedrine/hydrogen peroxide (TMB/H_2O_2) were procured from Bangalore Genei, India. Streptomycin, penicillin, Roswell Park Memorial Institute (RPMI) 1640 medium and HEPES buffer were procured from Himedia Pvt. Ltd. India. Fetal bovine serum (FBS) and Phytohemagglutinin-M (PHA) were procured from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Isolation of peritoneal macrophage and culture conditions

Peritoneal macrophages were isolated from mice which were injected intraperitoneally (i.p.) with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, and 100µg/ml streptomycin (complete RPMI). The macrophage count was determined by using hemocytometer and cell viability was tested by trypan-blue dye exclusion technique. Then the cells were adjusted to required cell count per ml and plated on a 96-well flat-bottom culture plate (Tarsons Products Pvt. Ltd., India) and then incubated for 2 h at 37^{0}C in a 5% CO_2 humidified incubator. After removing the nonadherent cells, the mono-layered macrophages were treated with MCM extract (832-6.5µg/ml) dissolved in complete RPMI medium containing 20% DMSO and maintained for 24 h at 37^{0}C in a 5% CO_2 humidified incubator[9]. Following *in vitro* assays were performed on these incubated cells. PHA (10µg/ml) was used as a positive control. All the experiments were performed in triplicate.

Nitrite assay

Nitrite accumulation was used as an indicator of nitric oxide (NO) production in the medium as per the procedure described earlier[10]. Cell-free supernatant (50µl) from 24h incubated macrophages (5x10^5 cells/ml) was mixed with 50µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. The optical density (OD) was measured at 540 nm with a microplate reader (ELX800MS, BioTek Instruments Inc., USA). Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) for nitrite release was calculated as the nitrite concentrations ratio of the treated and control macrophages.

NBT dye reduction assay

The NBT dye reduction assay was carried out as described previously. Briefly, 50µl of 0.3% NBT solution in PBS (phosphate buffered saline, pH 7.4) was added to the 24h incubated cells (1×10^6 cells/ml) with MCM extract, and the mixture was further incubated in CO_2 incubator. After incubation for 1h, the adherent macrophages were rinsed vigorously with complete RPMI medium, and washed four times with 200µl Methanol. After air-drying, formazan-deposits were solubilized in 120µl of 2M KOH and 140µl of DMSO. After homogenization of the contents of the wells, the OD was read at 630 nm by using a microplate reader. Stimulation index (SI) was calculated as the OD ratio of the treated and control macrophages[11].

Cellular lysosomal enzyme activity

The cellular lysosomal enzyme activity of macrophages was evaluated by measuring acid phosphatase activity as described earlier. Briefly, 24h after incubation of macrophages with MCM at 37°C in 5% CO_2, the supernatant was removed by aspiration and 20µl of 0.1% Triton X-100 (Himedia, India) were added to each well. A fter 15 minutes incubation, 100 µl of 10 mM *p*-nitrophenyl phosphate (*p*NPP) and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. Further the plates were incubated for 1h and 0.2 M borate buffer (150 µl, pH 9.8) was added. The OD was measured at 405 nm by using a microplate reader. The Phagocytic stimulation index (SI) was calculated as the OD ratio of the treated and control macrophages[11].

Myeloperoxidase activity assay

Myeloperoxidase activity was evaluated on isolated macrophages as per the earlier procedure[12]. Briefly, 24h incubated macrophages (5x10^{5}cells/ml) were washed three times with fresh complete RPMI medium. Then the mixture (100 µl) of *o*-phenylenediamine (0.4 g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well. The reaction was stopped after 10 min using 0.1 N H_2SO_4 and OD were measured at 490 nm. The myeloperoxidase stimulation index (SI) was calculated as the OD ratio of the treated and control cells.

In vivo phagocytic activity by carbon clearance assay

Phagocytic activity of MCM extract was determined as per the method described earlier[13]. Mice were divided into five groups, of six each. The control group received vehicle (0.5% NaCMC – sodium carboxy methyl cellulose). Mice in the treatment groups were

administered with orally MCM extract (50, 100 200 and 400mg/kg) suspended in vehicle daily for 20 days. Colloidal carbon solution, Rotring ink® (Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) was via tail vein to each mouse 24 h after last dose. Blood samples were drawn from retro-orbital plexus under ether anesthesia at 2 and 15 min after injection. Blood (25µl) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes OD was recorded at 660 nm. The phagocytic index (K) was calculated by using following equation:

K= (ln OD1-ln OD2) / (T_2-T_1) ,Where OD1 and OD2 are the optical densities at times T_1 and T_2, respectively[10].

