Metabolic profiling of *Lantana camara* L. using UPLC-MS/MS and revealing its inflammation-related targets using network pharmacology-based and molecular docking analyses

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Supplementary Table S1. XP G scores of *L. camara* top hit compounds in the compound–target network against the most enriched inflammation-associated target proteins.

| Protein kinase C alpha type (4RA4) | Transcription factor p65 (3QXY) | Interleukin-2 (1M49) | Mitogen-activated protein kinase 14 (6HWU) | Proto-oncogene c-Fos (1FOS) |
|------------------------------------|---------------------------------|----------------------|------------------------------------------|-----------------------------|
| Ferulic acid                      | -5.383                          | -2.237               | -5.602                                   | -8.770                      |
| Catechin gallate                  | -9.898                          | -4.660               | -6.664                                   | -9.901                      |
| Myricetin                          | -8.648                          | -3.933               | -6.014                                   | -11.928                     |
| Isoferulic acid                   | -7.710                          | -3.489               | -5.187                                   | -8.206                      |

Supplementary Table S2. Enrichment calculations for the investigated most enriched target proteins

| Enzyme     | Protein kinase C alpha type (4RA4) | Transcription factor p65 (3QXY) | Interleukin-2 (1M49) | Mitogen-activated protein kinase 14 (6HWU) | Proto-oncogene c-Fos (1FOS) |
|------------|------------------------------------|---------------------------------|----------------------|------------------------------------------|-----------------------------|
| AUC-ROC    | 0.992                              | 0.992                           | 0.995                | 0.992                                    | 0.989                       |
| EF(2%)     | 48                                 | 48                              | 50                   | 48                                        | 43                          |
| EF(5%)     | 19                                 | 19                              | 20                   | 19                                        | 19                          |
| EF(10%)    | 9.4                                | 9.4                             | 10                   | 9.4                                       | 9.6                         |
| BED-ROC    | 0.987                              | 0.987                           | 1                    | 0.987                                     | 0.996                       |
| $\alpha = 160.9$ | 0.950                              | 0.950                           | 1                    | 0.950                                     | 0.967                       |
| $\alpha = 8$ | 0.945                              | 0.945                           | 1                    | 0.945                                     | 0.962                       |
| No. of actives | 16                                 | 17                              | 13                   | 16                                        | 23                          |
| Ranked actives | 15                                 | 16                              | 13                   | 15                                        | 22                          |
| RMSD       | 1.172                              | -------                         | 0.386                | 0.558                                     | -------                     |
### Supplementary Table S3. ADME characteristics of *L. camara* top hit compounds

| Compound        | mol_MW  | donorHB | acceptHB | QPlogPo/w | Percent Human Oral Absorption |
|-----------------|---------|---------|----------|-----------|-----------------------------|
| Myricetin       | 318.239 | 5       | 6        | -0.299    | 37.45                       |
| Catechin gallate| 442.378 | 7       | 8        | 0.476     | 36.903                      |
| Ferulic acid    | 194.187 | 2       | 3.5      | 1.375     | 67.296                      |
| Isoferulic acid | 194.187 | 2       | 3.5      | 1.373     | 67.251                      |

### Supplementary Table S4. Brief literature survey on the top scoring *L. camara* constituents as anti-inflammatory candidates.

