Some newer marker phytoconstituents in methanolic extract of *Moringa oleifera* leaves and evaluation of its immunomodulatory and splenocytes proliferation potential in rats

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**OBJECTIVES:** The present study was undertaken to unravel the newer marker phytoconstituents in methanolic extract of *Moringa oleifera* leaves (MOLE) and evaluation of its immunomodulatory and splenocytes proliferation potential in rats.

**MATERIALS AND METHODS:** Hot methanolic extract of MOLE was subjected to gas chromatography-mass spectrometry (GC-MS) analysis. Immunomodulatory potential was studied in four groups of rats following administration of MOLE at 62.5 and 125 mg/kg for 21 days, followed by immunization with *Salmonella typhimurium* “O” antigen and antibody titer determined using indirect enzyme-linked immunosorbent assay kit. Total lymphocytes and T- and B-lymphocytes count were determined in control and after MOLE administration (62.5 and 125 mg/kg) to rats for 42 days. Splenocytes (2 × 10⁶ spleen cells/ml) from MOLE treated rats were harvested and stimulated using concanavalin A and optical density (OD) and stimulation index were determined. Splenocytes from healthy control rats were also collected and treated *in vitro* with different concentrations of MOLE (5, 10, 25, 50, and 100 µg/ml) and concanavalin A to determine effect of MOLE on OD and stimulation index.

**RESULTS:** GC-MS analysis revealed presence of 9,12,15-octadecatrienoic acid ethyl ester, 6-octadecenoic acid, cis-vaccenic acid and 2-octyl-cyclopropano-octanal in MOLE. MOLE at 125 mg/kg increased the antibody titer by 50%. Although there was slight decline in lymphocytes count (total, B- and T-lymphocytes) in MOLE treated rats, percentage of T-lymphocytes was increased nonsignificantly. *Ex vivo* and *in vitro* studies revealed marked increase in OD and stimulation index indicating MOLE-induced splenocytes proliferation.

**CONCLUSION:** GC-MS study revealed four new compounds in MOLE apart from promising its immunomodulatory potential based on humoral immune response, percentage increase in T-lymphocytes count, and induction of splenocytes proliferation.

**KEYWORDS:** Immunomodulation, lymphocytes count, *Moringa oleifera*, phytoconstituents, splenocytes proliferation

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Moringa plant provides a rich and rare combination of zeatin, quercetin, kempferol, and many other phytochemicals. Bioassay-guided analysis of ethanolic extract of leaves showed the presence of two nitrile glycosides, niazinrin and niazirinrin, and three mustard oil glycosides, 4-[(4’-0-acetyl-c-L-rhamnopyloxy) benzyl] isothiocyanate, niazimin A and B. Gas chromatography–mass spectrometry (GC–MS) analysis of methanolic extract of Moringa oleifera leaves (MOLE) and seeds revealed the presence of 16 chemical constituents in leaf extract with 9-octadecenoic acid (20.89%), L- (+) ascorbic acid, 2,6-dihexadecanolate (19.66%), and 14-methyl-8-hexadecenal (8.11%) as major ones while only five in seed extract and these were oleic acid (84%), L-(-)-ascorbic acid, 2,6-dihexadecanolate (9.80%), 9-octadecenoic acid (1.88%), methyl ester-hexadecanoic acid (1.31%). Monoterpenoid compounds (81.8%) in essential oil of M. oleifera extracted by hydrodistillation and analyzed by GC and GC-MS have been reported and its oil had highest percentage (25.2%) of alpha-phellandrene with p-cymene (24.9%). The presence of gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin, and vanillin in aqueous extract of leaves, fruits, and seeds of M. oleifera has also been documented using high-performance liquid chromatography (HPLC) and MS/MS techniques. Alcoholic extract of leaves has been reported to contain 15 components and major ones were hexadecanoic acid, ethyl palmitate, palmitic acid ethyl ester, 2,6-dimethyl-1, 7-octadiene-3-ol, 4-hexadecen-6-yne, 2-hexanone, 3-cyclohexyliden-4-ethyl, E2-dodecenylacetate, hi-oleic safflower oil, and safflower oil.

