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*Original Article*

# Standardized extract of *Mangifera indica* L. leaves as an antimycobacterial and immunomodulatory agent

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## ABSTRACT

**Background:** *Mangifera indica* L.; the largest fruit-tree found in India, is an important component of indigenous medical systems. *Mangifera indica* L. leaves (MIL) have been reported to possess various biological activities and thus, can be a useful source of substances for drug development. Although the leaves have been traditionally used as antibacterial and immunomodulatory agent, there is a paucity of scientific data in support of their efficacy. The purpose of this study was an in-depth evaluation of antimycobacterial and immunomodulatory activity of a standardized extract of MIL. **Material and Methods:** The hexane extract of *Mangifera indica* L. leaves (HEMIL) was prepared and standardized. Chromatographic characterization of HEMIL was done using validated HPLC and GC-MS/MS technique. HEMIL was evaluated for antimycobacterial and immunomodulatory activity by using various *in vitro* and *in vivo* assays. **Results and Discussion:** HEMIL showed lupeol and stigmasterol content of 21.04 ± 0.03 mg/g and 16.99 ± 0.04 mg/g, respectively; and total terpenoids content of 112.55 ± 2.16 mg LE/g. GC-MS/MS characterization of the extract confirmed the presence of lupeol and stigmasterol and revealed five other phytochemical constituents. The safety of HEMIL was established *in vitro* and *in vivo*. HEMIL showed concentration-dependent inhibition of MTB as evident in REMA and radiorespirometry. HEMIL was also found efficacious in immunomodulatory evaluations using RAW 264.7 cells, human PBMCs, cyclophosphamide induced myelosuppressed mice and SRBCs stimulated mice. **Conclusion:** The promising results not only support the traditional claim of MIL as antibacterial and immunomodulatory agent but also provide data on their use in food supplements for immuno-pharmacological use.

**Key words:** *Mangifera indica* L. Leaves, Triterpenoids, Chromatography, Safety, Efficacy.

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## INTRODUCTION

*Mangifera indica* L. (Anacardiaceae, Mango); the largest fruit-tree found in India,[1] is an important component of indigenous medical systems for over 4000 years.[2] It has been the focus of attention of many researchers searching for natural products as therapeutic agents.[3] Use of *M. indica* leaves (MIL) as a medicinal agent is dated back to as early as 327 BC.[4] The presence of some major groups of phytochemical constituents such as saponins, anthraquinones, terpenoids, etc and therapeutically active components such as mangiferin, stigmasterol, friedelin, lupeol, etc has also been reported from MIL.[5] Although the leaves have been traditionally used as antibacterial and immunomodulatory agent,[6,7] there is a paucity of scientific data in support of their efficacy.

Aqueous extracts of MIL showed significant impact on reproductive functions,[4] wound healing[8] and anti-diabetic activities.[9] Alcoholic extracts of MIL has been found to inhibit TNF-α and IL-1β expression[1] and β-lactamase producing enteric bacterial growth.[10] The extract has also been reported to possess antioxidant, cytoprotective,[3] analgesic, anti-inflammatory,[11] larvicidal and pesticidal[12] activities. Petroleum ether extracts of MIL demonstrated remarkable antiulcer[2] and antileishmanial activity.[13] An acetone extract of MIL has been found to have antibacterial activity.[14]

These studies indicate that MIL possesses numerous pharmacological properties. To date, no reports are available to provide empirical data for their use as an antimycobacterial and as an immunomodulatory agent in order to support their traditional claims. Therefore, keeping the above-mentioned facts in mind, in the present study an in-depth evaluation of antimycobacterial and immunomodulatory activity of MIL was carried out using various *in vitro* and *in vivo* assays.

In a preliminary study, different extracts of MIL were prepared using solvents of varying polarity and screened for antimycobacterial and immunomodulatory potential. Based upon the promising pharmacological effects shown by the hexane extract (inhibition of MTB growth and stimulation of NO production in macrophages), it was standardized and evaluated in detail for its antimycobacterial and immunomodulatory activities using different experimental models. Furthermore, the biological and chemical standardization of the hexane extract of *M. indica* leaves (HEMIL) is reported here for the first time.

## MATERIALS AND METHODS

### Chemicals and reagents

Whatman filter paper (no. 1), Griess reagent, Alsever's solution, MTT, LPS, resazurin sodium salt, streptomycin, vernier calliper, cyclophosphamide, linalool, levamisole, and leflunomide were obtained from Sigma-Aldrich Chemicals (St. Louis, USA). Nylon filter/fibre, hexane, glycerol, DMSO, and $NaNO_2$ were obtained from Merck Specialities (Mumbai, India). Different culture media from Himedia (Mumbai, India) and Gibco Co. (Grand Island, USA); mouse anti-SRBC IgG/IgM ELISA kits from Life Diagnostics, Inc. (West Chester, USA); standard pellet diet for animals from Amrit Feeds Ltd. (Kolkata, India) and radioactive source of carbon (1 μCi $^{14}$C-acetate) from the BRIT (Mumbai, India) were used in the study. Ultra-pure water was obtained using a Milli-Q purification system (Millipore, USA).

### Plant materials and extract preparation

MIL were collected from Varanasi (India) in the month of May and authenticated by Agharkar Research Institute, Pune (Auth 11-198). The leaves were shade dried for a week, kept in an oven preset at 40°C until completely dry, powdered, sieved (mesh 85) and stored in an airtight

container. The quality of MIL was evaluated in terms of physicochemical and phytochemical parameters as per the previously reported methods.[15] HEMIL was prepared by extracting an accurately weighed powdered sample of MIL (one kilogram) with hexane (5000 ml×3) on a horizontal shaker for six hour, standing overnight for 18 h, followed by extraction using an ultrasonic bath for 30 min (at 40-60°C). The mixture was filtered through Whatman filter paper no. 1 and the filtrate was evaporated at 40°C under reduced pressure to obtain HEMIL.

## Standardization of HEMIL

Standardization of HEMIL was carried out in terms of total terpenoids content as previously described.[16] Marker-based standardization of HEMIL was carried out by determining the content of lupeol and stigmasterol (Figure 1) using a validated HPLC method described earlier.[17] Chromatographic characterization of HEMIL was also done using GC-MS/MS technique as given in appendix-supplementary material.

## Bacterial culture and cell line

All procedures were performed in a Biosafety Level III laboratory. RAW 264.7 cells were obtained from NCCS (Pune, India) and maintained in RPMI medium with 10% FBS at 37°C and 5% $CO_2$. The laboratory strain of MTB (H37Rv-ATCC 35838) was grown in Middlebrook 7H9 broth supplemented with ADC and containing 0.05% glycerol at 37°C. Sterile drug solutions and extracts were prepared in DMSO and diluted in the respective media.