| Compound          | Inflammation- associated diseases | Model                                | PMID number | Mechanism                                                                                                                                                                                                 |
|-------------------|-----------------------------------|--------------------------------------|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. Ferulic acid   | Pre-eclampsia                     | NG-nitro-l-arginine methyl ester (L-NAME)-induced rats | 30183401    | - Decreased expression of circulating TNF-α, IL-6, IL-1β and PIGF. - Reduced placental TNF-α and NF-κB p65. - Rescued decreasing expression of IL-4 and IL-10 in the circulation and placenta of rats. - Ameliorated placental apoptosis by increasing Bcl-2 and decreasing Bax expression in placenta. |
|                   | Diabetic neuropathy               | Streptozotocin-administered rats     | 30804780    | - Modulated AGEs, MAPKs (p38, JNK, and ERK 1/2), NF-κB mediated inflammatory pathways, mitochondria-dependent and -independent apoptosis as well as autophagy induction.                                              |
|                   | Hepatotoxicity                    | Methotrexate (MTX) - administered rats | 31889292    | - Reduced serum TNF-α and IL-1β, and hepatic NF-κB p65, Bax, and caspase-3, whereas increased Bcl-2, Nrf2, NQO1, HO-1, and PPARγ.                                                                      |
|                   | Endometritis                      | LPS stimulated bovine endometrial epithelial cells (BEECs) | 31499425    | - Reduced mRNA expression of LPS-induced proinflammatory cytokines (IL1β, IL6, TNFA, and IL8) Inhibited the degradation of IκB and phosphorylation of NF-κB p65. - Suppressed the phosphorylation of MAPKs, including p38 and JNK. |
| Study Title                                                                 | Treatment/Model                                                                 | Key Findings                                                                                                                                                                                                 |
|----------------------------------------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Testicular inflammation                                                    | cadmium chloride (CdCl₂)-administered male rats.                                | alleviated apoptotic and inflammatory injuries in testicular tissue via Nrf2 activation.                                                                                                                     |
| Acute respiratory distress syndrome (ARDS)                                | LPS administered rats                                                          | It up-regulated the secretion of interleukin IL-1β, IL-6, tumor necrosis factor TNF-α, and IL-10 in BALF cells.                                                                                               |
| 2. Catechin gallate                                                        | human pancreatic ductal adenocarcinoma (PDAC) cells: PancTu-1, Panc1, Panc89 and BxPC3 | Inhibited TNFα-induced activation of NF-κB and consequently secretion of pro-inflammatory and invasion promoting proteins like IL-8 and uPA.                                                             |
| UV radiation-induced erythema                                              | healthy humans (phototype I/II)                                                 | Reduced levels of cyclooxygenase- and lipoxygenase-produced mediators of UVR inflammation, PGE2 and 12-hydroxy-eicosatetraenoic acid (12-HETE), respectively.                                         |
| 3. Myricetin                                                               | - C57BL/6J mice                                                                | Modulated the polarization of macrophages via inhibiting the TREM-1-TLR2/4-MyD88 signaling molecules in macrophages.                                                                                          |
| Non-alcoholic steatohepatitis (NASH)                                      | - NASH mice                                                                    | Suppressed the NF-κB p65 and AKT activation in NF-κB pathway and JNK, p-ERK and p38 in MAPK signaling pathway.                                                                                           |
| Acute lung injury                                                         | - RAW264.7 macrophages                                                          | Increased Nrf2 activity, decreased NF-κB activity, and inhibited TAK1/p38/JNK/1/2 MAPK signaling.                                                                                                           |
| Pathological cardiac hypertrophy                                          | - LPS-stimulated RAW 264.7 cells                                                | Abolished the levels of inflammatory factors TNF-α, IL-1β, IL-6, NF-κB, p-NF-κB, cyclooxygenase-2 (COX-2), PCNA and Cyclin D1 in the colonic tissues.      |
| colorectal tumorigenesis                                                  | - LPS-induced lung injury model.                                                | Decreased activity of myeloperoxidase (MPO) and the production of TNF-α, IL-6, and IL-1β triggered by LPS.                                                                                               |
| Mastitis                                                                  | - Wild type (WT) and cardiac Nrf2 knockdown (Nrf2-KD) mice                     | Inhibiting LPS-induced phosphorylation of AKT, IKK-α, IκB-α, and P65.                                                                                                                                     |
|                                                                           | AOM/DSS administered rats                                                       | inhibiting the AKT/IKK/NF-κB signaling pathway.                                                                                                                                                            |
**Nephrotoxicity**

Cisplatin administered rats - Decreased caspase-3, TNF-α, IL-6, COXI and COXII, MDA levels.