Immunization and treatment

Mice were divided into 4 groups of six each. The control group received vehicle (0.5% NaCMC); while mice in the treatment groups were administered with the MCM extract (50, 100 200 and 400mg/kg, p.o.) in vehicle daily for 20 days. On 14th day the animals were immunized subcutaneously with ovalbumin (3mg) dissolved in normal saline emulsified with equal volume of FCA.

Delayed type hypersensitivity (DTH) response

To assess the DTH response, mice were challenged subcutaneously with 25µg ovalbumin in 25µl normal saline in the left hind footpad 7 days after the immunization. The right hind footpad was injected with 25µl vehicle and served as control. The increase in footpad thickness was measured 24 h after the challenge with the help of a digimatic caliper (Mitutoyo Corporation, Japan)[14].

Detection of serum antibody response

Blood were collected from mice through retro-orbital plexus after seven days of immunization and serum were separated under centrifugation. Serum antibody titers for quantification of serum IgG to ovalbumin were estimated by ELISA as described earlier[14]. Flat bottom polystyrene plates were coated with 12.5µg of ovalbumin dissolved in 100µl of sodium carbonate buffer (pH 9.6) at 4°C for 12 h. The coated plates were washed three times with phosphate buffer saline (0.15M, pH 7.2) containing 0.05% TWEEN-20 (PBS-Tw). The wells were incubated with 100µl of 1% BSA in sodium carbonate buffer at 37°C for 1 h. Serial dilutions of mouse serum samples in PBS-Tw were prepared and 100µl was incubated with coated wells for 1 h at 37°C. After washing, diluted (1:2000) antimouse IgG conjugated with peroxidase (100µl) was added and the plates were incubated at 37°C for 1 h. The enzyme activity was determined by addition of TMB/H_2O_2. The enzyme reaction was stopped by addition of 50µl, 8N sulphuric acid and the absorbance was measured at 450nm. Endpoint antibody titers were expressed as log2 of the reciprocal of the highest dilution of the test serum samples showing three times more OD as compared with control samples.

Cyclophosphamide induced Myelosuppression

This experiment was studied according to the procedure described earlier with some modifications[15]. Mice were divided into five groups of six each. The control groups (vehicle and negative) received vehicle (0.5% NaCMC); while animals in treatment groups were given the MCM extract (50, 100, 200 and 400mg/kg, p.o.) in vehicle daily for 19 days. On 17th, 18th and 19th day of study all the animals except in the vehicle control group were injected with cyclophosphamide (25mg/kg, i.p.) 1 h after administration of the extract or vehicle. Blood samples were collected on day 20 and total white blood cell (WBC) count was determined using hematology analyzer (Arcus, Diatron, Wien Austria).

Statistical Analysis

Results are expressed as Mean ± SEM. Data was analyzed by one way ANOVA followed by Dunnet's multiple comparisons test with $P<0.05$ as the criteria for significance.

RESULTS

Extraction

The yield of the methanol extract of *Momordica charantia* fruits (MCM) was 22.5% w/w and stored at 4^0C till further use. The extract showed presence of phytosterols, saponins, proteins, alkaloids, glycosides and flavonoids.

Nitrite assay on isolated peritoneal macrophages

The nitrite level (nitric oxide) produced in cell culture supernatants was measured at 24 h of treatment, showing that MCM extract induced nitrite production in statistically significant higher ($P<0.05$) at 416µg/ml (SI 1.43), and 208µg/ml (SI 1.78) concentrations than controls in all three experiments performed. PHA (positive control) also showed Significant increase ($P<0.05$) in nitrite release (SI 2.36) (Fig. 1).

***In vitro* phagocytic assay on NBT dye reduction and cellular lysosomal enzyme activity**

The *in vitro* phagocytic effects of different concentrations of MCM extract on the reduction of NBT dye and cellular lysosomal enzyme activity of macrophages are presented in Fig. 1. The effect of MCM was not significant on NBT reduction but significant stimulation ($P<0.05$) was observed in case of lysosomal enzyme activity at 208µg/ml (SI 1.68) of MCM extract. Positive control, PHA showed significant stimulation ($P<0.05$) of NBT reduction (SI 1.73) and lysosomal enzyme release (SI 2.19).