## Evaluation of antimycobacterial activity using REMA

REMA was performed as described previously.[18] Briefly, dilutions of HEMIL (3.2-50 µg/ml) were prepared in Middlebrook 7H9 broth (100 µl each) and dispensed in sterile 96 well plates. Streptomycin was used as a positive control. The inoculum containing $5\times10^4$ cells of MTB (100 µl) was added to each well, the plate was covered, sealed with paraffin and incubated at 37°C with 5% $CO_2$ for seven days. After addition of resazurin solution (30 µl, 0.02 % in distilled water, w/v), the plate was incubated for two more days. The MIC and selectivity index was determined as previously described.[18,19] In addition, the total activity was calculated as the ratio of the total mass (mg) of extract and MIC value (mg/ml) as previously described.[19]

## Evaluation of antimycobacterial activity using radiorespirometry

The inoculum containing $5\times10^4$ cells of MTB (100 µl) was transferred into the radiorespirometry vial with 100 µl of $^{14}$C-acetate (1 µCi). To this, 100 µl of HEMIL (6.3-25 µg/ml prepared in Middlebrook 7H9 broth) was added. Streptomycin was used as a positive control. The assembly was incubated at 37°C. Generation of $^{14}CO_2$ was determined daily with a liquid scintillation analyzer (PerkinElmer, USA). The data was presented as CPM for five days.[20]

## Cell viability assay, NO production and myeloperoxidase activity in RAW 264.7 cells

An MTT assay was used to study the effect of HEMIL on the viability of RAW 264.7 cells and to determine the $IC_{50}$ value.[18] The effect of HEMIL and LPS on NO production in RAW 264.7 cells was studied using Griess reagent as described previously.[21] $NaNO_2$ was used as a standard to calculate the nitrite concentration in unknown samples. Myeloperoxidase activity in RAW 264.7 cells was measured in terms of stimulation index (ratio of absorbance of cells treated with HEMIL and control cells) as per the previously reported method.[22]

## Proliferation in PBMCs

PBMCs were separated from the blood sample of a healthy human donor and suspended in RPMI medium with 10% FBS at 37°C and 5% $CO_2$. Proliferation in PBMCs by HEMIL and LPS was studied as described previously.[23]

## Animals, test samples and acute oral toxicity study

The CPCSEA guidelines were followed for the animal study and prior permission was sought from IAEC (BT-130627-01/02). Female Swiss Albino mice (body weight: 21.8 ± 1.9 g) procured from Haffkine's Institute (Mumbai, India) were acclimatized in the animal house for one week prior to the commencement of the study, fed standard pellet diet and water was provided *ad libitum*. The test samples were premixed in 2% DMSO and orally administered to the overnight fasted animals. Blood samples were withdrawn under light ether anaesthesia from the retro-orbital plexus. The safety of HEMIL (oral administration at 2000 mg/kg body weight) was evaluated as per OECD test guidelines no. 420.[24]

## Evaluation of immunomodulatory activity

The immunomodulatory activity of HEMIL was evaluated at low, mid and high doses (100, 250 and 500 mg/kg body weight). Levamisole-25 mg/kg body weight[25] and leflunomide-20 mg/kg body weight[26] were used as positive controls.

## Effect on cyclophosphamide induced myelosuppressed mice (experiment 1)

Myelosuppression was induced by oral administration of cyclophosphamide in distilled water at 40 mg/kg body weight once daily for three consecutive days.[27] The animals were randomly divided into seven groups (six animals each), i.e. sham treated group I (administered with distilled water), cyclophosphamide induced group II-III (administered with distilled water and 2% DMSO respectively), group IV-VI (administered with three different doses of HEMIL) and group VII (administered with levamisole). The test samples were orally administered to the animals for fourteen days. On day fifteen, blood samples were withdrawn and the total blood count was evaluated. The animals were sacrificed by cervical dislocation and relative organ weights for spleen, thymus, liver and kidney were recorded.

## Effect on SRBCs immunized mice (experiment 2)

SRBCs were obtained from fresh sheep blood as described previously.[28] The animals were immunized by intraperitoneal administration of 0.2 ml SRBCs ($1\times10^9$ cells/ml) on day zero and day seven.[28] The animals were randomly divided into eight groups (six animals each), i.e. sham treated group I (administered with distilled water); SRBCs immunized group II-III (administered with distilled water and 2% DMSO respectively), group IV-VI (administered with three different doses of HEMIL), group VII-VIII (administered with levamisole and leflunomide respectively). The test samples were orally administered to the animals for fourteen days. On day fourteen, the thickness of right hind foot pad was measured using a vernier calliper and the animals were challenged with 0.02 ml of SRBCs ($1\times10^9$ cells/ml) in the sub-plantar region. The increase in the thickness of paw was measured on day fifteen and DTH response was determined as described previously.[25,28]

Blood samples were collected on day seven and fifteen (determination of primary and secondary antibody titre) and serum was separated. Estimation of antibody titres from serum was done using standard hemagglutination test[25,28] while serum IgG and IgM levels were determined using ELISA kits.[29] The animals were sacrificed on day fifteen by

**Table 1: Counts per minute (CPM) obtained for $^{14}CO_2$ released by MTB grown in the presence of HEMIL and streptomycin for five days during radiorespirometry**

| Samples | Concentration (µg/ml) | CPM on different days | | | | |
|---|---|---|---|---|---|---|
| | | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 |
| Growth control | -- | 1037.3 ± 44.05 | 2347.0 ± 34.21 | 8353.3 ± 530.74 | 20369.7 ± 1169.50 | 52959.3 ± 2389.41 |
| Streptomycin | 10 | 1057.0 ± 52.97 (0.00%) | 1357.0 ± 28.62[c] (42.18%) | 1202.7 ± 27.81[c] (85.60%) | 1135.7 ± 19.78[c] (94.42%) | 1115.3 ± 42.35[c] (97.89%) |
| HEMIL | 6.3 | 1100.7 ± 43.38 (0.00%) | 1370.0 ± 63.17[c] (41.63%) | 1918.3 ± 72.57[c] (77.04%) | 3060.7 ± 358.59[c] (84.97%) | 3784.7 ± 80.90[c] (92.85%) |
| | 12.5 | 1017.7 ± 32.54 (0.00%) | 1377.0 ± 21.79[c] (41.33%) | 1738.3 ± 112.24[c] (79.19%) | 2590.3 ± 129.38[c] (87.28%) | 7561.0 ± 33.50[c] (85.72%) |
| | 25 | 1071.3 ± 74.67 (0.00%) | 2269.0 ± 31.56[c] (3.32%) | 7418.3 ± 73.20[b] (11.19%) | 14402.0 ± 628.24[c] (29.30%) | 44411.7 ± 350.85[c] (16.14%) |

Data expressed as mean ± SE, n=3. Data of streptomycin and HEMIL treatment is supported with their respective %growth inhibition value in the bracket. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001 when compared with growth control.