**DM-associated kidney injury**

Nrf2 knockdown (Nrf2-KD) mice - Prevented DM-associated decreased expression of Nrf2 and inhibited IκB/NF-κB (P65) signaling pathway.

**4. Isoferulic acid**

**Rheumatism**

LPS-stimulated human blood - Inhibited levels of IL-6, TNF-α, and IFN-γ.

**Neuritis**

LPS-stimulated BV2 microglial cells - Suppressed NO and PGE2 production through the induction of nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent heme oxygenase-1 (HO-1).

**Respiratory Syncytial Virus Infection**

Murine macrophage cell line (RAW264.7) - Inhibited the production of macrophage inflammatory protein-2 (MIP-2).

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**Supplementary Table S5. Detailed interactions of the highest scoring compounds with their respective target proteins**

| Compound          | Protein target                                      | Interactions                                   | Amino acid residues (distances, Å)          |
|-------------------|-----------------------------------------------------|-----------------------------------------------|--------------------------------------------|
|                   |                                                     | Type                                          |                                             |
|                   |                                                     | Hydrogen bonds                                | GLU418(1.34,1.37), ASP424 (1.04,1.08)       |
|                   |                                                     | Hydrophobic                                   | PHE350, ALA480, MET417, TYR419, VAL420, ALA366, MET470, LEU345, VAL353 |
| Catechin gallate  | Protein kinase C alpha type (4RA4)                   | Hydrogen bonds                                | LEU362(1.23,1.25), ARG208(1.34), GLU197(1.57, 1.64) |
|                   |                                                     | Hydrophobic                                   | LEU362, LEU205, LEU193, TYR188, LEU210       |
|                   | Transcription factor p65 (3QXY)                      | Hydrogen bonds                                | ARG38(0.94,0.96,1.64,1.65), LYS43(1.01), GLU68(1.47) |
|                   |                                                     | Hydrophobic                                   | MET39, PHE42, PRO65, PHE44                   |
|                   | Interleukin-2 (1M49)                                 | Hydrogen bonds                                | ALA51(1.34), MET109(1.09,1.53), VAL30(1.09) |
|                   |                                                     | Hydrophobic                                   | VAL30, VAL38, VAL52, VAL105, ALA51, LEU104, LEU75, PHE169, LEU108, MET109, LEU171 |
|                   | Mitogen-activated protein kinase 14 (6HWU)          | Hydrogen bonds                                | SER154(1.21), SER278(1.01,1.34), ARG158 (1.32), DG30(1.56), DG8(1.24), DA9(1.29) |
|                   |                                                     | Hydrophobic                                   | ALA151                                       |
Supplementary Table S6: Structural features of active compounds used in enrichment studies for each of the investigated target proteins

