

Loranthus Micranthus Extracts Suppress the Expression and Enzymatic Activity of Indoleamine 2,3-Dioxygenase in Human Breast Cancer Cells via the Inhibition of JAK/STAT and NF- κ B Pathways

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Research

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Abstract

Background: *Loranthus micranthus* (African mistletoe) is a woody parasitic plant. It is found in Africa, Asia, Europe, and America. Its leaves and stem/twigs have been used in traditional medicine in various continents for the treatment of metabolic diseases: diabetes, hypertension, and cancer. The present study is designed to investigate the effect of *Loranthus micranthus* leaves and stem (LML and LMS) extracts on indoleamine 2,3-dioxygenase (IDO); a key regulator of cancer immunity; and the possible molecular mechanism(s) involved.

Methods: Human breast cancer cells; MDA MB-231 and MCF-7 were pre-treated with LML and LMS at doses of 1-30µg/mL for 2 hours. This was followed by interferon-γ (INF-γ) stimulation at 50 ng/mL for 24 hours. After the treatment period, the activity of indoleamine 2,3 dioxygenase 1 (IDO-1) and T cell proliferation were determined using a spectrophotometric assay. Protein expression of IDO-1, IDO-2, tryptophan-2,3-dioxygenase-2 (TDO-2), Bridging inhibitor 1 (BIN-1), Janus kinase/signal transducers and activators of transcription (JAK/STAT), Inhibitor of κ B (IκBα) and Nuclear factor-κB (NF-κB) pathway were analyzed using the western blotting technique. The mRNA expression of IDO-1 was quantified using the qRT-PCR technique.

Results: MDA MB-231 and MCF-7 cells pre-treated with LML and LMS exhibited lower kynurenine synthesis than that of INF-γ treatment. We observed downregulation of IDO-1, IDO-2, and TDO-2 protein expressions, and lower IDO-1 catalytic activity in LML and LMS pretreated cells than those of INF-γ treated cells alone. Cells pretreated with LML and LMS exhibited significant downregulation of phosphorylation of JAK-1, JAK-2, STAT-1, and STAT-3 than those of IFN-γ treatment alone. Furthermore, LML and LMS pre-treated cells exhibited upregulation of IκBα and BIN-1 protein expression compared with IFN-γ treated cells alone. Additionally, LML and LMS pretreatment significantly improved T cell viability when compared with IFN-γ treated cells alone.

Conclusion: These findings indicate that LML and LMS extracts regulate immune responses in cancer cells by inhibiting IDO activity/expression in a JAK/STAT and NF-κB-dependent manner. This accounts for their anticancer effects in traditional medicine, hence a promising candidate for future drug development.

Background

Humans are endowed with myriads of medicinal remedies from nature's treasure chest of green gold (herbs). Herbal products and their derivatives have been reported to represent more than 50% of the drugs in modern therapeutics [1]. Since ancient times, research and development of therapeutic agents have focused on drug discovery from herbal medicines or botanical sources [2]. This is because synthetic agents have more intense adverse effects than the natural/plant agents [3]. Thus, some plant-derived drugs such as vincristine, vinblastine, Paclitaxel (Taxol), Myriocin among others have been introduced for the treatment/management of cancer and various ailments [4].

Loranthus micranthus (African mistletoe) belongs to the family of Loranthaceae, and is used in Nigerian folklore medicine as immune-enhancer and antioxidant especially in some of the immuno-related pathological diseases including but not limited to cancer, diabetes, flu and other viral infections [5]. Earlier studies demonstrate preliminary scientific evidence for this practice [6]. Several experimental shreds of evidence demonstrating anticancer potentials of mistletoe plant extract from different continents have been reported [7, 8, 9, 10, 11, 12]. However, there is no report linking mistletoe's pharmacological effects to indoleamine 2,3 dioxygenase and kynurenine pathways. Following preliminary scientific evidence and traditional healing claims of *Loranthus micranthus*; its phytoconstituents have been structurally elucidated [5, 10, 13]. The various compounds, like quercetin, rutin, and epicatechin identified in this plant are known to possess anti-inflammatory, antioxidant, antitumor and immunomodulatory properties principally by regulating immune cell proliferation [5, 10, 13]. Although Immunomodulatory activity of *Loranthus micranthus* on mice splenocyte, was reported by Ogechukwu et al. 2011; studies on the molecular mechanisms linking the immunomodulatory potentials of this plant to IDO regulation remain to be clarified. Hence the need for the current study.

Studies on genetic and pharmacological assessment of IDO and TDO have confirmed that this tryptophan metabolism enzymes are a central driver of immunologic processes including autoimmunity, cancer growth/advancement, diabetes among others [14, 15]. The catabolic metabolite of the IDO enzyme- kynurenine, has received increasing attention owing to its link with inflammation, the immune system, cancer progression and neuro-toxicological conditions [15, 16]. To this end, therefore, this study for the first time designed to evaluate the possible effects of *Loranthus micranthus* leave and stem (LML and LMS) extracts on IDO expression, is very important to further understand the underlying mechanisms of its therapeutic influence particularly in autoimmune disorders like cancer, and the need for future drug development from its Phytoconstituents.

Materials And Methods

Chemicals and Reagents

Antibodies used for this study and their sources are as follows: IDO 1 #13268-I-AP, IDO 2 #25053-1-AP, TDO 2 #15880-I-AP, BIN 1 #15880-I-AP, JAK 2 #17670-1-AP, and I κ B- α #10268-1-AP, were purchased from Proteintech, San Ying Biotechnology, Wuhan, China. JAK 1 (3332S), and p-JAK 2 (3776S), from Cell Signaling Technology. STAT 1 (ET1606-39), and STAT 3 (ET1607-38) from HangZhou HuaAn Biotechnology Co., Ltd. p-JAK 1 #11149-1, Tyr 1022, p-STAT 1 #11044, Tyr 701, p-STAT 3 #11045, Tyr 705, and NF κ B-p65 #21014, from Signalway antibody, Baltimore Ave. USA. IDO 1 primers were obtained from Bioengineering (Shanghai) Co., Ltd. Interferon- γ was obtained from GenScript Piscataway, NJ USA. Nylon wool fiber for T-cell purification (#18369-10) from Polysciences, Inc. Warrinton PA. USA. Every other chemicals and solvents were purchased from Sigma-Aldrich. Reagents used in this study were of the highest available analytic grade (purity \geq 98%).

Authentication of Plant Material

LML and LMS, collected from trees around the eastern part of Nigeria, in July 2017, authenticated by the Department of Botany herbarium team, the University of Ibadan, where a voucher specimen exists in the herbarium (UIH-22812). Subsequently, leaves (LML) and stem (LMS) were separated, air-dried in a room protected from light.

Preparation of plant Extract

Dried leaves and stem, were crushed separately in an electric grinder. From the powder of LML and LMS: 10 g was extracted with 100 mL of 75% aqueous ethanol by maceration for 48 hours at 25 °C. The extract was filtered, and dried by low pressure, and stored at 4°C, protected from light.

