**IN VITRO EVALUATION OF IMMUNOMODULATORY ACTIVITY OF ETHANOLIC EXTRACT OF MORINGA CONCANENSIS NIMMO**

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**ABSTRACT**

**Objective:** The present study was undertaken to evaluate immunomodulatory activity of ethanolic extract of Moringa concanensis Nimmo.

**Methods:** Immunomodulatory activities were determined by in vitro models-plaque-forming cell assay, nitric oxide (NO) radical scavenging activity, inhibit RAW 264.7 macrophage cell line from generating harmful NO induced by lipopolysaccharide (LPS) and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

**Results:** Showed significant (P<0.01) phagocytic effect on human neutrophils in the parameters studied. Scavenged nitric oxide radical by ethanolic extract of Moringa concanensis inhibition 3% and isolated compound scavenged nitric oxide radical inhibition 44% respectively compared to control group exerted 52% decrease of nitric oxide radical respectively concentration at 0.1,0.01 and 0.0 mg/ml LPS in murine macrophage, RAW 264.7 cells culture medium induced a high release of NO but pretreatment with Moringa concanensis extract and isolated compound resulted in inhibition of NO production by 29,37,95 % and 32,44,96 % at concentrations of 0.5, 0.1 and 1.0 mg/ml compared to LPS-induced sample. Ethanolic extract and isolated compound significantly stimulate RAW 264.7 cell viability (P<0.001) by MTT assay.

**Conclusion:** The present experimental finding demonstrated that isolated compound has superior immunomodulatory activity than the ethanolic extract of Moringa concanensis this effect presumably due to the greater ability of an isolated compound to boost the innate and adaptive immune system.

**Keywords:** Moringa concanensis, Ethanolic extract, Immunomodulatory, Plaque forming cell

**INTRODUCTION**

A wide vary of medicative plants square measure used as AN extract for raw medication and that they possess varied medicative properties. a number of these medicative square measure measures collected in smaller quantities by the native communities and folks healers for native use; several different raw medication square measure measures collected in giant quantities and listed within the market because the material by several herbal industries [1]. The system is concerned within the etiology moreover as pathophysiologic mechanisms of the many diseases. It is often modulated and this involves induction, expression, amplification, or inhibition of any half or part of the reaction [2]. Immunomodulation may be a procedure which will alter the system of an organism by interfere with its functions; if it leads to an improvement of a reaction, it’s named as an immunostimulatory drug that primarily implies stimulation of non-specific system. Medicinal drug implies primarily to cut back resistance against infections, stress and will occur on account of environmental or chemotherapeutical factors. Immunostimulation and immunological disorder each got to be thought of to control the traditional immunologic functioning. Therefore each immunostimulating agents and immunosuppressing agents have their standing; therefore hunt for higher agents exerting these activities is changing into the sphere of major interest everywhere the planet. many Indian medicative plants and varied ‘Rasayana’ are claimed to possess Immunomodulatory activity [3].

**MATERIALS AND METHODS**

The whole plant of Moringa concanensis was collected from native Mandleshwar, Dist Khargone, Madhya Pradesh, India. The plant was known and genuine by Dr. S K. Mahajan, Ex. Faculty member, biological science Department of Govt PG College, Khargone (M. P.). A
voucher specimen (Ref. No. SKM/PGC/Herbarium/2017/A-1)) has been deposited at the division herbarium.

**Preparation of extracts**

About 500g of the dried plant powder was placed in the soxhlet apparatus (Perfert, India) and subjected to extraction using ethanol, extracts were filtered and the filtrate was evaporated using a vacuum evaporator (Perfert, India) under reduced pressure at ± 50 °C temperature. The crude extract obtained after evaporation was stored in desiccators. After extraction with a remaining solvent residue of the plant was discarded and the extract was weighed.

**Experimental animals**

Albino mice (Swiss) of either sex were used in the present study. The animals were fed with commonplace pellet diet, water spontaneously, and maintained beneath commonplace atmosphere conditions (22±5 °C with twelve h of light/dark cycle). All experimental protocols have been approved by the Institutional Animal Ethical Committee of BN College of Pharmacy, Bhopal Nobles University, Udaipur (Reg. No B70/PO/Re/S/05/CPSEA) (Approval Number of IAEC is 15/BNPC/IAEC/2018).