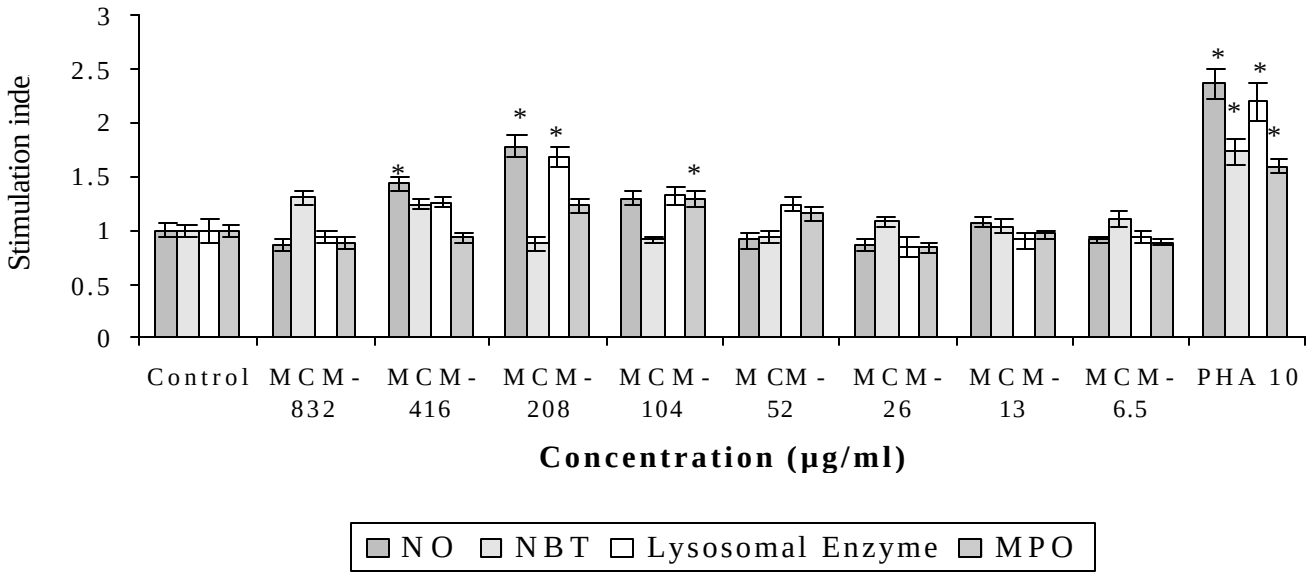
Myeloperoxidase activity assay

The effect of MCM extract on myeloperoxidase activity of macrophages is presented in Fig. 1. The extract showed significant ($P<0.05$) stimulation of myeloperoxidase activity of macrophages at 104µg/ml (SI 1.29) as compared to control wells. Positive control, PHA showed significant stimulation with SI value 1.59.

***In vivo* phagocytic activity by carbon clearance assay**

Macrophages accomplish nonspecific immune function through phagocytosis. *In vivo* phagocytic activity of MCM extract was determined by the carbon clearance assay in mice. The results of this assay are presented in Table 1. The phagocytic index (K) for MCM extract was Significantly higher ($P<0.01$) at 100mg/kg (42%), 200mg/kg (50%) and 400mg/kg (55%) dose levels as compared to control group.

Fig. 1: *In vitro* effect of MCM on release of nitric oxide, NBT reduction, lysosomal and myeloperoxidase activity of isolated macrophages



*The data plotted represent Mean ± SEM (n=3). * P<0.05 and ** P<0.01 vs control group.*

Table 1: *In vivo* effects of MCM extract on phagocytosis (phagocytic index), DTH reaction and antibody titre in ovalbumin immunized mice

Treatment group (dose mg/kg)	Phagocytic index (K)	DTH in mm ($x10^{-2}$)	Antibody Titre (Log 2 of reciprocal of dilution)
Control	0.062±0.004	21.34±1.75	11.98±0.49
MCM (50)	0.067±0.008	24.61±1.88	12.64±0.26
MCM (100)	0.088±0.005**	25.94±3.20	13.14±0.43
MCM (200)	0.093±0.004**	27.02±1.62	14.31±0.56*
MCM (400)	0.096±0.005**	28.85±2.13	14.48±0.65**

*The data is expressed as Mean ±S.E.M; n=6. *P<0.05 and **P<0.01 vs control group*

DTH response in ovalbumin immunized mice

To examine effect of MCM on cellular immune system, its activity was investigated as DTH reaction to ovalbumin immunized mice. The DTH response i.e. difference in footpad thickness of mice is shown in Table 1. MCM have shown to no significant increase the DTH response to ovalbumin at all the tested dose levels.

Detection of serum antibody response to ovalbumin

Humoral response to ovalbumin was studied by ELISA antibody titer assay. Mice treated with different doses of the MCM extract showed an increase in the antibody titer in a dose dependent manner. There was significant increase in serum antibody titer at 200mg/kg (14.31) (P<0.05) and 400mg/kg (14.48) (P<0.01) of MCM extract compared to control group (11.98) (Table 1).

Cyclophosphamide induced Myelosuppression

There was significant reduction (P<0.01) in total WBC count of cyclophosphamide (25mg/kg) treated mice (6.02 $x10^3$ cells/cmm) as compared to vehicle control group (14.43 $x10^3$ cells/cmm). No significant increase in total WBC count was observed with MCM plus cyclophosphamide treated groups as compared to cyclophosphamide alone treated group. The values of total WBC count for MCM (50, 100, 200, 400mg/kg) with cyclophosphamide (25mg/kg) treated groups were 7.22 $x10^3$; 7.85 $x10^3$, 8.37$x10^3$ and 9.38$x10^3$ cells/cmm respectively.