HPLC Analysis of Extract

According to the method described by Vijay et al. [17] with slight modification as described in brief; 10 mg of powdered plant extracts of LML and LMS were dissolved in 10 mL of methanol to get a concentration of 1mg/mL, subsequently, the solution was filtered using a 0.45µm syringe filter (Millipore) for sterilization. 1 mg of each standard (epicatechin, quercetin, and rutin) was dissolved individually in 1mL of methanol and sterile filtered through 0.45 µm syringe filter (Millipore), before HPLC analysis. The prepared samples of extracts and standards were used for HPLC. The SSI 1500 HPLC series equipped with a DAD detector connected to the system processor was used for analysis. The system explored Empower software with standard certification for analysis of the results; maximum pressure of 2500 psi and a minimum of 1500 psi was maintained. The HPLC of solvents (60:40 ratio of methanol and water) was run at 300 nm wavelength using reverse phase C-18 column (5 µm, 250 mm X 4.6 mm). During the run, a flow rate of 1mL/min was maintained using a binary mode of gradient system. To, identity the compounds, standards of epicatechin, quercetin, and rutin were used. The peaks were identified by comparing the retention time (RT) of the standard with that of different peaks obtained in HPLC analysis of extracts confirmed by UV-spectra online.

Enzyme assay for IDO1 activity

MDA-MB 231 and MCF-7 cells were seeded in a 96-well plate (1×10^5 cells/well) and treated with LML and LMS doses of 1µg/mL, 5µg/mL, 10µg/mL, 15µg/mL, 20µg/mL and 30µg/mL for 2 h before addition of INF-γ (50 ng/mL) for another 24 h to determine the IC₅₀. Dimethylsulfoxide (DMSO, 0.5%) and epacadostat (25 nM) were used as negative and positive controls respectively. IDO1 activity was estimated by measuring the concentration of L-kynurenine in cell culture media according to the method described by Travers et al. [18]. In Brief; 100µL of culture medium was incubated with IDO buffer and then mixed with 25µL of 30 % trichloroacetic acid, incubated for 30 minutes at 50 °C and thereafter centrifuged at 10,000 rpm for 5 min. 100 µL of the supernatant was transferred into a new 96 well plate, and an equal volume of freshly prepared 2% w/v *p*-dimethylaminobenzaldehyde in acetic acid was added. The optical density was measured at 480 nm using Thermo Scientific VarioskanFlash Multimode Reader. The kynurenine concentration was determined from L-kynurenine standard curve. Each assay was performed in triplicate and data presented as mean ± standard error of mean.

Cell viability assay

MDA-MB 231 and MCF-7 cells were seeded in a 96-well plate (1×10^5 cells/well) and treated with LML and LMS doses of 1, 5, 10, 15, 20 and 30 $\mu\text{g}/\text{mL}$ for 2 h. INF- γ (50 ng/mL) was added for another 24 h at 37 °C in a humidified incubator with 5% CO_2 , to determine the cell viability. Dimethylsulfoxide (DMSO, 0.5%) and epacadostat (25 nM) were used as negative and positive controls respectively. The cell viability was determined by replacing the initial culture medium with 100 μL of fresh culture medium containing 10% of the Cell Counting Kit-8 (CCK-8, Beyotime) in each well of the microplate. After 1 hour of incubation at 37 °C in a humidified incubator with 5% CO_2 , the absorbance was read at 450 nm on a Thermo Scientific VarioskanFlash Multimode Reader. Cell viability was calculated as a percentage of control (DMSO, 0.5%).

Western blotting

Cells were harvested in RIPA buffer supplemented with a protease inhibitor cocktail (Sigma, Shanghai, China) at the end of the treatment period. The concentration of protein was measured by using a BCA protein assay kit (Bestbio, Shanghai, China). Western blotting was conducted according to standard procedures, as described in our earlier study, Wonganan et al. [19]. In brief; well-mixed aliquots of total cell lysates with loading buffer were boiled for 3-5 min, and separated on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, gels were cut according to molecular weight with the aid of Blue Plus® II Protein Marker (TransGen Biotech Co., LTD Cat. No. DM111-01) and proteins were transferred to nitrocellulose membranes and 5% bovine serum albumin or 5% defatted milk powder were used to block for 1 h at room temperature and then incubated overnight at 4 °C with each of the following antibodies: anti-IDO1, anti-IDO2, anti-TDO2, anti-BIN1, anti-phospho-STAT1, anti-STAT1, anti-phospho-STAT2, anti-STAT2, anti-phospho-STAT3, anti-STAT3, anti-NF κ B-p65, anti-I κ B- α and GAPDH across all gel for each protein. Afterward, the membranes were incubated with an appropriate peroxidase-conjugated secondary antibody 511203 (Zen Bioscience, Chengdu). The protein bands were determined by the detection of enhanced chemiluminescence solution (Amersham Biosciences, Piscataway, NJ, USA).

Evaluation of IDO1 activity/expression in pCMV3-IDO1-transfected HEK293A cells

HEK293A cells were seeded in a 6-well plate, overnight for proper attachment. pCMV3-IDO1 plasmids (0.94 $\mu\text{g}/\mu\text{L}$) were transfected into cells by using lipofectamine® 2000 according to the manufacturer's instructions. After 6 h of incubation, the transfection complex was replaced with fresh medium, and the transfected cells were used for subsequent experiments 12 h later. Transfected cells were treated with different concentrations of LML and LMS (5, 10 and 20 μM) and epacadostat (25 nM) served as a positive control. After 2 h of incubation, the transfected and treated cells were lysed with RIPA lysis buffer, and IDO 1 activity was estimated while the same amount of protein samples were separated on SDS-PAGE to determine the IDO1 expression by Western blot analysis.

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total cellular RNA isolated from treated MDA-MB 231 and MCF-7 cells using TRIzol reagent (Invitrogen) by following the manufacturer's protocol was reverse-transcribed using SuperScript III Reverse Transcriptase (Invitrogen) with oligo dT18 primer. Equal amounts of cDNA were subjected to real-time quantitative PCR with the fluorescent dye SYBR Green I by following the manufacturer's protocol (TransGen Biotech, Beijing, China). The primer pairs used in the assay for IFN- γ induced IDO 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: IDO 1, (forward); 5'-AA C AGC GCC TTT AGC AAA GTG TCC CGT TCT TG-3' (reverse); 5'-AGC GCC TTG CAC GTC TAG TTC TGG GAT GC-3' and GAPDH, (forward); 5'-TGC ACC ACC AAC TGC TTA GC-3', (reverse); 5'-GGC ATG GAC TGT GGT CAT GAG-3'. The samples were run in triplicate and the relative expression levels of IDO 1 were determined by normalizing the expression of each target gene to that of IFN- γ using the $2^{-\Delta\Delta Ct}$ method.