**Antigen**

Fresh sheep blood was collected from a local slaughterhouse in sterile Alsever’s solution (1:1 proportion). Sheep red blood cells (SRBCs) were washed thrice in pyrogens free traditional saline and centrifuged at 2500-3000 rpm for 10 min. The supernatant was removed with Alsever’s solution (1:1 proportion). Sheep red blood cells (SRBCs) were prepared from it in the RPMI-1640 medium. For PFC assay, the SRBC prepared from it in the RPMI-1640 medium. For PFC assay, the SRBC has been prepared at a density of 5 x 10^8 cells/ml in PBS. One milliliter of SRBC in the medium along with 0.5 ml of diluted rabbit serum complement (1:10 diluted with normal saline) was added to 1 ml of spleen cell suspension. Cuningham chambers were prepared using glass slide, coverslips, and double-sided tape. The chambers were loaded with a known volume of assay mixture, sealed with petroleum jelly, and incubated at 37 °C for 1 h. The plaques were counted under a light microscope and expressed as PFC per 106 spleen cells.

**Evaluation of nitric oxide (NO) radical scavenging activity** [12]

NO generated from sodium nitroprusside (SNP) was measured using the Griess reagent method whereby 0.5 ml of the test sample was added to 0.2 ml of SNP (10 mmol) and 1.8 ml of phosphate buffer (pH 7.4). The reaction mixture was turned into allowed to incubate at 37 °C for 3 h. Thereafter, 1.0 ml of the reaction mixture containing nitrate was pipette and mixed with 1.0 ml of Greiss reagent and allowed to stand for 30 min in diffused light solution. The absorbance of the pink-colored chromospheres was measured spectrophotometrically at 540 nm in opposition to the corresponding clean solution.

**Cell culture**

The murine macrophage, RAW 264.7, cells, were purchased from American Type Culture Collection and cultured in Dulbecco’s modified essential media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37 °C in a 5% CO2 atmosphere. Cells were seeded at a density of 2 x 10^6 onto each well in a 6-well plate for 24 h before drug treatment.

**Measurement of nitric oxide**

The cells were incubated with the test sample at different concentrations (1.0, 0.1, and 0.01 μg/ml). Four hours later, the cells were stimulated with lipopolysaccharides (LPS, 1 µg/ml) except for the control group for 20 h. The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent assay. The absorbance of the pink-colored chromospheres was measured spectrophotometrically at 540 nm against the corresponding blank solution and results were expressed as mmol nitric oxide.

**Mtt assay test** [13, 14]

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of Pavonia odorata roots plant extract and isolated compound on RAW264.7 cell proliferation. The MTT test measures the capability of cells to convert MTT to formazan. The cells were plated in 96-well tissue culture plates at a density of 5 x 10^5 cells/ml in complete DMEM medium and incubated in triplicate in a 96-well plate at a final volume of 100 µl for 24 h at 37 °C and 5% CO2 conditions. The cells were treated with Pavonia odorata plant extract and isolated compound at the final concentrations of 25, 50, and 100 μg/ml were incubated for 24 h at 37 °C and 5% CO2. Then, 10 µl of 5 mg/ml phosphate-buffered saline (PBS) MTT solution was added to each well. After 4 h of incubation at 37 °C, the media and MTT were aspirated, and 100 μl of dimethyl sulfoxide was added to dissolve the yellow MTT tetrazolium salt produced by metabolism to acquire purple MTT formazan salt. The amount of MTT formazan salt produced is proportional to the number of viable cells, and the cell proliferation rate is determined by measuring the absorbance at 570 nm using a microplate reader.

**RESULTS AND DISCUSSION**

**Plaque forming cell (PFC) method**

EEMC and ICMC were evaluated for in vitro immunomodulatory assay, where ICPO showed good immunomodulatory activity with plaque-forming cells (PFC) (table 1). The number of antibody-secreting cells from spleen was determined using plaque-forming cell assay. The effect of ethanolic extract and isolated compound of *Moringa concanensis* on antibody-secreting cells of mouse spleen have indicated that immunostimulation was achieved through humoral immunity. The isolated compound effect was significant (“*P<0.01” compared to control and methanolic extract effect was significant (“*P<0.05”). The results obtained in the PFC given in table 1.
Table 1: Effect of ethanolic extract and isolated compound of *Moringa concanensis* in vitro immunomodulatory assays

| Groups | Treatment | PFC 10⁶ cells | O.D 10*10⁶ |
|--------|-----------|---------------|-------------|
| I      | CONTROL   | 474.8±0.703   | 0.623±0.008 |
| II     | STANDARD  | 624.8±0.703   | 0.831±0.007 |
| III    | EEMC      | 564.5±0.587*  | 0.645±0.007* |
| IV     | ICMC      | 642.0±0.573** | 0.720±0.007** |