Immunomodulatory studies on MOLE ethanolic extract in normal and immune-suppressed mice model revealed significant rise (P < 0.05) in phagocytic index and hematological and serum enzyme levels. Moringa leaf powder supplementation has been observed to stimulate immune response in HIV-positive people and lectin present in M. oleifera pods has been reported to modulate the immune system. Many workers observed immunomodulatory effect of alcoholic and hydro-alcoholic extracts of Moringa leaves and roots. The present study was undertaken to investigate the major marker phytoconstituents in methanolic extract of MOLE using GC–MS technique and evaluation of its immunomodulatory potential employing humoral immune response and splenocytes proliferation assays.

Materials and Methods

Plant Material
Leaves of M. oleifera were collected from Veterinary College Campus, Mathura. The identity of the plant material was confirmed by Department of Botany, RBS College, Bichhipuri, Agra, India, based on taxonomic features of whole plant material.

Extraction of Plant Material
Hot-methanolic extract of shade-dried and coarsely powdered MOLE was prepared in Soxhlet apparatus by hot percolation method. MOLE extract was concentrated to dryness using rotary evaporator under reduced pressure and low temperature (<40°C). The extract was kept in air-tight containers and stored at 4°C for further studies.

Phytochemical Studies

Gas Chromatography–Mass Spectrometry Analysis of Crude Methanolic Extract
GC-MS analysis of the crude methanolic extract of MOLE was carried out using GC-MS (Agilent 7890A GC system and 5975C VL MSD) with triple axis detector and column (Agilent HP-5) having length, internal diameter and thickness of 30 m, 0.320 mm, and 0.25 µm, respectively. Suitable GC column conditions were set based on the information available in literature. Injector temperature was set at 270°C, and the pressure in column was 80 kPa. Carrier gas used was hydrogen, and the split ratio was 1:10. Total GC program time was 32.33 min, solvent cut off time 2.5 min, MS start time 2.5 min and MS end time 32.33 min.

Twenty milligrams each of the crude extracts were dissolved in 5 ml of HPLC grade methanol and filtered through 0.22 µm membrane filter. One microliter of the test extract was injected into the system using specific syringe. The identity of the compounds was confirmed by comparing mass spectral data from National Institute of Standards and Technical 2008 library.

Immunomodulation Studies

The experimental protocol was approved by the Institutional Animal Ethics Committee as per the approved Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals. Experimental rats were maintained in the Departmental Animal House and provided ad libitum pelleted feed. Rats had free access to clean drinking water and light and dark cycle of almost 12 h was maintained.

Humoral Immune Response Studies

Feeding of Extract and Immunization of Animals
Twenty adult Wistar rats of either sex weighing between 110 and 125 were randomly divided into four groups of five animals each. Rats of Group I (negative control) received only distilled water; Group II served as positive control and immunized with S. typhimurium “O” antigen. Rats of Groups III and IV were orally administered MOLE at 62.5 and 125 mg/kg for 21 days and immunized with S. typhimurium “O” antigen (1 ml subcutaneously at 3–4 different sites) on day 0, 7, and 15 (day 0 as the 1st day of start of feeding of extract).

Detection of Antibody Titer
Blood samples were collected 15 days after the second booster dose of antigen from retro-orbital plexus of rats.
Serum was separated for determining antibody titer using indirect enzyme-linked immunosorbent assay (ELISA) test using commercially available kits (Bangalore Genei, Bengaluru, India). Optical density (OD) of each well was measured at 450–570 nm using ELISA reader.