Co-culture and T cell Proliferation Assay

Primary T cells were isolated from mouse spleen by following the method described by Flaherty and Reynolds [20], with little modifications as follows in brief: Spleens from freshly sacrificed mouse were collected into a 60 mm dish containing 3 ml of PBS+. Afterward, a square of sterile nylon mesh was placed over the tissue using sterilized forceps. The thumb side of a syringe plunger (3-10 mL) was used to gently smash the tissue against the mesh. This was followed by pipetting the suspension up and down a few times to break up the remaining soluble clumps and a new square piece of nylon mesh was placed over the opening of a 15 mL conical tube and the suspension filtered through to remove the remaining debris of splenic tissues. Cells were centrifuged at 475 x g for 5 min at 4°C, then the supernatant discarded and re-suspend the cell pellet in 1 mL of ice-cold 1X cell lysing solution per spleen for 1 min to lyse erythrocytes. Then 10 ml of PBS⁺ was added on top of the lysing solution, and the tube inverted twice, and centrifuged at 475 x g for 5 min at 4°C. After centrifugation, the supernatant (splenocytes) was aspirated and re-suspended in another 10 mL of PBS⁺. Primary T cells from the splenocytes were purified by using nylon wool mesh (Polysciences, Inc. USA).

MDA MB-231 cells were incubated with T-Cells isolated from mice as described above. Cancer cells were incubated with T-cells at a ratio of 1:10 or 1:5 (cancer cells: T-Cells) for 48 h at 37 °C in a humidified incubator with 5% CO₂. At the end of co-culture experiments, conditioned medium (Dulbecco's Modified Eagle Medium (DMEM) plus 10% Fetal Bovine Serum (FBS) was discarded by pipetting, and cell viability was estimated using CCK 8 counting kit as described above.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad, La Jolla, CA, USA). Experiments were repeated at least three times and representative results presented. The data were compared by the Student-T test for each concentration of LML and LMS and one-way ANOVA across the groups followed by Dunnett's post-hoc test. Differences were considered statistically significant for values at $p < 0.05$.

Results

HPLC analysis of LML and LMS extracts

Earlier study by Omeje et al. [5] indicated that flavonoids like epicatechin, rutin and quercetin were present in the plant (LML and LMS) extracts. The HPLC analysis of LML and LMS in the current study confirmed the presence of epicatechin, rutin and quercetin in both LML and LMS extracts as shown in Fig. 1A, and 1B.

Effects Of Lml And Lms Extracts On Kynurenine Synthesis

To examine the effect of LML and LMS extracts on kynurenine synthesis; breast cancer cells were incubated with various concentrations (5, 10 and 20 $\mu\text{g}/\text{mL}$) of LML and LMS for 2 h. This was followed by addition of $\text{INF-}\gamma$ (50 ng/mL) and incubated for 24 h. As shown in Fig. 2A and 2D; $\text{INF-}\gamma$ induced kynurenine synthesis in MDA MB-231 and MCF-7 cells were significantly inhibited by Epacadostat (a specific inhibitor of IDO1 activity). Additionally, both LML and LMS at 5–20 $\mu\text{g}/\text{mL}$ also significantly inhibited $\text{INF-}\gamma$ induced kynurenine synthesis in both breast cancer cells. To examine the effect of extracts on kynurenine synthesis in more detail; breast cancer cells were treated with LML and LMS at concentrations of 1 to 30 $\mu\text{g}/\text{mL}$ as shown in Fig. 2B and 2E. LML and LMS extracts inhibited kynurenine synthesis in a concentration-dependent manner in MDA MB-231 and MCF-7 cells. The IC_{50} values for LML and LMS extract were calculated as 4.05 and 12.13 $\mu\text{g}/\text{mL}$ in MDA-MB 231 cells and 10.7 and 8.9 $\mu\text{g}/\text{mL}$ in MCF-7 cells respectively. The cut-off limit for relative cell viability for cytotoxicity is 50%; therefore, tested concentrations of LML and LMS were not cytotoxic to MDA MB-231 and MCF-7 cells (Fig. 2C and 2F). These findings indicate that LML and LMS extracts inhibit kynurenine synthesis in breast cancer cells.

Influence Of Lml And Lms Extracts On Ido Expression/activity

Furthermore, we examined whether LML and LMS have efficient inhibitory effects on IDO1, IDO2 and TDO2 expression following $\text{INF-}\gamma$ stimulation in MDA-MB 231 and MCF-7 cells. Pre-treated cells particularly at 10 and 20 $\mu\text{g}/\text{mL}$ of extracts exhibited a lower IDO1, IDO2 and TDO2 protein expression compared with $\text{INF-}\gamma$ treated cells alone as shown in Figs. 3A, B, C and D. Furthermore, $\text{INF-}\gamma$ induced IDO 1 mRNA expression in MDA MB-231 and MCF-7 cells were significantly suppressed by LML and LMS pre-treatment (Figs. 3E, F, G and H). These results indicate that, treatment with extracts inhibit $\text{INF-}\gamma$ induced IDO expression at the transcriptional level. To further investigate whether LML and LMS extracts possess direct inhibitory activity on IDO1 protein; IDO1 was transiently over-expressed in HEK293A cells. As shown in Figs. 3I and J; LML and LMS extracts significantly inhibited relative IDO 1 catalytic activity in IDO1-overexpressing HEK293A cells, indicating that the extracts also directly inhibit the catalytic activity of

IDO1. These findings suggest that extracts inhibit kynurenine synthesis in cancer cells by the suppression of IDO1 catalytic activity and its expression both at the translational and protein levels. Figures 3K and L showed no effect on IDO 1-overexpressing HEK 293A confirming the direct inhibitory effect on the catalytic activity and not degradation of IDO 1 protein.

Influence Of Lml And Lms Extracts On Jak/stat Pathway

To further expound the molecular mechanism controlling the suppression of IDO expression by LML and LMS; JAK/STAT signaling pathway was evaluated in MDA MB-231 cells. LML and LMS at 10 and 20 µg/mL pre-treated cells for 2hour inhibited IFN-γ phosphorylation of STAT1 and STAT3. Similarly, IFN-γ induced-phosphorylation of JAK1 and JAK2 was also inhibited by LML and LMS pre-treatment at 10 and 20 µg/mL for 2hour (Fig. 4A, B, C and D). There was remarkable inhibition in the cells treated with JAK inhibitor 1. These results suggest that LML and LMS extracts can suppress INF-γ induced IDO expression via the inhibition of JAK/STAT pathway.