Values are expressed as mean±SEM, (n=6). All the groups were compared with control and standard groups using ANOVA followed by Dunnett’s t-test. Significant values are expressed as (p<0.01).

Nitrite scavenging assay

Table 2 and fig. 2 depicts that at 0.01, 0.1, and 1.0 mg/ml, the *Moringa concanensis* ethanolic extract and isolated compound of PO scavenged nitric oxide radicals by 15, 23 and 34 %, and 22,25,40 respectively, compared to control. Vitamin C at concentrations of 0.01, 0.1, and 1 mg/ml exerted 24, 34, and 44 % decrease of nitric oxide radicals, respectively both the sample was the same significant level (**p≤0.001) compared to control. Although ICMC is more effective than EEMC.

Table 2: Percentage of NO inhibition by *Moringa concanensis* ethanolic extract and isolated compound

| Concentration in µg/ml | NO Scavenging by Vit C | NO Scavenging by EEMC | NO Scavenging by ICMC |
|------------------------|------------------------|------------------------|------------------------|
| 0.01                   | 20±1.73                | 15±2.88                | 24±1.15                |
| 0.1                    | 27±2.30                | 23±0.57                | 34±2.30**              |
| 1                      | 52±3.46                | 34±1.73**              | 44±2.88***             |

Inhibition of NO production by the *M. concanensis* ethanolic extract and isolated compound. Data are mean±SEM (n = 3); **p<0.01.

Fig. 1: NO scavenging activity of ethanolic extract *Moringa concanensis* and isolated compound, data are mean±SEM (n = 3); *p<0.05 and **p<0.01

LPS induced nitrite oxide assay

Table 3 and fig. 2 and 3 shows that treatment with LPS induced a high release of NO to the culture medium but pretreatment with *Moringa concanensis* ethanolic extract and isolated compound resulted in inhibition of NO production by EEMC 29, 37 and 95 % and ICMC 32,44 and 96% at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample. ICMC is somewhat more effective than EEMC, but significant (*p<0.01) levels are the same.

Fig. 2: LPS Induced Inhibition of NO production by the *Moringa concanensis* ethanolic extract, data are mean±SEM (n = 3) ***p<0.001
Table 3: LPS Induced release of NO by *Moringa concanensis* ethanolic extract and isolated compound

| Concentrations in µg/ml | LPS | EEMC+LPS | ICMC+LPS |
|------------------------|-----|----------|----------|
| 1                      | -   | 17±1.73*** | 21±2.30*** |
| 0.5                    | -   | 29±1.15**  | 32±2.46*** |
| 0.1                    | -   | 37±4.05**  | 44±1.73**  |
| 0.01                   | -   | 95±1.73    | 96±0.57   |
| 1                      | 100 |           |          |

LPS Induced inhibition of NO by *Moringa concanensis* ethanolic extract and isolated compound Data are mean±SEM (n=3); **p<0.01

![Bar graph showing NO release](image)

**Fig. 3**: LPS Induced Inhibition of NO production by the *Moringa concanensis* isolated compound, data are mean±SEM (n=3) ***p<0.001

MTT assay

Table 4 and Fig. 4 shows the immunomodulatory effect of the methanolic Pavonia odorata roots extract and isolated compound on the RA264.7 macrophage cell line was investigated by MTT assay.

The results prove that *Moringa concanensis* stimulates RAW264.7 cell proliferation in a dose-dependent manner. Cell viability significantly increased (p<0.001) with mean viable cell percent±SEM values of EEMC 137±4.04 and ICMC 158±2.88 at concentration 100µg/ml, ICMC is more cell viability than EEMC.