| #  | name                        | Structure | #  | name                        | Structure | #  | name                        | Structure |
|----|-----------------------------|-----------|----|-----------------------------|-----------|----|-----------------------------|-----------|
| 1  | Calphostin \(^1\)           | ![Calphostin](image1) | 2  | Enzastaurin \(^1\)          | ![Enzastaurin](image2) | 3  | Riluzole \(^1\)             | ![Riluzole](image3) |
| 4  | Staurosporine \(^1\)        | ![Staurosporine](image4) | 5  | Tamoxifen \(^2\)            | ![Tamoxifen](image5) | 6  | Ruboxistaurin \(^3\)        | ![Ruboxistaurin](image6) |
| 7  | 7-hydroxystaurosporine \(^3\)| ![7-hydroxystaurosporine](image7) | 8  | N-benzyladriamycin-14-valerate \(^2\) | ![N-benzyladriamycin-14-valerate](image8) | 9  | Safingol \(^1\)             | ![Safingol](image9) |
|   |   |   |   |
|---|---|---|---|
| 1. | Glusodichoto-mine AK |   |   |
| 2. | 3-hydroxy-beta-carboline |   |   |
| 3. | Arenarine D |   |   |
| 4. |   |   |   |
| 5. | Transcription factor p65 (3QXY) |   |   |
| 6. |   |   |   |
| 7. |   |   |   |
| 8. |   |   |   |
| 9. |   |   |   |
| 10. | Auranofin |   |   |
| 11. | Resveratrol |   |   |
| 12. | Sodium aurothiomalate | Balanol |   |
| 13. | NPC-15437 |   |   |
| 14. | Sotrastaurin |   |   |
| 15. | Chelerythrine |   |   |
|   |   |   |   |
|---|---|---|---|
| 4. | Arenarine B<sup>4</sup> | 7. | Caffeic acid phenethyl amide (CAPA)<sup>9</sup> |
| 5. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> | 8. | Barbolin<sup>6</sup> |
| 6. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> | 9. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |
| 7. | Trichostatin A<sup>5</sup> | 10. | Curcumin<sup>8</sup> |
| 8. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> | 11. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |
| 9. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 10. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 11. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 12. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 13. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 14. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
| 15. | 1,2,3,4-dihydro-1,3,4-trioxo-β-carboline<sup>4</sup> |   |   |
|   | Interleukin-2 (IM49) |   |   |
|---|---------------------|---|---|
| 16. | Silymarin | Galgravin | Pentoxifylline |
| 17. | DOPA | Nectandrin A | A17B284 |
| 2. | Nectandrin A | A17B284 | Dexamethasone |
| 1. | 1 | 2 | 1 |
|   |   |   |
|---|---|---|
| 1. | Pamapimod⁴³ | Genistein⁴⁶ |
| 2. | FR 167653⁴⁶ | BIRB 795⁴³ |
| 3. | Momelukast²⁵ |   |
| 4. | SB239063²⁸ |   |
| 5. |   |   |
| 6. |   |   |
| 7. |   |   |
| 8. |   |   |
| 9. |   |   |
| 10. | Tyrophosin²² |   |
| 11. | Futokalurin⁶ |   |
| 12. | Veraguensin⁶ |   |
| 13. |   |   |

Mitogen-activated protein kinase 4 (6HWU)
|    |    |    |
|----|----|----|
| 7. |    | SB202190[^29] |
| 8. |    | SB203580[^30] |
| 9. |    | Losmapimod[^31] |
| 10.|    | SCIO-469[^34] |
| 11.|    | Na salicylate[^33] |
| 12.|    | VX-702[^37] |
| 13.|    | RWJ 67657[^36] |
| 14.|    | Skepinone-L[^32] |
| 15.|    | Ralimetinib[^35] |
|   | Proto-oncogene c-Fos (1FOS) |
|---|---------------------------|
| 1. | BMS-582949 $^{38}$        |
| 2. | momordin $^{39}$          |
| 3. | Acteoside $^{40}$         |
| 4. | Curcumin $^{39}$          |
| 5. | Kaempferol $^{39}$        |
| 6. | Nagilactone $^{39}$       |
| 7. | Citrifolin A $^{39}$      |
|   |          |          |
|---|----------|----------|
| 16. | Glaucarubinone |  6-gingerol |
| 17. | Isosteviole | T-5224 |
| 18. | Isosteviole lactone |  K1115 A |
| 19. | Ailanthinone | 6-shogaol |
| 20. |              |          |
| 21. |              |          |
| 22. |              |          |
Supplementary Figure S1. Base peak chromatogram of the extract of *L. camara* leaves.

Supplementary Figure S2. Network of compound–target gene interactions for *L. camara* constituents by linking 39 compounds (presented in violet color) and 35 target proteins (presented in blue color).
**Supplementary Figure S3.** Protein–protein interaction (PPI) network of identified inflammation-related targets.