Effects of LML and LMS extracts on NF-κB and BIN 1 expression

As shown in Fig. 5A; INF-γ induced downregulation of IκB-α expression was upregulated in MDA MB-231 cells by LML and LMS pre-treatment. Conversely, pre-treatment with LML and LMS at 10 and 20 µg/mL did not show noticeable change in the expression level of total NF-κB p65 compared with INF-γ treated cells alone in MDA MB-231 cells. However, LML and LMS pre-treated cells exhibited augmentation in expression of BIN 1 relative to INF-γ treated cells alone (Fig. 5B). These findings indicate that LML and LMS extracts suppress INF-γ induced IDO expression via the inhibition of NF-κB pathway and up-regulation of BIN1.

Effects of LML and LMS extracts on T cell viability

To determine the possible influence of LML and LMS pre-treatment on T cell survival, we isolated primary T cells from the spleen of female C57 mice and co-cultured them with MDA MB-231 cells. As shown in Fig. 6; IFN-γ treatment of MDA MB-231 cells decreased the proliferation of T cells, whereas LML and LMS pre-treatment of MDA MB-231 cells significantly restored the proliferation of T cells to near control cells. These results indicate that suppression of INF-γ induced IDO expression by LML and LMS pre-treatment in breast cancer cells, enhanced T cell survival.

Discussion

IDO is overexpressed in several human cancers, such as prostate, breast, brain, and hematologic malignancies [21, 22, 23]. The current study is consistent with earlier reports. However, pretreatment with LML and LMS suppressed both IDO1 mRNA and protein expression. Additionally, pCMV3-IDO1-transfected HEK 293A cells also showed a decrease in IDO1 catalytic activity following treatment with LML and LMS extracts. This is an indication that the extracts are directly interacting with IDO1 protein.

Interestingly, the corresponding suppression of IDO2 protein expression by LML and LMS treatment further increased the potentials of this plant to assuage compromised immune responses associated with cancer progression. The Previous report on detailed preclinical studies showed that epacadostat acts as a kynurenine/tryptophan-competitive inhibitor of the catabolic activity of human IDO1 in cell-based assays. It was also observed that epacadostat showed no effect on IDO1 protein expression [24, 25, 26]. The current study corroborated earlier report; epacadostat elicited no inhibitory effects on IDO1 protein expression. TRP metabolism is a substitute path to IDO1 activity engaged by tumors. Hence, there is increasing interest in pharmacological targeting of IDO for cancer immunotherapy [27]. Interestingly this study revealed that LML and LMS pre-treatment elicited a significant inhibition in IDO1, IDO2, and TDO2 protein expressions, following IFN- γ stimulation in the two breast cancer cells. These findings have further strengthened the earlier report on the immunomodulatory effects of the studied plant.

Induction of IDO expression by Inflammatory mediators such as IFN- γ is mediated by JAK-STAT dependent mechanism(s) [28] and NF κ B signaling pathways in cells [29]. The current findings revealed the influence of LML and LMS treatment in downregulating IDO expression by blocking the JAK-STAT and NF κ B signaling pathways. This is in agreement with other reports that IDO downregulation is mediated by JAK/STAT and NF κ B pathways [30]. BIN 1 is a cancer suppressor protein that controls the expression of IDO1 [31]. Suppression of BIN 1, a tumor suppressor gene in tumor cells correlates with the up-regulation of IDO expression [32]. In the current study, LML and LMS extracts up-regulated BIN 1 expression in breast cancer cells, compared with INF- γ treatment alone.

Up-regulation of IDO has been implicated in cancer progression by blocking T-cell activation ultimately leading to T-cell death. Thus, there is a need for immunomodulatory agents targeting this enzyme [33], to enhance T-cell viability. Therefore, the suppression of L-kynurenine concentration by LML and LMS pre-treatment, after IFN- γ stimulation in MDA-MB 231 cells also contributed to the inhibition of IDO expression in this study. Hence resulting in the improved survival rate of primary T cells. These findings indicate that LML and LMS exert immunomodulatory properties by inhibiting IDO activity/expression and thus boosting immune response. The earlier report highlighted the potentials of phytochemicals to assuage compromised immune responses associated with cancer progression and other immune-related diseases [34] and the current report has further supported the claims.

Conclusion

LML and LMS extract inhibited kynurenine synthesis in breast cancer cells. This was followed by suppressing the expression of IDO1, IDO2, and TDO2. The underlying mechanism involves direct and indirect interaction between extracts and IDO1, blocking of JAK/STAT and NF- κ B pathways. Having identified a novel immunosuppressive activity of this plant; a further study on the assessment of the various phytochemicals with the aim of finding a novel compound and to identify the components that are responsible for the IDO regulation is an ongoing study in our laboratory.

Abbreviations

IDO; Indoleamine 2, 3-dioxygenase, LML; *Loranthus micranthus* leaves, LMS; *Loranthus micranthus* stems, TDO; TRP-2,3-dioxygenase, IFN- γ ; Interferon- γ , JAK/STAT; Janus kinase/signal transducers and activators of transcription, NF- κ B; Nuclear factor κ B, I κ B- α ; Inhibitor kappa B- α , BIN1; Bridging integrator 1.

Declarations

Ethics approval and consent to participate

All experiments with mice followed the regulations of Chinese Academy of Science guidelines for the care and use of Laboratory Animals and approved by the Institutional Animal care and use committee of Chengdu Institute of Biology, Chinese Academy of Science (protocol number: CIBCAS-2019-0139).

Consent for publication

All the participating authors read the manuscript and give consent for publication.

Availability of data and materials

Please contact the corresponding author for data request

Competing interests

The authors have no real or potential conflicts of interest to declare.

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Authors' contributions

APE, GZ, LL, and WF conceptualized and designed the experiment; APE, TP, EMN, and ZZ performed the experiment; APE, and WF interpreted the results; APE, AAH and WF prepared the manuscript. All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work.