**Enumeration of Total Lymphocytes and T- and B-lymphocytes**

Adult Wistar rats (100–120 g) of either sex were randomly divided into three groups of five animals each. Group I served as control while rats of Groups II and III were daily administered MOLE at 62.5 and 125 mg/kg body weight, respectively for 42 days by oral route. Blood samples from rats of all the groups were collected in sterile test tubes containing ethylenediaminetetraacetic acid at 1–2 mg/ml of blood and immediately processed for total lymphocytes count and separation of T- and B-lymphocytes using commercially available nylon wool fiber column (Polyscience Inc., Nulife, USA) employing the standard experimental protocol.

**Splenocytes Proliferation Assay**

*Ex vivo* and *in vitro* effects of MOLE on splenocytes proliferation were tested employing splenocytes proliferation assay.[10]

### Ex vivo studies

Adult rats of either sex weighing 100–120 g were randomly divided into three groups of eight animals each. Rats of Group I (control) were administered triple glass distilled water while those of Groups II and III were orally administered MOLE at 62.5 and 125 mg/kg body weight, respectively, for 42 days. After 42 days, animals of the all the three groups were sacrificed, and spleen were aseptically collected. Splenocytes were harvested (2 × 10⁶ spleen cells/ml) in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum. Two hundred microliters of the cell culture were transferred to each well in a flat bottom culture plate. Two micrograms of concanavalin A, a conventionally used stimulating agent for splenocytes, were added to each well except blank which contained only cell culture. Concanavalin A (2 µg) was added in all the wells except blank. Rest of the procedure was same as described in *ex vivo* studies.

### In vitro studies

MOLE was filtered through 0.2 µ membrane filter (Waters) and different dilutions of the test extract (5, 10, 25, 50 and 100 µg/ml) were added into wells of different rows of culture plate containing 200 µl of spleen cells (2 × 10⁶ cells/ml) in RPMI-1640 medium, except control which contained only splenocytes culture. Concanavalin A (2 µg) was added in all the wells except blank. Rest of the procedure was same as described in *ex vivo* studies.

**Results**

**Phytochemical Analysis**

GC-MS chromatogram of crude methanolic extract of *Moringa* leaves extract [Figure 1] revealed 14 peaks suggesting the presence of 14 different compounds having different functional groups. But the major ones were 9,12,15-octadecatrienoic acid ethyl ester (area %20.50; retention time (RT): 14.248 min), 6-octadecenoic acid (area %20.01; RT: 14.195 min), cis-vaccenic acid (area %19.94; RT: 14.055 min), and 2-octyl-cyclopropaneoctanal (area 9.37%; RT: 14.073 min) as shown in Table 1.

**Figure 1:** Gas chromatography-mass spectrometry chromatogram of *Moringa oleifera* leaves hot methanolic extract

| Peak number | RT (min) | Area % | Library/ID       | Quality assurance |
|-------------|----------|--------|-----------------|------------------|
| 1           | 14.055   | 19.94  | cis-vaccenic acid | 98               |
| 2           | 14.195   | 20.01  | 6-octadecenoic acid | 99             |
| 3           | 14.248   | 20.50  | 9,12,15-octadecatrienoic acid ethyl ester | 99            |
| 4           | 14.073   | 9.37   | 2-octyl-cyclopropaneoctanal | 78          |

MOLE= *Moringa oleifera* leaves, RT=Retention time
**Immunomodulatory Studies**

**Humoral immune response**

Antibody titer against *Salmonella* antigen in rats treated with methanolic extract of *Moringa* leaves is presented in Table 2. Results reveal that compared to the antibody titer of positive control group (640.0 ± 240.0), there was no alteration in antibody titer of rats of in Group III (640 ± 240.0) treated with MOLE at 62.5 mg/kg. At higher dose (125 mg/kg), there was 50% increase in antibody titer (960 ± 160.00); however, the increase was not statistically significant.