Table 4: Percent cell viability of ethanolic extract and isolated compound *Moringa concanensis* and isolated compound their various concentrations by MTT assay

| Concentration in µg/ml | Control | MTT+EEMC | MTT+ICMC |
|------------------------|---------|----------|----------|
| 25                     | -       | 110±5.77* | 116±1.73* |
| 50                     | -       | 117±1.73** | 143±0.57*** |
| 100                    | -       | 137±4.04*** | 158±2.88*** |
| 100                    | 100     | **       | **       |

![Bar graph showing cell viability](image)

**Fig. 4**: Each value represents the mean percent±SEM significantly different versus control group, P ≤ 0.05*, P≤0.01**, P<0.001***

Ethanol extracts and the isolated compound was evaluated for in vitro immunomodulatory assay wherever ICMC and EEMC showed smart immunomodulatory activity with plaque-forming cell (PFC) (table 1) the amount of antibody-secreting cells from spleen was firm mistreatment plaque-forming cell assay. The result of the ethanol extract of *Moringa concanensis* and Isolated compound on antibody-secreting cells of mouse spleen have indicated that immunostimulation was achieved through humoral mediated immunity. The humoral mediated immunity includes the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions because the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by
cross-linking to form clusters that are more readily ingested by phagocytic cells [15]. The effect was significant (P<0.01) compared to control. The humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effector of the humoral immune response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. The effect of ethanolic extract of Moringa concanensis and the isolated compound has indicated that immunostimulation achieved through humoral immunity.

Table 2 and fig. 1 shows the dose-response result of the NO scavenging activity of the Moringa concanensis and isolated compound. These results suggest that ethanolic extract of Moringa concanensis and isolated compound posses hydrogen donating abilities to act as an antioxidant property [16]. Fig. 1 depicted that the 0.1,0.01 and 1.0 mg/ml of the M concanensis ethanolic extract scavenged nitric oxide radical by 15,23,34% and isolated compound scavenged NO by 24,34,44% respectively compared to control. Vit C concentration at 0.1,0.01 and 1.0 mg/ml exerted 24,34 and 52% decrease of nitric oxide radical, respectively. The isolated compound shows the more significant inhibit NO production then methanolic extracts.

Fig. 2 and 3 shows that treatment with LPS induced a high release of NO to the culture medium but pretreatment with Moringa concanensis ethanolic extract and isolated compound resulted in inhibition of NO production by 29,37, 95% and 32,44,96% at concentrations of 0.5, 0.1 and 1.0 mg/ml, compared to LPS-induced sample. In macrophages, high concentrations of nitric oxide radical can be converted into peroxynitrates, which cause diverse chemical reactions in a biological system, including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport, and oxidation of biological thiol compounds.[17] The findings of the present study strongly suggest that Pavonia odorata and isolated compound can reduce nitric oxide production by LPS in the RAW 264.7 macrophages.

The immunomodulatory effect of the ethanolic Moringa concanensis extract and isolated compound on RAW264.7 macrophages cell line was investigated by MTT assay. The results prove that the M concanensis and isolated compound stimulate RAW264.7 cell proliferation in a dose-dependent manner. Cell viability significantly increased (**P<0.01) with mean viable cell percent±SEM values of EEMC 110±5.77, 117±1.73, 137±4.04 and ICMC 116±1.73, 143±0.57, 158±2.88 for 25, 50 and 100 μg/ml. All the doses after incubation after 24 h compared with control fig. 4.

RAW 264.7 macrophage cells were used in this study to determine the immunomodulatory activities. The results of this study show that the M concanensis and its isolated compound modulate immunity by increasing RAW 264.7 macrophage cell proliferation in a dose-dependent manner. These findings indicate the significant immunomodulatory effect of both the plant and its active fraction as immunostimulators. These findings importantly show that the isolated compound of Moringa concanensis very suitable candidate for modulating macrophage function and inducing the immune system.

CONCLUSION

This study concluded that the ethanolic extract of Moringa concanensis and isolated compound both possess significant immunomodulatory potential depends on increases antibody-secreting cell of plaque-forming cell and inhibition of NO production of nitric oxide scavenging which confers the immunostimulating activity. Hence, these plant additional scientific attention to realize its potentialities within the field of drugs and health sciences.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFICT OF INTERESTS

The authors hereby declare that they have no conflicts of interest either to disclose

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