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Figures

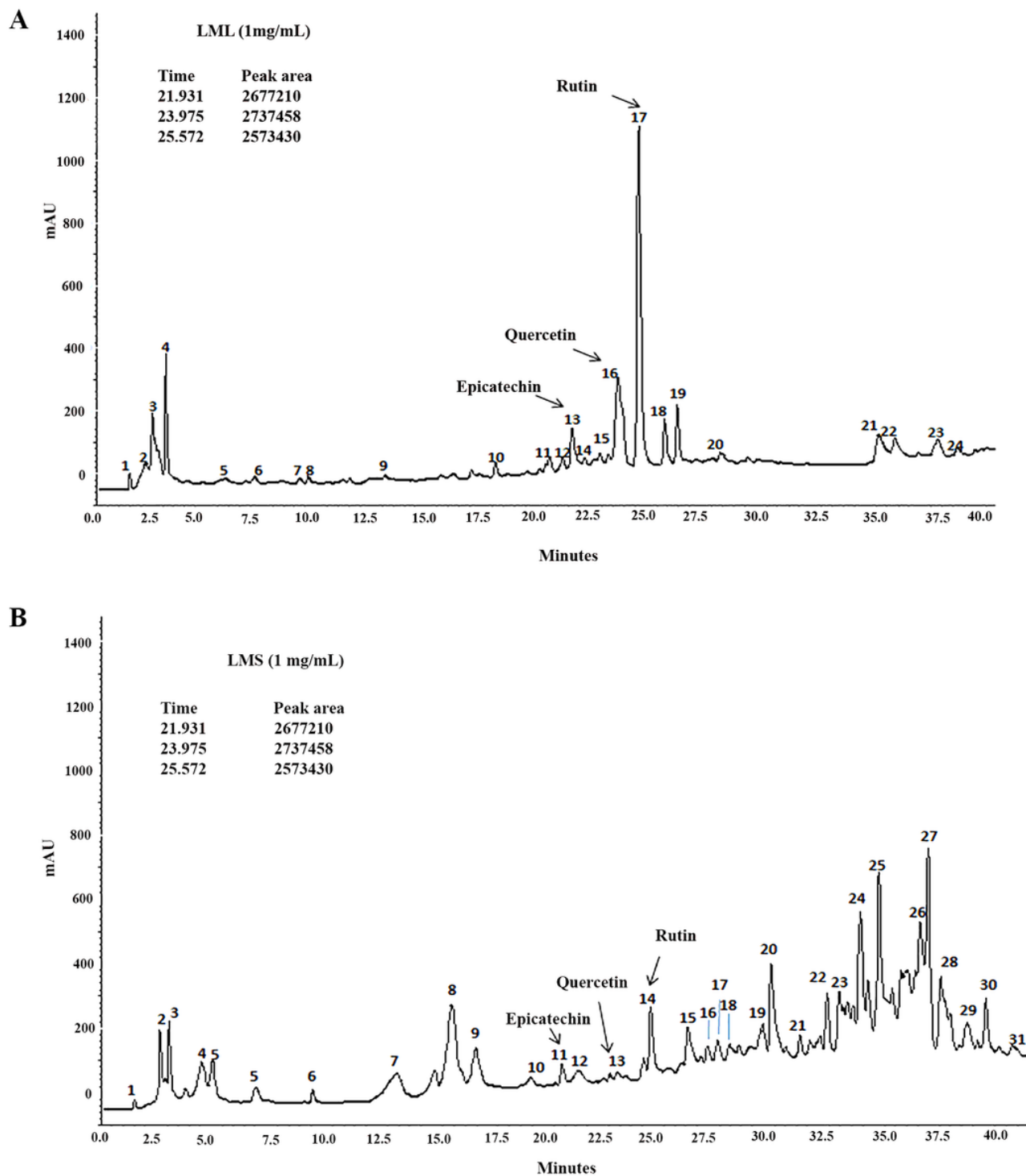


Figure 1

HPLC analysis of *L. micranthus* extracts. (A and B) represent the time and peak areas of the standards (Epicatechin, rutin and quercetin) against LML and LMS respectively.

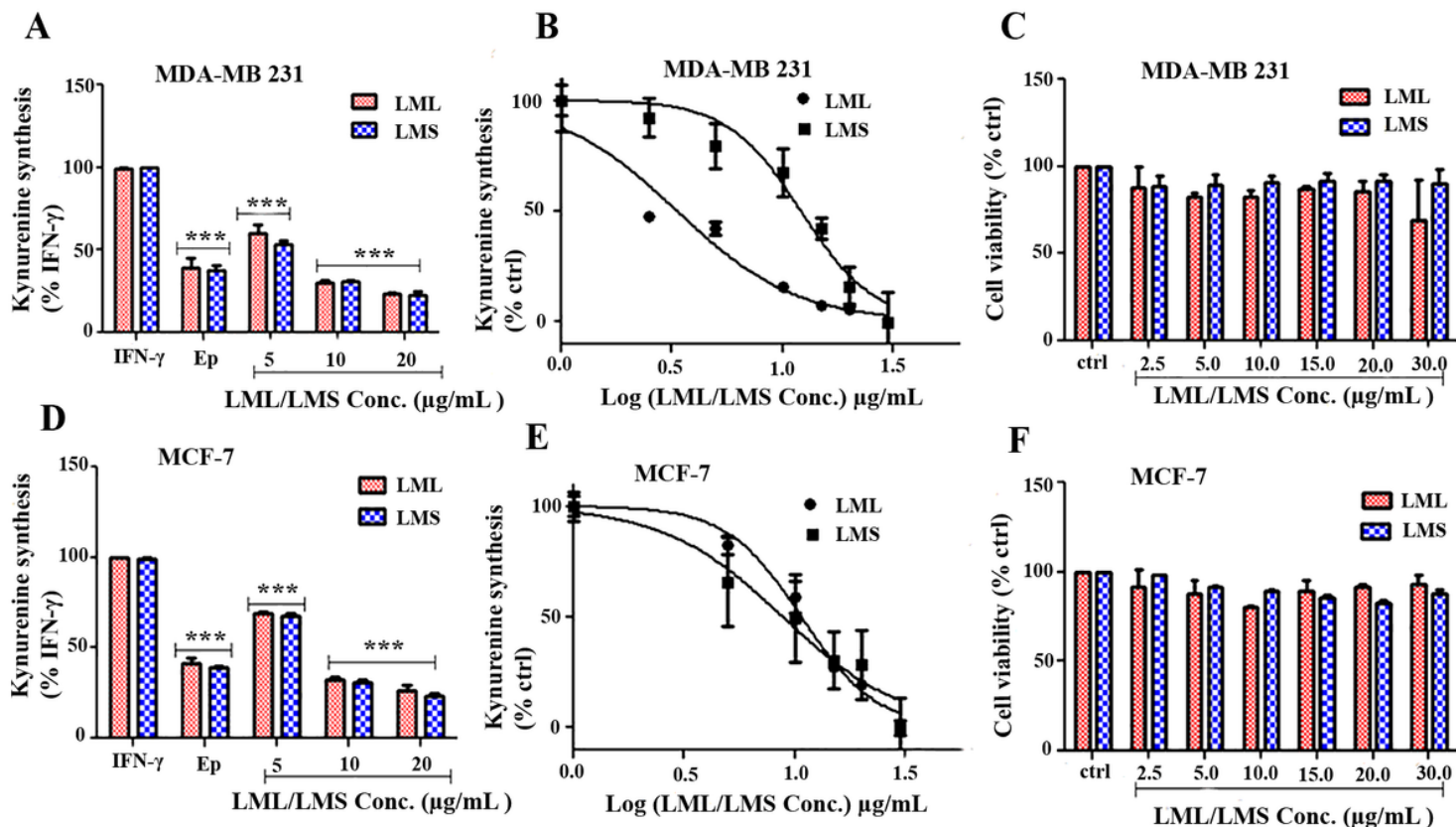


Figure 2

Effects of *L. micranthus* extracts on kynurenine synthesis in human breast cancer cells. MDA-MB 231 and MCF-7 cells were incubated with various concentrations of *L. micranthus* extracts for 2 h followed by addition of IFN- γ (50 ng/mL) for another 24 h. Epacadostat (25nM), a specific inhibitor of IDO1, and DMSO served as positive and negative control respectively. (A and D) Represent MDA-MB 231 and MCF-7 cells pretreated with *L. micranthus* extracts at 5, 10 and 20 $\mu\text{g/mL}$ prior to IFN- γ stimulation, followed by evaluation of kynurenine synthesis in the two breast cancer cells. (B and E) Depict IC₅₀ values for *L. micranthus* extracts on MDA-MB 231 and MCF-7 cells respectively. Their IC₅₀ values were calculated as 4.05 and 12.13 $\mu\text{g/mL}$ respectively for LML and LMS for MDA-MB 231 cells and 10.7 and 8.9 $\mu\text{g/mL}$ respectively for LML and LMS for MCF-7 cells. (C and F) Represent the viability of MDA-MB 231 and MCF-7 cells following LML and LMS pretreatment. ***= $p < 0.001$ compared with the control ($n = 3$).