**Supplementary Figure S4.** Gene–pathway network (genes are presented in blue color, pathways are presented in red color).
Supplementary Figure S5. Superimposition of co-crystallized ligand (red) and re-docked ligand (green) at the binding site of the proteins (a) Protein kinase C alpha type (4RA4), (b) interleukin-2 (1M49) and (c) Mitogen-activated protein kinase 14 (6HWU).
Methods:

Analysis of *L. camara* extract using UPLC-MS/MS technique:

ESI-MS conditions and metabolites annotation:

Electrospray ionization (ESI) source in conjunction with triple quadrupole (TQD) mass spectrometer were utilized to analyze the samples in a negative ionization mode. ESI operating conditions briefly were: capillary voltage of 3 kV, cone voltage; 35 V, the ion source temperature was 150°C, the nebulizer (nitrogen gas) pressure was 35 psi, drying and sheath gas (N₂) temperatures were 440°C and 350°C, respectively. The drying and sheath gas flows were applied at 900 L/h and 50 L/h, respectively. The analytical run time was extended to 30 min. MS spectra were achieved by full range acquisition covering 100-1000 m/z. For automatic MS/MS fragmentation analyses of the precursor ions which were mass-selected by the first quadrupole (Q1), the collision-induced dissociation (CID) energy was ramped from 30 to 70 eV using nitrogen gas as a collision gas in the second quadrupole collisional cell (Q2). Finally, the daughter ions yielded from CID are consequently related to the molecular structure of the precursor ions and can be monitored by a third quadrupole mass analyzer (Q3). Assignment of the metabolites was accomplished by comparison of their retention times to external standards. Furthermore, quasi-molecular ions in addition to the characteristic MS/MS fragmentation pattern were used for metabolite annotation in comparison to our in-house database, reference literature and phytochemical dictionary of natural products database (CRC) in order to get metabolite annotation with a high level of confidence 44.

*In vitro* cytotoxicity and anti-inflammatory activity testing

It was carried out according to the method described by Darwish et al. 45 as following:

**Isolation and cultivation of human white blood cells**

A blood specimen was provided from Alexandria Regional Blood Transfusion Center (63 Ahmed Soliman El-Shaikh Street, Kom Ad Dakah Sharq, Al Attarin, Alexandria Governorate, Egypt). This blood specimen was placed in a sterile heparin tube, from which 1 mL was drawn into 15 mL centrifuge tube which was then filled with 10% v/v fresh cold lysing solution prepared from stock solution containing NH₄Cl 8.02g, NaHCO₃ 0.84g and EDTA 0.37g. The centrifuge tube was then inverted at room temperature for about 10 min till the liquid turned into clear red. After that, the
blood specimen was centrifuged at 2000 rpm and 4°C for 10 min and the supernatant was decanted. The pellets (WBCs) were suspended in 10 mL cold PBS (phosphate-buffered saline containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 10 mM KH2PO4, pH 7.4) and centrifuged. Then they were resuspended in RPMI-1640 medium, containing 10% fetal bovine and 2% L-glutamine. The evaluation of WBCs viability and counting was carried out using dye exclusion method. Fifty µL of cell suspension was blended with equal volume of 0.5% trypan blue staining solution then loaded onto hemocytometer. Eventually, counting of viable "unstained" and nonviable "stained" cells in each of the four corner quadrants (A, B, C, D) was carried out.

**Calculation**

N / mL = mean of WBCs counting x 10⁴ × D

N: Number of viable or nonviable cells
D: Sample dilution (1:1 with the trypan blue).

% Cell viability = (Number of viable cells / Total number of cells) × 100

% Cell viability must be at least 90% in order to perform the assays. WBCs were cultured in RPMI media and incubated in CO₂ incubator at 37°C, 5% CO₂, and 90% relative humidity for six days. Then they were seeded in 96 well cell culture plate (100,000 cells/ well).

**Assessment of cytotoxicity of the crude extracts compared to piroxicam (MTT assay)**

Treatment of 200 µL cultured medium containing 100,000 WBCs / well with different concentrations (0, 3.125, 6.25, 12.5, 25 and 50 µg/mL) of the crude extracts was carried out in RPMI medium without fetal bovine serum, using the known NSAID drug ‘Piroxicam’ as a positive control. Plates were then incubated in CO₂ incubator for 72 h in the same conditions, then 20 µL of MTT solution was added to each well and re-incubated to allow MTT reaction to be accomplished. Afterwards, the plates were centrifuged at 1650 rpm for 10 min and the medium was discarded. The MTT byproducts (formazan crystals) were suspended in 100 µL DMSO and measurement of absorbance was performed at a wavelength of 570 nm using optima spectrophotometer, in order to detect the safe dose which causes 100% cell viability.