**Total lymphocytes and T- and B-lymphocytes count**

Data on effect of test extract on total lymphocytes and T- and B-cells counts in rats are presented in Table 3. Results revealed that compared to the control Group I (75.20 ± 8.13 × 10⁴/cmm), there was 23% decline in total lymphocytes count in animals of Group II (57.80 ± 12.97 × 10⁴/cmm), and 34% in Group III (49.50 ± 5.57 × 10⁴/cmm) but decrease was not statistically significant. Similarly, T-lymphocytes count of Group II (38.20 ± 1.08 × 10⁴/cmm) and Group III rats (35.50 ± 4.55 × 10⁴/cmm) was also found to be lower by 13% and 19%, respectively, compared to the corresponding value of 44.00 ± 4.78 × 10⁴/cmm in animals of control group. However, percentage of T-lymphocytes was higher in Group II (63.00% ± 3.50%) and Group III (72.00% ± 4.83%) compared to that of 58.60% ± 3.35% in control group (higher by 10.74% ± 4.79% and 24.56%, respectively). On the contrary, values of B-lymphocytes in rats of Group II (7.00 ± 0.70 × 10⁴/cmm) and Group III (6.50 ± 1.19 × 10⁴/cmm) were significantly (P < 0.05) lower than in control group (15.20 ± 3.73 × 10⁴/cmm). Compared to the control group, percentage B-lymphocytes count in animals of Groups II and III were lower by 19.78% and 28%, respectively.

**Splenocytes proliferation response**

**Ex vivo effects**

Mean OD and stimulation index values of splenocytes proliferation following pretreatment of rats with MOLE for 42 days at 62.5 and 125 mg/kg are summarized in Table 4. OD values were found to be 0.28 ± 0.06 and 0.31 ± 0.07 in animals of Groups II and III, respectively, and these were 48.93% and 62.76% higher compared to the value of 0.19 ± 0.01 in control group. Although effect of MOLE on splenocytes proliferation was concentration-dependent, OD values did not differ significantly between control and treatment groups. Almost similar trend in stimulation index was also observed as the stimulation index values were found to be 1.46 ± 0.33 and 1.59 ± 0.35 in Groups II and III, respectively.

**In vitro effects**

Following in vitro treatment of splenocytes with MOLE at 5, 10, 25, 50, and 100 µg/ml, mean OD and stimulation index values were determined, and the data are summarized in Table 5. Following treatment of splenocytes with MOLE at 5, 10, 25, 50, and 100 µg/ml, compared to that in control group, mean OD values were found to be increased by 17.55, 18.0, 20.0, 45.21, and 59%, respectively, but these were not statistically significant. Similarly, stimulation index values were also found to be increased by MOLE but the increase was marked only at 50 and 100 µg/ml.

**Discussion**

GC-MS analysis of MOLE in the present study revealed the presence of 14 different compounds in our test extract but the major marker compounds identified were cis-vaccenic acid, 6-ocdecenoic acid, 9,12,15-ocdecatrienoic acid ethyl ester, and 2-octyl-cyclopropanoanotal. Earlier studies by various other workers have also reported that *Moringa* is rich in several phytoconstituents. [1,10-12] GC-MS analysis data of our study have revealed three newer phytoconstituents in MOLE methanolic extract, and these have not been reported earlier by any other researcher. Therefore, further studies are indicated for establishing their structure and also use in drug development program.

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**Table 2:**

| Groups/treatment | Antibody titer |
|------------------|----------------|
| Negative control | 240.0±40.0     |
| MOLE (62.5 mg/kg)+ Ag treatment | 640.0±240.0 |
| MOLE (125 mg/kg) + Ag treatment | 960.0±160.0 |
| Positive control (Ag treatment only) | 640.0±240.0 |

Data presented are mean±SE of five animals in each group. Data with similar superscripts in a column did not differ significantly (P<0.05). SE=Standard error, MOLE=Moringa oleifera leaves, ELISA=Enzyme-linked immunosorbent assay