DISCUSSION

Immunomodulation through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy[16]. There is a growing interest in identifying herbal immunomodulators ever since their possible use in modern medicine has been suggested[17]. The main objective of the study was to investigate the immunomodulatory effects of methanol extract of *Momordica charantia* (MCM).

Macrophages have been known to play an important role in the host protection against a wide range of tumors and microorganisms. Macrophages also presents antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out non-specific immune responses. There has been great interest in reactive nitrogen intermediates, nitric oxide (NO), because of its antibacterial and antitumor

effect[18]. NO mediates diverse functions, including vasodilatation, neurotransmission and inflammation [19]. A very high NO production indicates increased phagocytosis and bactericidal activity, which is supported by the data, presented in Fig. 1. Macrophages play an important role in defense mechanism against host infection and in killing tumour cells. Higher reduction of NBT dye by MCM represents a higher activity of oxidase enzyme, reflecting stimulation of phagocytes in proportion to intracellular killing. For lysosomal enzyme activity, the transformation of *p*-NPP to coloured compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytisis [20]. Phagocytosis of particles by macrophages is usually accompanied by a burst of oxidative metabolism allowing the generation of reactive oxygen species which can be detected through an assay based on the reduction of NBT [21]. The effect of various concentrations of MCM on the reduction of NBT dye and lysosomal enzyme activity response of macrophages were studied for phagocytic assay. MCM extract appeared to produce phagocytic stimulation without dose response relationship in lysosomal enzyme activity evaluation.

Myeloperoxidase, a heme protein secreted by neutrophils and macrophages, which uses the oxidizing potential of H_2O_2 to convert chloride ion into hypochlorous acid (HOCl). A potent bactericidal agent, HOCl is a critical component of host defenses against invading bacteria, fungi, and viruses [22]. The increase in the stimulation index of myeloperoxidase by the exposure of MCM extract indicates enhanced defense capability of these cells to pathogenic organisms. The release of immune mediators from murine peritoneal macrophages was significantly stimulated by the exposure of the extract. Murine isolated peritoneal macrophages incubated with the MCM extract at different concentrations ranging between 832 – 6.5 µg/ml for 24h, showed a significant activation of macrophages by modulating the secretion of various mediators including nitric oxide (NO), lysosomal enzyme and myeloperoxidase activity. This suggests that MCM can effectively strengthen innate immunity against foreign particles [23].

The process of phagocytosis involves certain body cells, known as phagocytes, which ingest and removes microorganisms, malignant cells, inorganic particles and tissue debris [24]. Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defense. Macrophages are an integral part of the immune system, acting as phagocytic, microbicidal and tumoricidal effector cells [25]. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response[18]. In view of the pivotal role played by the macrophages, MCM extract was also evaluated for its *in vivo* effect on macrophage phagocytic activity. The increase in carbon clearance i.e. phagocytic index by MCM reflects the enhancement of phagocytic function of mononuclear macrophage and thus non-specific immunity. This indicates that MCM was able to activate murine peritoneal macrophages, and hence phagocytic assays *in vitro* and *in vivo*.

The effect of the MCM extract on cell-mediated immunity (CMI) was evaluated through delayed-type hypersensitivity reaction to ovalbumin, a T cell dependant antigen. As the effect of the extract was not significant at all the tested dose levels, we can say that it may not be acting through CMI. The TB cell cognate interaction provides an optimal signal for B cell differentiation and antibody production towards T-dependent antigen (ovalbumin)[26]. The augmentation of the humoral immune response to ovalbumin by MCM, as evidenced by increase in the antibody titre in mice indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis.

A high degree of cell proliferation renders the bone marrow a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppressive therapy especially with cyclophosphamide. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leucopenia [27]. Concomitant treatment of MCM extract in CYP treated mice resulted in non-significant increase in total WBC count of mice in dose dependent manner till 400mg/kg dose. This suggests that further studies at higher dose levels and/or therapeutic treatment studies needs to be carried out to evaluate its further effects in myelosuppressive conditions. The immunomodulatory activity of methanol extract of *Momordica charantia* fruits could be attributed to various phytoconstituents present in it. The fraction specific studies of the extract can clarify the active constituent(s) responsible for immunomodulatory activity.

CONCLUSION

The studies have demonstrated non-specific immunostimulating properties of the methanol extract of *Momordica charantia* fruits in various *in vitro* and *in vivo* experimental methods. This suggests its therapeutic usefulness in immunocompromised conditions.

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