The % viability was calculated as follow: (A_T-A_b/A_C-A_b) x 100

A_T = mean absorbances of cells treated with a certain concentration of the plant extract.
\[ A_C = \text{mean absorbances of control untreated cells with culture medium only} \]
\[ A_b = \text{mean absorbances of cells treated with vehicle of plant extract (RPMI without fetal bovine serum)} \]

The cytotoxicity assay of the compound was expressed as \( CC_{50} \), which is the drug concentration required for reducing the cell viability by 50%, and it was calculated by the Graphpad Instat software (https://www.graphpad.com/scientific-software/instat/) by interpolation from the plot of % cell viability vs. serial dilutions of the plant extract.

**Detection of the effective anti-inflammatory concentrations (EAICs) of the tested extract in lipopolysaccharides (LPS)-stimulated human WBC's culture**

In a 96 well plate, dispensing of 50 µL of the culture medium containing 100,000 of human WBCs per well was carried out. Induction of inflammation was accomplished by adding 50 µL of LPS to the plated cell, then they were incubated in CO\(_2\) incubator. After 24 h, centrifugation of the plate at 1650 rpm for 5 min was performed, then the supernatants were discarded. Afterwards, addition of 200 µL of serial concentrations (0, 3.125, 6.25, 12.5, 25 and 50 µg/mL in culture media) of the crude extracts or the standard anti-inflammatory drug piroxicam was carried out. The blank was wells containing untreated cells in culture medium only. The plates were then incubated for another 72 h in CO\(_2\) incubator. Thereafter, measurement of cells proliferation was carried out using MTT test and it was expressed as stimulation index (SI).

**Stimulation index** = \((\text{mean absorbance of LPS-stimulated cells (negative control) or LPS-stimulated cells treated with different concentrations of plant extract} / \text{absorbance of control untreated cells (blank)})\).

Graphpad Instat software was used to calculate the effective anti-inflammatory concentration (EAIC) of the extract that was able to resume the abnormal proliferation of LPS-stimulated cells to normal proliferation of control untreated cells (SI = 1).

**Extraction of RNA of untreated and treated LPS-stimulated human white blood cells**

Cell pellets were suspended in 50 µL of solution R1 (qiagen RNA extraction kit). They were mixed for 30 s, then incubated for 1 min at room temperature. Afterwards, 300 µL of solution R2 (qiagen RNA extraction kit) were added and blended for 30 s. Centrifugation was accomplished for 3-5
min at 4ºC. Next, the supernatant was transferred into a spin column and centrifuged at 14000 rpm for 30 s at 4ºC. Then, addition of 300 µL of working wash buffer was done after removing the flow-through and centrifugation was repeated for 30 s. This step was repeated twice. Subsequently, the spin column was centrifuged at 10,000 rpm for 1 min then delivered to a sterile 1.5 mL micro centrifuge tube. Then, 30 µL of elution buffer were added to the membrane center and incubated for 1 min at room temperature, then centrifuged at 14000 rpm for 30 s at 4ºC. Eventually, the optical density (OD) of the extracted RNA was determined via absorbance and purity measurement at A260 and A260/A280 nm, respectively. Then it was kept at -80ºC until real time polymerase chain reaction (PCR) was performed.