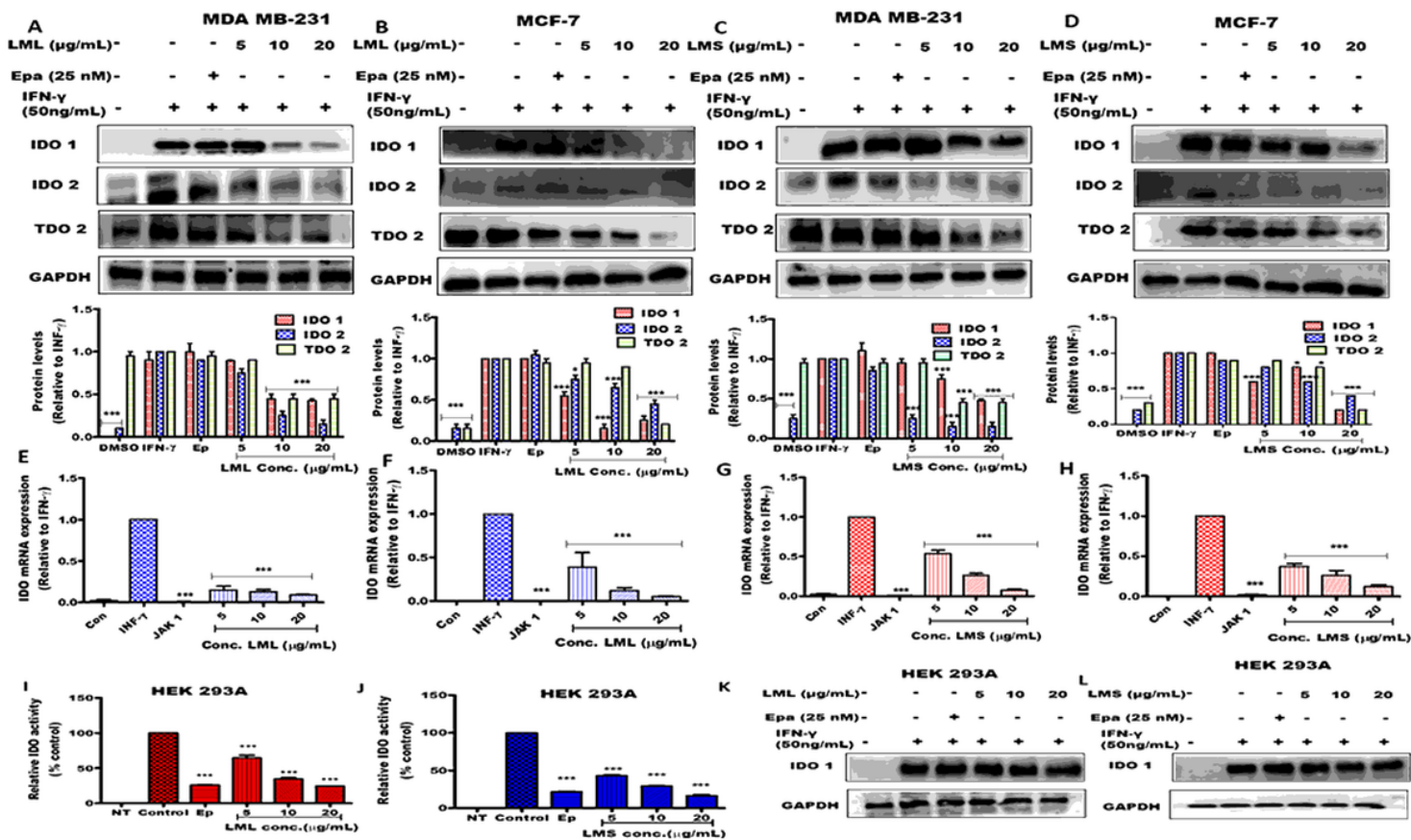


Figure 3

Influence of *L. micranthus* extract on IFN- γ induced expression of IDO and TDO. (A, B, C and D) Represent protein expression of IDO1, IDO2 and TDO2 following *L. micranthus* extracts pretreatment prior to induction by IFN- γ in MDA-MB 231 and MCF-7 cells. (E, F, G and H) Illustrate the influence of *L. micranthus* extracts pretreatment on IFN- γ induced IDO 1 mRNA expression in both MDA-MB 231 and MCF-7 cells. (I, J, K and L) represent the influence of *L. micranthus* extracts on IDO 1 protein expression in pCMV3-IDO1-transfected HEK 293 A cells. ***= $p < 0.001$ compared with control ($n = 3$).