**Table 2: Effect of HEMIL at different concentrations on RAW 264.7 cells (NO production and myeloperoxidase activity) and PBMCs**

| Samples | Concentration (µg/mL) | NO production (Nitrite content in µM) | Myeloperoxidase activity (Stimulation index) | PBMCs proliferation (% cell viability) |
|---|---|---|---|---|
| Control | | 0.450 ± 0.0045 | 1.000 ± 0.0000 | 100.00 ± 0.000 |
| LPS | 1 | 7.035 ± 0.1065[c] (15.63 fold) | 1.808 ± 0.0254[c] (80.80%) | 118.70 ± 1.667[a] (18.70%) |
| HEMIL | 2.5 | 0.853 ± 0.0236[c] (1.90 fold) | 1.034 ± 0.0100 (3.40%) | 103.00 ± 0.909 (3.00%) |
| | 5 | 1.043 ± 0.0274[c] (2.32 fold) | 1.111 ± 0.0022[b] (11.07%) | 108.11 ± 0.849[c] (8.11%) |
| | 10 | 1.424 ± 0.0093[c] (3.16 fold) | 1.245 ± 0.0226[c] (24.47%) | 117.76 ± 1.038[c] (17.76%) |

Data expressed as mean ± SE, n=3. Data of LPS and HEMIL treatment is supported with their respective fold increase or %stimulation value in the bracket. [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001 when compared with control.

**Table 3: Effect of HEMIL at different doses on the number of leucocytes, neutrophil cells, haemoglobin and relative weight of the organs of cyclophosphamide induced myelosuppressed mice**

| Groups | Number of leucocytes (thousand cells/µl) | Neutrophil cells (%) | Haemoglobin (g/dl) | Relative organ weight (g/25 g body weight) | |
|---|---|---|---|---|---|
| | | | | Spleen | Thymus |
| I (Normal control) | 9.370 ± 0.1259 | 43.0 ± 1.03 | 15.49 ± 0.083 | 0.137 ± 0.0016 | 0.015 ± 0.0009 |
| II (Cyclophosphamide control) | 3.216 ± 0.0771* | 24.5 ± 0.43* | 9.24 ± 0.226* | 0.030 ± 0.0013* | 0.003 ± 0.0002* |
| III (Vehicle control) | 3.275 ± 0.0348 (0.95%) | 25.0 ± 0.82 (2.70%) | 9.28 ± 0.093 (0.64%) | 0.025 ± 0.0007 (0.86 fold) | 0.003 ± 0.0004 (0.98 fold) |
| IV (HEMIL 100 mg/kg body weight) | 4.943 ± 0.0784[c] (28.06%) | 26.0 ± 0.86 (8.11%) | 9.74 ± 0.073 (7.97%) | 0.062 ± 0.0016[c] (2.10 fold) | 0.010 ± 0.0008[b] (3.53 fold) |
| V (HEMIL 250 mg/kg body weight) | 5.698 ± 0.0782[c] (40.33%) | 31.2 ± 0.65[c] (36.04%) | 11.15 ± 0.085[c] (30.59%) | 0.140 ± 0.0030[c] (4.72 fold) | 0.024 ± 0.0007[c] (8.44 fold) |
| VI (HEMIL 500 mg/kg body weight) | 6.214 ± 0.0319[c] (48.71%) | 36.5 ± 0.76[c] (64.86%) | 12.89 ± 0.086[c] (58.40%) | 0.194 ± 0.0027[c] (6.55 fold) | 0.032 ± 0.0008[c] (11.00 fold) |
| VII (Levamisole 25 mg/kg body weight) | 8.786 ± 0.1802[c] (90.50%) | 41.5 ± 0.76[c] (91.89%) | 15.31 ± 0.063[c] (97.07%) | 0.239 ± 0.0086[c] (8.06 fold) | 0.075 ± 0.0040[c] (25.94 fold) |

Data expressed as mean ± SE, n=6. Data of group III-VII is supported with their respective fold increase or %protection value in the bracket. *P<0.001 when compared with group I; [a]*P*<0.05, [b]*P*<0.01, [c]*P*<0.001 when compared with group II.

**Table 4: Effect of HEMIL at different doses on humoral and cell mediated response in SRBCs immunized mice**

| Groups | Change in paw thickness (%) | Hemagglutination titre ($log_2$ of the titre value) | | Serum IgM level (µg/ml) | Serum IgG level (µg/ml) | Number of PFCs/$10^5$ spleen cells |
|---|---|---|---|---|---|---|
| | | Primary | Secondary | | | |
| I (Normal control) | 1.6 ± 0.15 | 0.0 ± 0.00 | 0.0 ± 0.00 | 10.7 ± 0.77 | 155.0 ± 1.94 | 29.3 ± 3.60 |
| II (SRBCs control) | 8.4 ± 0.59* | 4.0 ± 0.00* | 5.5 ± 0.22* | 140.8 ± 2.69* | 1133.6 ± 28.58* | 280.7 ± 9.35* |
| III (Vehicle control) | 8.1 ± 0.37 (0.96 fold) | 4.0 ± 0.00 (0.00%) | 5.7 ± 0.21 (3.03%) | 138.0 ± 5.21 (-2.12%) | 1137.9 ± 33.13 (0.44%) | 279.2 ± 7.70 (-0.61%) |
| IV (HEMIL 100 mg/kg body weight) | 11.3 ± 0.23 (1.35 fold) | 5.5 ± 0.34[a] (37.50%) | 6.7 ± 0.21[a] (21.21%) | 149.4 ± 1.01 (6.62%) | 1241.1 ± 8.79[c] (10.98%) | 323.8 ± 2.50[c] (17.16%) |
| V (HEMIL 250 mg/kg body weight) | 25.4 ± 0.48[c] (3.02 fold) | 7.7 ± 0.21[c] (91.67%) | 7.7 ± 0.21[c] (39.39%) | 154.3 ± 0.84[c] (10.40%) | 1320.4 ± 2.71[c] (19.09%) | 356.0 ± 5.14[c] (29.95%) |
| VI (HEMIL 500 mg/kg body weight) | 43.8 ± 0.30[c] (5.21 fold) | 8.7 ± 0.21[c] (116.67%) | 9.0 ± 0.26[c] (63.64%) | 162.1 ± 2.27[c] (16.35%) | 1390.5 ± 9.04[c] (26.25%) | 424.7 ± 5.32[c] (57.27%) |
| VII (Levamisole 25 mg/kg body weight) | 108.0 ± 4.02[c] (12.86 fold) | 9.3 ± 0.21[c] (133.33%) | 9.8 ± 0.17[c] (78.79%) | 210.7 ± 2.25[c] (53.75%) | 1346.7 ± 17.34[c] (21.77%) | 665.7 ± 4.33[c] (153.13%) |
| VIII (Leflunomide 20 mg/kg body weight) | -38.3 ± 1.05[c] (-4.56 fold) | 2.3 ± 0.21[c] (-41.67%) | 3.3 ± 0.21[c] (-39.39%) | 122.8 ± 1.42[c] (-13.81%) | 861.9 ± 24.56[c] (-27.77%) | 141.5 ± 3.02[c] (-55.37%) |

Data expressed as mean ± SE, n=6. Data of group III-VIII is supported with their respective fold increase or % stimulation value in the bracket.*$P$<0.001 when compared with group I; [a]$P$<0.05, [b]$P$<0.01, [c]$P$<0.001 when compared with group II.