**cDNA synthesis from RNA extracted from untreated and treated LPS-stimulated human white blood cells**

In PCR tubes, 2 µg of total RNA or nuclease-free water and 1 µL of oligo dT primer were added to nuclease-free water in a total volume of 12 µL, and they were gently mixed. Centrifugation was performed before incubation for 5 min at 65ºC in PCR machine, then the mixture was set immediately on ice. Four microliters of 5X reaction buffer, 1 µL of RNase inhibitor, 2 µL of dNTPs mix and 1 µL of reverse transcriptase or 1 µL of nuclease-free water instead of reverse transcriptase for reverse transcriptase negative control were gently mixed with previous mixture. Afterwards, the PCR tubes were spined down and incubated for 60 min at 42ºC, then they were heat inactivated at 70ºC for 5 min in PCR machine

**Determination of IL-1β, IL 6, TNF and INF-γ expression level by real time polymerase chain reaction (PCR)**

In PCR tubes, admixture of 13 µL of 2 X SYBR green master mix with 5 µL of cDNA, 0.5 µL of 10 pmoles/mL forward primer and 0.5 µL of 10 pmoles/mL reverse primer for each primer was carried out. The same as in the reference tube, addition of 0.5 µL of 10 pmoles/mL forward primer of β-actin and 0.5 µL of 10 pmoles/mL for reverse primer of β- actin was done. Another tube was utilized as a non-template control (NTC) to assess reagent contamination or primer dimers, by inserting 1 µL of nuclease-free water as a substitute of template used. Afterwards, the tubes were subjected to gentle mixing with 6.5 µL nuclease free water without bubbles formation and subsequently subjected to spinning for few seconds. Samples were set in the cycler and the program was initiated as following; initial denaturation (1 cycle of 95ºC for 10 min), then
denaturation (40 cycles of 95°C for 15 sec), annealing (at 60°C for 30s) and extension (at 72°C for 30s). Fold change in gene expression was used to assess the influence of LPS and extracts on the expression of genes.

**Calculation**

**Expressions fold levels of gene are computed by**

\[ \Delta Ct_{normal} = Ct_{normal\ untreated\ cells} - Ct_{reference} \]
\[ \Delta Ct_{tested\ plant\ extract} = Ct_{tested\ plant\ extract-treated\ cells} - Ct_{reference} \]
\[ \Delta Ct_{induced} = Ct_{LPS-exposed\ cells} - Ct_{reference} \]

**In case of genes:**

\[ \Delta \Delta Ct_{tested\ plant\ extract} = \Delta Ct_{tested\ plant\ extract} - \Delta Ct_{normal} \]
\[ \Delta \Delta Ct_{induced} = \Delta Ct_{induced} - \Delta Ct_{normal} \]

**In case of GAPDH:**

\[ \Delta \Delta Ct_{tested\ plant\ extract} = \Delta Ct_{normal} - \Delta Ct_{tested\ plant\ extract} \]
\[ \Delta \Delta Ct_{induced} = \Delta Ct_{normal} - \Delta Ct_{induced} \]

**Fold change in gene expression = \log (2^{\Delta \Delta Ct})**

Where:

- \(Ct_{tested\ plant\ extract}\): threshold cycle value of genes of extracted mRNA of plant extract treated-LPS-stimulated WBCs which is defined as the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold.
- \(Ct_{reference}\): threshold cycle value of GAPDH which is used for normalization.
- \(Ct_{normal}\): threshold cycle value of genes of extracted mRNA of untreated control WBCs
- \(Ct_{induced}\): threshold cycle value of gene of extracted mRNA of LPS-stimulated WBCs

**Primers**

| Gene   | Forward          | Reverse          |
|--------|------------------|------------------|
| TNF-α  | F-CTCTTCTGCCTGCTGCACTTTG | R- ATGGGCTACAGGCTTGTCACTC |
| IL-6   | F, 5’-TGAACCTCCTTCTCCACAAGCG-3’ | R, 5’-TCTGAAGAGGTGAGTGCTGTC-3’ |
| IL-1β  | F, CCACAGACCTTCCAGGAGAATG | R, GTGCAGTTTGAGTGATCGTACAGG |
| Gene  | Forward Sequence | Reverse Sequence |
|-------|------------------|------------------|
| INF-γ | F, GAGTGTGGAGACCATCAAGGAAG | R, TGCTTTGCGTTGGACATTCAAGTC |
| GAPDH | F, GGATTGTTGCTATGGTATTGGG | R, GGAAGATGGTGATGGGATT |

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