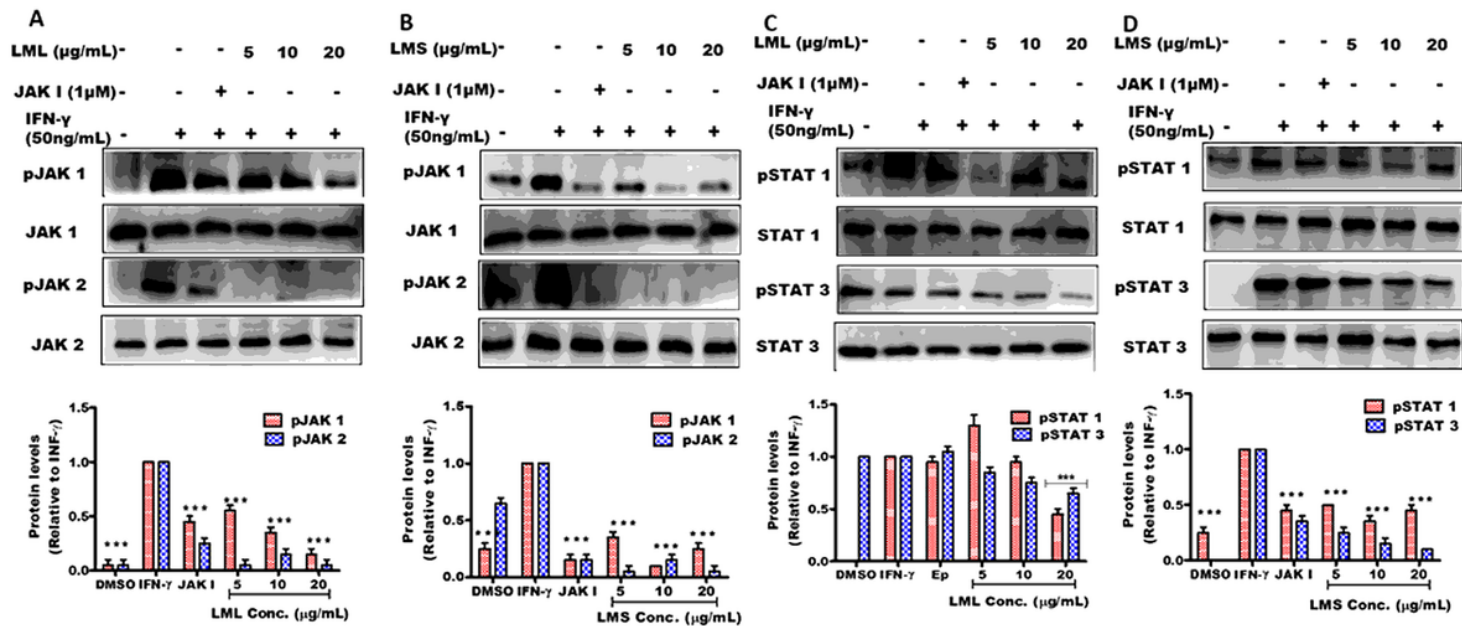


Figure 4

Influence of *L. micranthus* extract on JAK/STAT pathway. (A, B, C and D) Represent the effects of *L. micranthus* extracts on JAK/STAT pathway after IFN-γ induction in MDA-MB 231 cells. (n = 3).

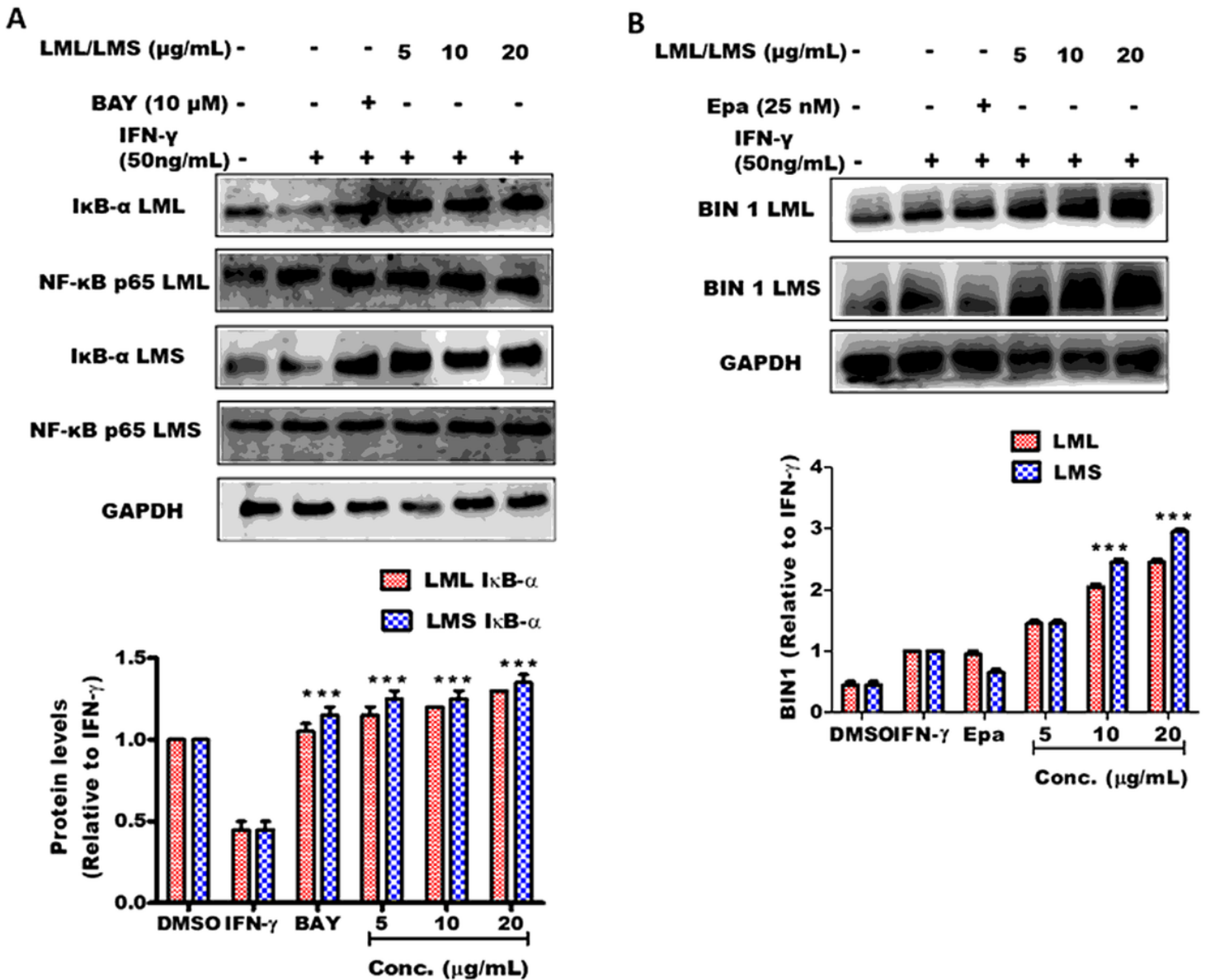


Figure 5

Influence of *L. micranthus* extract on IFN- γ induced NF κ B pathway. (A) Depicts the effects of *L. micranthus* extracts pretreatment on the expression of total NF κ B and I κ B- α after IFN- γ stimulation in MDA-MB 231 cells. (B) Represents the effects of LML and LMS pretreatment on expression of BIN 1 after IFN- γ stimulation in MDA-MB 231 cells. ***= $p < 0.001$ compared with the control ($n = 3$).