**Table 3:**

| Lymphocytes   | Control       | MOLE (62.5 mg/kg) | MOLE (125 mg/kg) |
|---------------|---------------|-------------------|-------------------|
| Total lymphocytes | 75.20±8.13    | 57.80±12.97       | 49.50±15.57      |
| T lymphocytes  | 44.00±4.78    | 38.20±1.08        | 35.50±4.55       |
| Percentage of T lymphocytes | 58.60±3.35 | 63.00±4.55 | 72.00±4.83 |
| B lymphocytes  | 15.20±3.73    | 7.00±0.70         | 6.50±1.19        |
| Percentage of B lymphocytes | 18.20±3.20 | 14.60±2.65 | 13.00±1.47 |

Data presented are mean±SE of five animals in each group. Data with different superscripts in a row differed significantly (P<0.05). SE=Standard error, MOLE=Moringa oleifera leaves

**Table 4:**

| Parameters | Control       | MOLE (mg/kg) |
|------------|---------------|--------------|
| OD         | 0.19±0.01     | 0.28±0.06    |
| Stimulation index | -      | 1.47±0.33   |

Data presented are mean±SE of eight animals in each group. Data with similar superscripts in a row did not differ significantly (P>0.05). SE=Standard error, MOLE=Moringa oleifera leaves, OD=Optical density

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Compared to the antibody titer against *S. typhimurium* “O antigen” in rats of negative and positive control groups, antibody titer was found to be increased by almost 50% in MOLE-treated rats (125 mg/kg). Thus, suggesting moderate immunomodulatory potential of MOLE against *S. typhimurium* “O antigen.” Our results on humoral immune response are in agreement with the observations of other researchers who observed significant increase in white blood cells, percent neutrophils, and serum immunoglobulins following treatment with methanolic and ethanolic extracts of MOLE and enhanced humoral immune response in rats and mice.1,11,15,17,19

Compared to the total lymphocytes count of control group, there was 23% and 34% decline in total lymphocytes count in rats of Group II (57.80 ± 12.97) and Group III (49.50 ± 5.57), respectively, treated with MOLE at 62.5 and 125 mg/kg for 42 days but the decline in cells count was not statistically significant. Although T-lymphocytes count in Group II (38.20 ± 1.08) and Group III rats (35.50 ± 4.55) was lower by 13% and 19%, respectively, compared with the corresponding value of 44.00 ± 4.78 in rats of control group, percentage T-lymphocytes were higher in MOLE-treated rats of Group II (63.00% ± 3.50%) and Group III (72.00% ± 4.83%) compared to 56.60% ± 3.35% in control group (higher by 10.74 and 24.56%, respectively). The values of B-lymphocytes were significantly (*P* < 0.05) lower in rats of Group II (7.00 ± 0.70) and Group III (6.50 ± 1.19) compared to those of 13.20 ± 3.73 × 10^3/cm^3 in control group. Following administration of MOLE for 42 days, MOLE possibly seems to adversely affect B-lymphocytes production but increases percentage T-lymphocyte count. Therefore, based on our limited observations, it may not be unreasonable to attribute the moderate increase in humoral immune response to significant increase in percentage of T-lymphocytes, especially over active T-helper cells (T^h^).20

Results of ex vivo and in vitro studies on concanavalin A-induced splenocytes proliferation also revealed moderate and concentration-dependent increase in optimal density and stimulation index; thereby supporting immunomodulatory potential of MOLE may be attributed different phytochemicals including flavonoids, abundance of calcium and vitamin A present in *Moringa*21 which activate lymphocytes,22 enhance immune function and interleukin (IL)-2 production,23 and improve lymphocytes proliferation and antibody production.24 MOLE extract has been reported to inhibit nitric oxide production by macrophage cells by attenuating expression of inducible nitric oxide synthase and production of IL-1β and tumor necrosis factor-α.25 The possibility of immunomodulatory and other pharmacological actions due to the presence of some more marker compounds, which are yet to be unraveled including those being reported by us in the present study, cannot be ruled out. Therefore, further studies are warranted for titration of dose and duration of exposure including mechanism of immunomodulation and cytokines assay, etc.

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### Conflicts of Interest
There are no conflicts of interest.

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