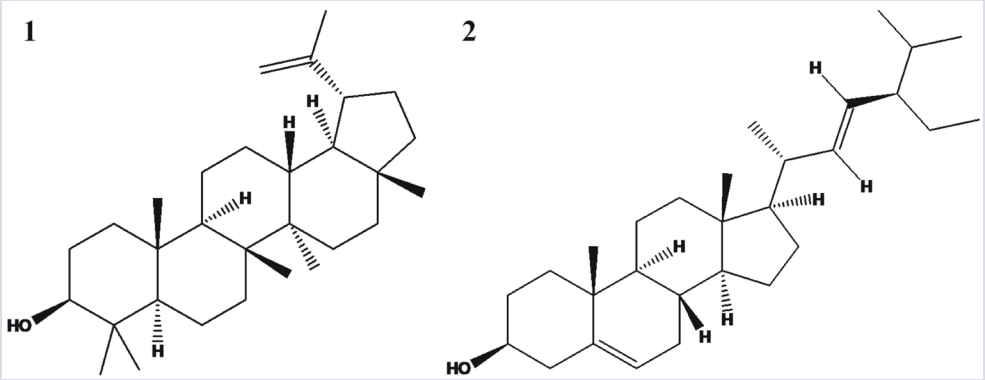



**Figure 1:** Structure of lupeol (1) and stigmasterol (2).

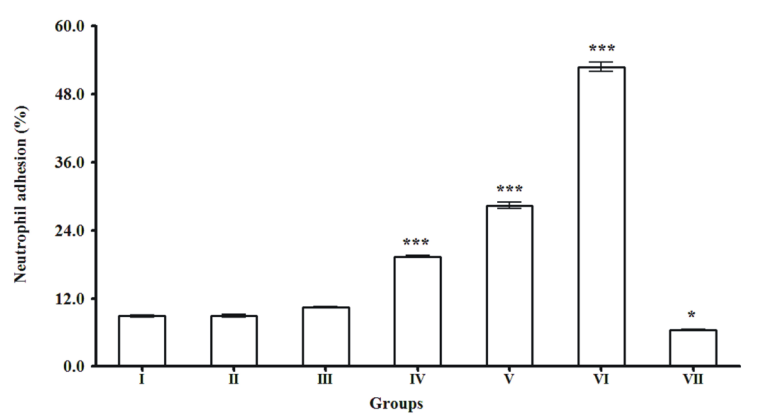



**Figure 2:** Effect of HEMIL on neutrophil adhesion in mice. Group details: I (Normal control), II (Vehicle control), III-V (HEMIL 100, 250 and 500 mg/kg body weight, respectively), VI (Levamisole 25 mg/kg body weight), VII (Leflunomide 20 mg/kg body weight). Data expressed as Mean ± SE, n = 6. [a]$P$< 0.05, [b]$P$<0.01, [c]$P$<0.001 when compared with group I.

cervical dislocation. The spleen from each animal was removed and single cell suspensions were prepared in the RPMI-1640 medium. The number of PFCs/$10^5$ spleen cells was determined as previously described.[30]

### Effect on neutrophil adhesion (experiment 3)

The animals were randomly divided into seven groups (six animals each), i.e. group I-II (administered with distilled water and 2% DMSO respectively), group III-V (administered with three different doses of HEMIL) and group VI-VII (administered with levamisole and leflunomide respectively). The test samples were orally administered to the animals for fourteen days. On day fifteen, blood samples were withdrawn to evaluate neutrophil index and neutrophil adhesion as per the method reported.[28]

### Statistical analysis

GraphPad Prism 5 software version 5.03 (GraphPad Software Inc., California, USA) was used to statistically evaluate the results. Data were analyzed using one-way ANOVA and Tukey's test. A value of $P<0.05$ was considered to be statistically significant.

## RESULTS AND DISCUSSION

### Standardized HEMIL

Quality control parameters for MIL were established (appendix-supplementary material) and HEMIL was prepared. The extraction yield for HEMIL was found to be 2.58 ± 0.07%. Amongst the total terpenoids present (112.55 ± 2.16 mg LE/g of dry extract) in HEMIL, the content of lupeol and stigmasterol were found to be 21.04 ± 0.03 and 16.99 ± 0.04 mg/g respectively. Using GC-MS/MS technique, five other phytochemical constituents namely pentadecanal; hexadecanoic acid, methyl ester; 16-heptadecenal; squalene and lupenyl acetate were also detected in HEMIL.

### Establishment of safety profile of HEMIL in RAW 264.7 cells and mice

The $IC_{50}$ value of HEMIL for RAW 264.7 cells using MTT assay was found to be similar to streptomycin. In this assay, the concentration of HEMIL showing percent cell viability greater than 85% (2.5-10 µg/ml) were opted for subsequent *in vitro* studies. The safety of HEMIL was also established in mice using acute oral toxicity study wherein its oral administration at 2000 mg/kg body weight did not cause any toxicological effects or mortality as compared with the control animals (appendix-supplementary material). Thus, HEMIL can be considered to have a wide margin of safety for oral use in mice.

### HEMIL as an antimycobacterial agent

In REMA, the MIC value of HEMIL and streptomycin were 12.5 and 3.2 µg/ml, respectively. The selectivity index of HEMIL and streptomycin was found to be more than 10, indicating their potential antimycobacterial effect.[19] HEMIL showed a total activity of 2088 ml/g which indicates that the hexane extract derived from one gram of MIL can be diluted to approximately 2.1 l of the solvent and still inhibit the growth of MTB. In radiorespirometry assay, at lower concentrations of HEMIL, the inhibition of MTB growth observed was comparable to streptomycin (nearly 40%) until day two. However, from day three onwards, there was an increase in the CPM. On comparison with the CPM of growth control, significant inhibition of MTB growth was observed at each concentration of HEMIL ($P<0.001$). At the highest concentration, there was an increase in the growth of MTB (Table 1). The reason for this increase has not been established.