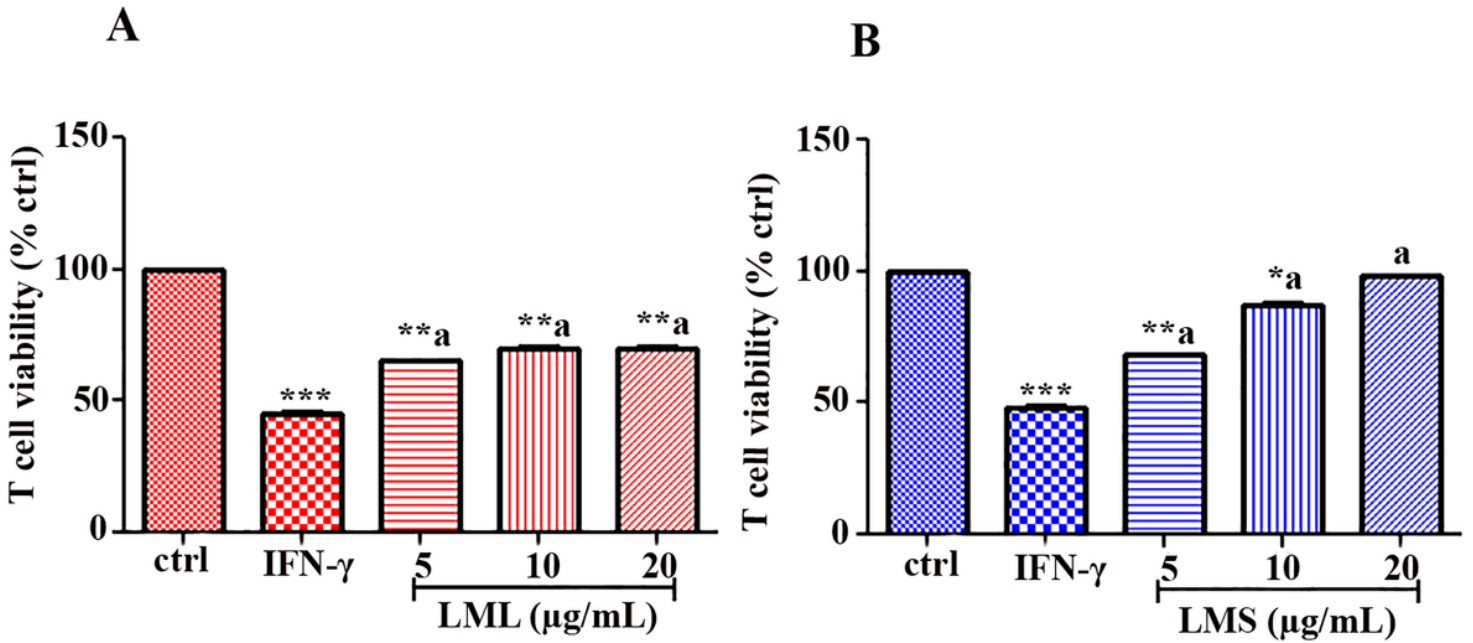


Figure 6

Effect of *L. micranthus* extracts on T-Cell viability. Isolated T cells from mouse were co-cultured with MDA-MB 231 cells, followed by *L. micranthus* extracts treatment. (A) Represents effects of LML treatment on T-cell viability. (B) Represents effects of LMS treatment on T-cell viability. *** = $p < 0.001$ compared with control ($n = 3$). a = $p < 0.05$ compared with IFN- γ ($n = 3$).

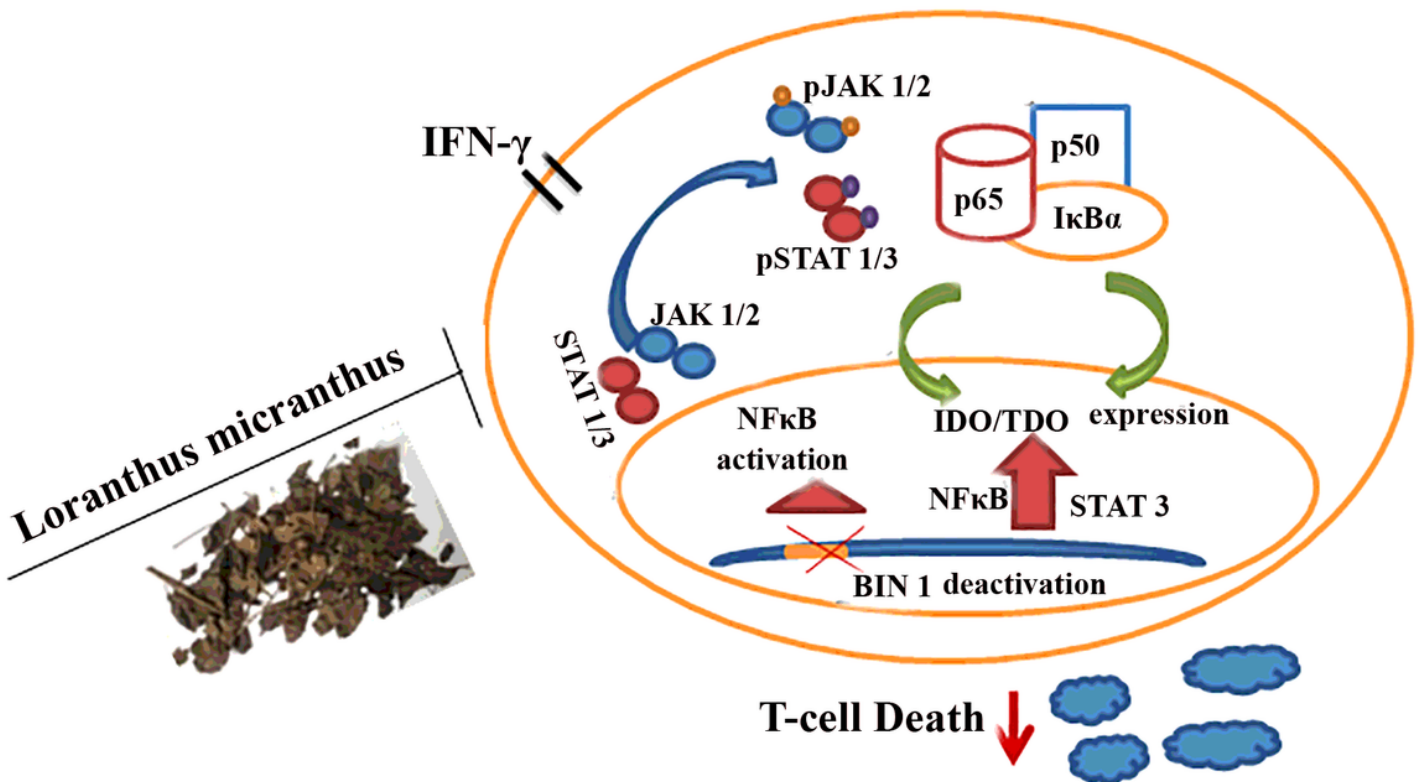


Figure 7

Mechanism of regulation of IDO 1 by *L. micranthus* treatment in human breast cancer cells. INF- γ stimulation of breast cancer cells resulted in activation of JAK/STAT pathway via phosphorylation and NF κ B pathway by degradation of I κ B- α , ultimately leading to inactivation of BIN 1 and decrease in T-cell viability. On the other hand, treatment with *L. micranthus* extract reversed the entire episode of the INF- γ alterations in breast cancer cells relative to control.