The hexane extract of plants from South Africa,[19] India,[31] Mozambique[32] and America[33] have been screened for their antimycobacterial activity. The MIC value of HEMIL was found to be lower than those previously reported from the hexane extract of other plants. Terpenoids and other phytochemical constituents including squalene, β-sitosterol, oleanolic acid, ursolic acid, farnesol, methylic ester of hexadecanoic acid, etc have been reported for their inhibitory activity against MTB.[34] Therefore, the effect shown by HEMIL may be attributed to the terpenoids present in it. These findings need to be confirmed in MDR strains of MTB along with the evaluation of MTB inhibition within macrophages.

### HEMIL as an immunomodulatory agent

Incubation of PBMCs with HEMIL for 72 h showed a significant increase ($P<0.001$) in their proliferative response at 5 and 10 µg/ml concentrations (Table 2). At the 10 µg/ml concentration, HEMIL showed 17.76% stimulation of PBMCs compared to the control and this increase was comparable to the reference compound LPS (18.70% stimulation). This clearly suggests that the extract is non-toxic to the human immune cells and modulates the cellular immune response.[23] Similar results have been published from the crude extracts, flavonoids and terpenoids of *Cuscuta campestris*[35] and flavonoids of *Phyllanthus niruri*.[23]

In addition, HEMIL stimulated the NO production in RAW 264.7 cells in a concentration-dependent manner (Table 2). At 10 µg/ml concentration, HEMIL significantly increased the NO production (3.16 fold) compared to the untreated control ($P<0.001$), although this value was not comparable to NO stimulated by the potent macrophage activator LPS (15.62 fold). HEMIL showed a similar effect on myeloperoxidase activity in macrophages (Table 2). Similar results have been reported for polysaccharides isolated from *Ganoderma lucidum*[21] and the ethanolic extract of *Capparis mooni*.[22] This effect of HEMIL on macrophages suggests its possible role in killing the microbes by inducing the innate immunity against them and significantly increasing the phagocytic activity of the macrophages.[21]

In the present study, a significant reduction in the number of leucocytes, haemoglobin content, percent neutrophils and the relative weight of spleen and thymus ($P<0.001$, Table 3) was observed in myelosuppressed mice as reported previously.[25,27,36] Oral administration of HEMIL significantly increased ($P<0.001$) the above-mentioned parameters in dose-dependent manner and the response to the treatment was found to be similar to that observed with levamisole (Table 3). The effect of HEMIL on the relative weight of spleen and thymus may be correlated to its stimulatory effect on the lymphocytes and hematopoietic cells of bone marrow, which ultimately home in the thymus or spleen.[36] However, this may be temporary and in due course of time normalcy may ensue. Similar immune boosting and immune restorative effects have been reported from the aqueous methanolic extract of *Loranthus micranthus*,[23] ethanolic extract of *Moringa oleifera*[36] and carnosine[27] to overcome the toxic effects of cyclophosphamide (in mice). Stimulation of the hematopoietic system by HEMIL suggests that it is a rich source of phytochemical constituents which can induce the non-specific immunity of granulocytes, macrophages, natural killer cells and complement functions.[36]

The DTH response of HEMIL is represented as percentage difference in Table 4. Treatment of SRBCS immunized mice with HEMIL for fourteen days at three different doses produced 11.3% to 43.8% increase in DTH response. The effect was significant ($P<0.001$) at mid and high doses. Levamisole produced 108.0% increase and leflunomide showed decrease in DTH response by 38.3% ($P<0.001$, Table 4). The data suggests that HEMIL stimulates T-cells, macrophages, monocytes, neutrophils etc, which may result in an enhanced DTH response and increased infiltration of macrophages to the inflammatory site.[28] In a similar manner, HEMIL produced a significant ($P<0.001$) and dose-dependent increase in the circulating primary and secondary antibodies which lead to enhanced hemagglutination titre compared to the control (Table 4).

# APPENDIX - SUPPLEMENTARY MATERIAL

## 1. Quality control data on MIL

**Physicochemical and preliminary phytochemical analysis of MIL**

| Parameters | | Results (%Mean ± SD, n=3) |
|---|---|---|
| Foreign matter | | 0.218 ± 0.0035 |
| Loss on drying | | 12.172 ± 0.0560 |
| Moisture content (%) | | 11.759 ± 0.0559 |
| Ash content | Total | 14.155 ± 0.0923 |
| | Acid insoluble | 4.236 ± 0.0355 |
| | Water soluble | 1.146 ± 0.0177 |
| Extractive value | Ethanol soluble | 7.451 ± 0.0432 |
| | Water soluble | 21.660 ± 0.2138 |
| | Ether soluble | 3.728 ± 0.0258 |
| Phytochemical fractions | Fats and waxes | 2.360 ± 0.0392 |
| | Terpenoids and phenolics | 5.223 ± 0.0720 |
| | Alkaloids | 0.094 ± 0.0015 |
| | Quaternary alkaloids and n-oxides | 13.258 ± 0.2402 |
| | Fibers | 66.327 ± 1.0711 |

**Heavy metal analysis of MIL using ICP-OES technique**

| Details | Content of the heavy metals (µg/g) | | | |
|---|---|---|---|---|
| | Lead | Arsenic | Cadmium | Mercury |
| Permissible limits as per WHO/AYUSH | 10 | 10 | 0.3 | 1 |
| Detection limit of the instrument | 1.0 | 1.0 | 0.25 | 0.5 |
| Heavy metal content in MIL | 8.77 ± 0.041 | Not detected | Not detected | Not detected |

Values are %Mean ± SD, n=3.

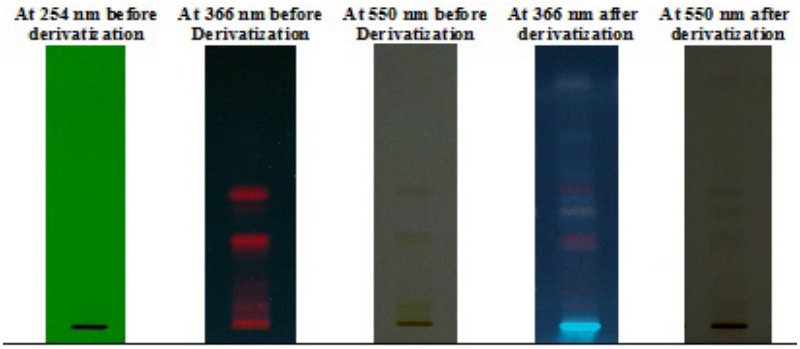
## Chromatographic fingerprint of MIL

**Sample preparation:** The plant powder (2 g) was extracted with ethanol (20 mL), vortex mixed for a minute and sonicated for 20 min followed by filtration through Whatman filter paper (no. 1). The filtrate was used for the development of chromatographic fingerprint using HPTLC and HPLC.

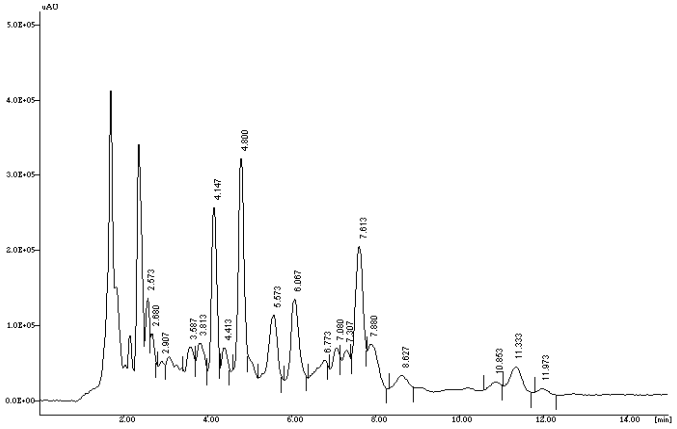
**Chromatographic conditions (HPTLC):** The HPTLC system used consisted of TLC Scanner 4 (CAMAG, Muttenz, Switzerland) supported by winCATS software version 1.4.7 equipped with Linomat 5 sample spotter (CAMAG, Muttenz, Switzerland) and Reprostar 3 system (CAMAG, Muttenz, Switzerland) for photo-documentation. Chromatographic separation of the phytochemical constituents was achieved on HPTLC plates (Merck, Darmstadt, Germany) pre-coated with silica gel 60 F254 (0.2 mm thickness) on aluminium sheet support. The sample (10 µL) was applied to the plate as 8 mm band and at a distance of 15 mm from the edges. One of the walls of twin trough chamber (CAMAG, Muttenz, Switzerland) was lined with Whatman filter paper (no. 1) and the mobile phase (toluene: chloroform: ethyl acetate: glacial acetic acid, 10: 2: 1: 0.03, v/v/v/v) was poured on the same side, in order to soak the filter paper completely with the mobile phase. The chamber was tilted at 45° to distribute the mobile phase equally on the both sides and allowed to saturate for 15 min. The plate was then developed up to a distance of 85 mm. After development, the plate was dried in a current of air at room temperature, derivatized using 10% methanolic sulphuric acid and dried in an oven preset at 110°C for 10 min. For densitometric scanning, the source of radiation was a mercury lamp (366 nm). All the measurements were performed at 22 ± 1°C. Plate was photo-documented at 254 nm (before derivatization), 366 nm (before and after derivatization) and 550 nm (before and after derivatization).

**Chromatographic conditions (HPLC):** Chromatographic analysis was performed using HPLC system (Jasco, Easton, USA) comprising of two PU-1580 pumps (HG-1580-31), rheodyne injector (20 µL loop) and photo diode array detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on C18-column (150 × 4.6 mm, 5 µm, Cosmosil, Nacalai Tesque, USA) using mobile phase acetonitrile: ethanol (40: 60, v/v) delivered at a flow rate of 1 mL/min. After 30 min of equilibration period, the samples were injected into HPLC system. Peaks were recorded at 205 nm.

**HPTLC plate photo of MIL under different wavelengths and plate development conditions**




**RP-HPLC chromatograms as fingerprints of the plant samples at 205 nm**




## 2. Standardization of HEMIL

### HPLC analysis of HEMIL

**Sample preparation:** HEMIL (40 mg) was dissolved in ethanol (10 mL) by vortex mixing, filtered through nylon micro filter paper (0.45 µm) and the filtrate was subjected to HPLC analysis.

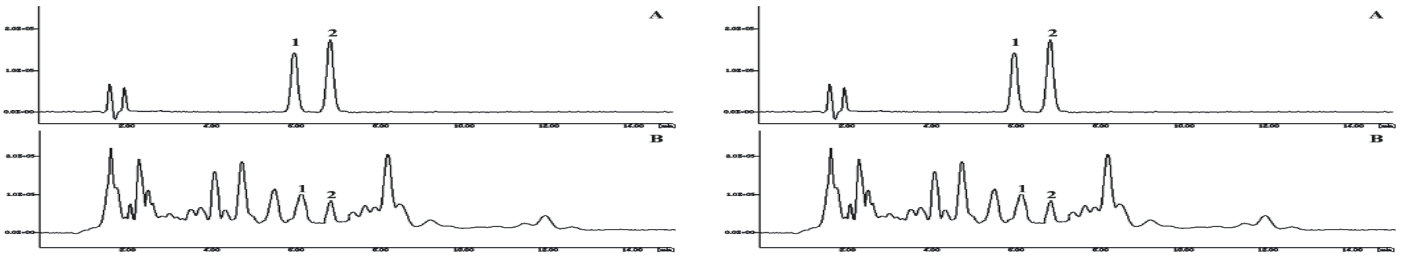
**Chromatographic conditions:** Chromatographic analysis was performed using HPLC system (Jasco, Easton, USA) comprising of two PU-1580 pumps (HG-1580-31), rheodyne injector (20 µL loop) and photo diode array detector (MD-1510). Chromatograms were recorded by means of Jasco-Borwin chromatography software version 1.50. Separation was achieved on $C_{18}$-column (150 × 4.6 mm, 5 µm, Cosmosil, Nacalai Tesque, USA) using mobile phase acetonitrile: ethanol (40: 60, v/v) delivered at a flow rate of 1 mL/min. After 30 min of equilibration period, the samples were injected into HPLC system. Peaks were recorded at 205 nm.

### GC-MS/MS analysis of HEMIL:

**Sample preparation:** HEMIL (100 mg) was dissolved in ethanol (10 mL) by vortex mixing, filtered through nylon micro filter paper (0.45 µm) and the filtrate was subjected to GC-MS/MS analysis.

**Chromatographic conditions:** The analysis was performed using Agilent 7890A/7000 GC-MS/MS system. Separation was achieved using a DB5-MS capillary column (15 m × 0.25 mm, 0.25 µm). Helium was used as a carrier gas with a flow rate of 1.2 mL/min. Sample (2 µL) was injected in splitless mode. GC temperature program was 120°C for 1 min, ramped to 200°C at 10°C/min and finally to 300°C at 4°C/min. The GC injector temperature was 300°C; the transfer line temperature was held at 280°C. The mass spectrometer parameters for EI mode were as follows: ion source temperature: 280 °C; electron energy: 70 eV; filament current: 34.6 µA; electron multiplier voltage: 1300 V; mass analyser: triple quadrupole; mass detector: photomultiplier tube. The total run time was 42 min. The spectra were analyzed in scan mode and the mass range

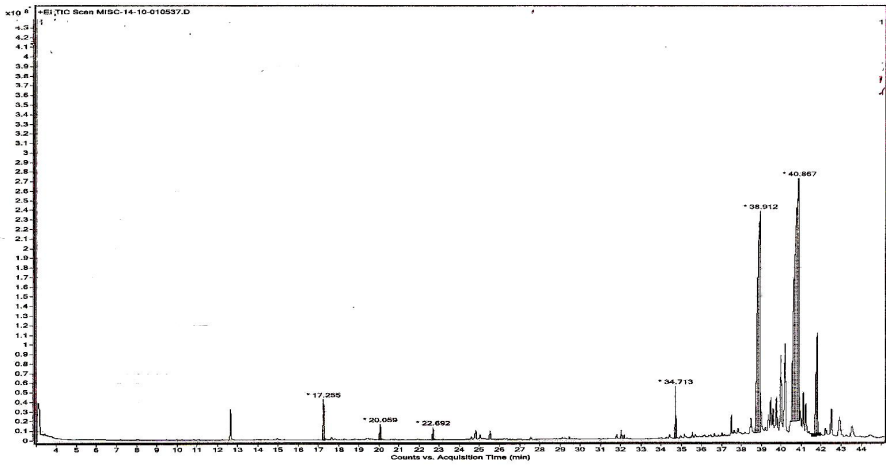
**HPLC chromatograms of standards (A) and HEMIL (B) showing lupeol (1) and stigmasterol (2)**



was m/z 40-500 amu. Compounds were identified in terms of Rt values and mass spectra with those obtained from authentic samples / certified reference material/the NIST library.

## 3. Data on acute toxicity study of HEMIL

**GC chromatogram of HEMIL**




**GCMS-MS CHROMATOGRAM OF SAMPLE (C)**

**Phytochemical constituents detected using GC-MS/MS analysis in HEMIL**

| Name of the compound | $R_t$ | Molecular weight | Molecular formula | High intensity peaks observed at m/z |
|---|---|---|---|---|
| Pentadecanal | 17.255 | 226 | $C_{15}H_{30}O$ | 41, 57, 69, 82, 96 |
| Hexadecanoic acid, methyl ester | 20.059 | 270 | $C_{17}H_{34}O_2$ | 43, 55, 87, 143, 227 |
| 16-heptadecenal | 22.692 | 252 | $C_{17}H_{32}O$ | 43, 57, 68, 82, 96 |
| Squalene | 34.713 | 410 | $C_{30}H_50$ | 41, 69, 81, 95, 121, 137 |
| Stigmasterol | 38.912 | 412 | $C_{29}H_{48}O$ | 41, 55, 69, 83, 95, 133, 255 |
| Lupeol | 40.867 | 426 | $C_{30}H_{50}O$ | 43, 55, 68, 81, 95, 109 |
| Lupenyl acetate | 41.794 | 468 | $C_{32}H_{52}O_2$ | 43, 55, 69, 81, 95, 121, 189 |

**Daily body weight record of the animals from group I (normal control) showing the percent mean difference between consecutive days**

| Animal no. | Body weight (g) on different days | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Mean (n=5) | 20.9 | 21.4 | 21.5 | 21.6 | 21.8 | 21.6 | 21.6 | 21.5 | 21.7 | 22.0 | 22.4 | 22.3 | 22.5 | 22.8 | 23.2 |
| SE | 0.33 | 0.33 | 0.32 | 0.24 | 0.25 | 0.37 | 0.29 | 0.32 | 0.46 | 0.42 | 0.37 | 0.41 | 0.42 | 0.34 | 0.37 |
| % Mean difference | -- | 2.39 | 0.47 | 0.47 | 0.93 | -0.92 | 0.00 | -0.46 | 0.93 | 1.38 | 1.82 | -0.45 | 0.90 | 1.33 | 1.75 |

**Daily body weight record of the animals from group II (vehicle control) showing the percent mean difference between consecutive days**

| Animal no. | Body weight (g) on different days | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Mean (n=5) | 20.7 | 20.8 | 21.2 | 21.0 | 21.2 | 21.5 | 21.8 | 21.8 | 22.3 | 22.2 | 22.3 | 22.7 | 22.9 | 23.1 | 23.5 |
| SE | 0.49 | 0.56 | 0.49 | 0.42 | 0.34 | 0.39 | 0.37 | 0.41 | 0.41 | 0.34 | 0.44 | 0.34 | 0.37 | 0.43 | 0.42 |
| % Mean difference | -- | 0.48 | 1.92 | -0.94 | 0.95 | 1.42 | 1.40 | 0.00 | 2.29 | -0.45 | 0.45 | 1.79 | 0.88 | 0.87 | 1.73 |

**Daily body weight record of the animals from group III (administered with the standardized HEMIL at 2000 mg/kg body weight) showing the percent mean difference between consecutive days**

| Animal no. | Body weight (g) on different days | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Mean (n=5) | 19.8 | 19.9 | 20.0 | 19.9 | 20.2 | 20.3 | 20.6 | 20.9 | 21.0 | 21.1 | 21.2 | 21.1 | 21.4 | 21.6 | 22.1 |
| SE | 0.46 | 0.43 | 0.47 | 0.46 | 0.34 | 0.51 | 0.37 | 0.29 | 0.22 | 0.37 | 0.54 | 0.70 | 0.89 | 0.81 | 0.80 |
| % Mean difference | -- | 0.51 | 0.50 | -0.50 | 1.51 | 0.50 | 1.48 | 1.46 | 0.48 | 0.48 | 0.47 | -0.47 | 1.42 | 0.93 | 2.31 |

**Daily food intake record of the animals from group I-III showing the percent difference between consecutive days**

| Group | Parameters | Different days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| I | Food intake (g)* | 24.5 | 25 | 26 | 25 | 26 | 25 | 26 | 25 | 26 | 27 | 26 | 25.5 | 26.5 | 26 |
| | % Difference | -- | 2.04 | 4.00 | -3.85 | 4.00 | -3.85 | 4.00 | -3.85 | 4.00 | 3.85 | -3.70 | -1.92 | 3.92 | -1.89 |
| II | Food intake (g)* | 23 | 23.5 | 23 | 24 | 24 | 24 | 25 | 25.5 | 25 | 25.5 | 26 | 26 | 27 | 27.5 |
| | % Difference | -- | 2.17 | -2.13 | 4.35 | 0.00 | 0.00 | 4.17 | 2.00 | -1.96 | 2.00 | 1.96 | 0.00 | 3.85 | 1.85 |
| III | Food intake (g)* | 26 | 26 | 26.5 | 27 | 27 | 27.5 | 26.5 | 26 | 25 | 26 | 26.5 | 26 | 26.5 | 26 |
| | % Difference | -- | 0.00 | 1.92 | 1.89 | 0.00 | 1.85 | -3.64 | -1.89 | -3.85 | 4.00 | 1.92 | -1.89 | 1.92 | -1.89 |

**Daily water intake record of the animals from group I-III showing the percent difference between consecutive days**

| Group | Parameters | Different days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| I | Water intake (mL)* | 35 | 35 | 36.5 | 35 | 36 | 35 | 36 | 36.5 | 35.5 | 35.5 | 36 | 36.5 | 37 | 37.5 |
| | % Difference | -- | 0.00 | 4.29 | -4.11 | 2.86 | -2.78 | 2.86 | 1.39 | -2.74 | 0.00 | 1.41 | 1.39 | 1.37 | 1.35 |
| II | Water intake (mL)* | 40 | 38.5 | 38 | 38 | 39.5 | 40 | 40.5 | 40 | 39.5 | 41 | 41.5 | 40.5 | 41 | 42 |
| | % Difference | -- | -3.75 | -1.30 | 0.00 | 3.95 | 1.27 | 1.25 | -1.23 | -1.25 | 3.80 | 1.22 | -2.41 | 1.23 | 2.44 |
| III | Water intake (mL)* | 36.5 | 38 | 37 | 37 | 38.5 | 38 | 39 | 39 | 38.5 | 39 | 40 | 41 | 41 | 41.5 |
| | % Difference | -- | 4.11 | -2.63 | 0.00 | 4.05 | -1.30 | 2.63 | 0.00 | -1.28 | 1.30 | 2.56 | 2.50 | 0.00 | 1.22 |

Group I: Normal control, group II: Vehicle control, group III: Administered with the standardized HEMIL at 2000 mg/kg body weight. *As animals of respective groups were kept in separate cages, record of food intake was cumulative for all the animals (n=5) of respective group.

**Data on mortality record, mortality latency and cage side observation during acute oral toxicity study of HEMIL for fourteen days**

| Groups | Sample details | D/T | Mortality latency* | Symptoms of toxicity** |
|---|---|---|---|---|
| I | Normal control administered with distilled water | 0/5 | -- | No toxic symptoms during the observation period |
| II | Vehicle control administered with 2% DMSO | 0/5 | -- | |
| III | Administered with the standardized HEMIL at 2000 mg/kg body weight | 0/5 | -- | |

D/T = Dead/treated mice. *Time to death (in h) after oral administration. **Changes in skin and fur, teeth, eyes and mucous membrane (nasal) and also autonomic changes (salivation, lacrimation, perspiration, piloerection, urinary volume, breathing abnormalities, abdominal distension and defecation) and alterations to the central nervous system (ptosis, drowsiness, gait, tremors and convulsion).

Similar observations have been reported in the previously published studies.[25] This suggests that HEMIL markedly augments the antibody response to SRBCs in mice[28] which in turn supports its effect on cytokines and cytokine producing cells like macrophages, T-and B-cells which are an essential part of the humoral immunity.[25] The mid and high doses of HEMIL significantly increased the serum IgG and IgM levels compared to the SRBCs control ($P$<0.001, Table 4). This supports the ability of HEMIL to stimulate the B-cells in response to the SRBCs antigen and improve the primary humoral immune response.[34] Similar results have been reported for the polysaccharide from *Angelica sinensis*[29] and aqueous extract of *Gynostemma pentaphyllum*.[37] Augmentation of the humoral response to SRBCs was also evident from the significantly increased ($P$<0.001) number of PFCs in the spleen of mice (Table 4) which can be correlated to the number of B-cells producing SRBC-specific IgM or antigen presenting cells.[30] Comparable data were reported for a polyherbal combination Trikatu[38] and an aqueous extract of *Glycyrrhiza glabra* in combination with Zinc.[28] The trend shown by levamisole and leflunomide on hemagglutination titre, serum immunoglobulin and number of PFCs was found to be similar to the one observed in DTH response.

The effect of HEMIL on neutrophil activation in mice is shown in Figure 2. The mid and high dose of HEMIL significantly increased ($P$<0.001) the neutrophil adhesion as compared to the control. A similar trend was observed with levamisole treatment. On the contrary, leflunomide significantly decreased ($P$<0.05) the neutrophil adhesion. Similar observations on neutrophil adhesion were reported for the gum polysaccharide of *Terminalia bellerica*.[39] The significant increase in the neutrophil adhesion by HEMIL correlates the process of margination of cells in blood vessels and the number of macrophages reaching the site of inflammation.[28] Thus, HEMIL may regulate the synthesis of β2 integrins and secrete the cytokines for stimulation of neutrophils which results in their increased adhesion to nylon fibres.[28,39]

## CONCLUSION

This study validates the potential use of HEMIL in pharmacological applications. Although, it is not possible to single out the most effective constituent of the plant, the findings of this study indicate that terpenoids may play the most significant role in the elucidation of these activities. Further, bioactivity-guided fractionation, isolation, pharmacological and pharmacodynamic studies should be conducted to find lead compound(s) and determine the mechanisms underlying its therapeutic effect.

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## CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

## ABBREVIATIONS USED

**ADC:** Albumin-Dextrose Complex; **ATCC:** American Type Culture Collection; **B-Cells:** B-lymphocytes; **BRIT:** Board of Radiation and Isotope Technology; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals; **CPM:** Counts Per Minute; **DTH:** Delayed Type Hypersensitivity; **FBS:** Foetal bovine serum; **H37Rv:** Virulent strain of MTB; **IAEC:** Institutional Animal Ethics Committee; **IgG:** Immunoglobulin G; **IgM:** Immunoglobulin M; **IL-1β:** Interleukin-1β; **LE:** Linalool Equivalent; **LPS:** Lipopolysaccharide; **MTB:** *Mycobacterium tuberculosis*; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **m/z ratio:** mass to charge ratio; **MDR:** Multi Drug Resistant; **NCCS:** National Centre for Cell Science; **NO:** Nitric Oxide; **PBMCs**: Peripheral Blood Mononuclear Cells; **PBS:** Phosphate Buffered Saline; **PFCs:** Plaque Forming Cells**; RAW 264.7 cells:** Macrophage cell line; **REMA:** Resazurin Microtitre Assay; **RPMI:** Roswell Park Memorial Institute (a medium used during *in vitro* studies); **SRBCs:** Sheep Red Blood Corpuscles; **T-cells:** T-lymphocytes; **TNF-α:** Tumour Necrosis Factor-α.

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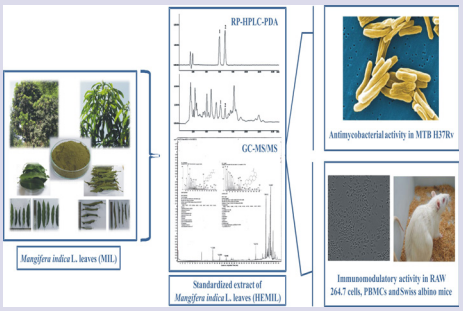
## PICTORIAL ABSTRACT




## SUMMARY

- *Mangifera indica* leaves have been standardized using spectroscopic/chromatographic techniques and their antimycobacterial and immunomodulatory activities have been evaluated.
- Results support their traditional claim and provide data on their use in food supplements.

## ABOUT AUTHOR

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