**Materials and methods**

1. **Chemicals and plant materials**

The utilized chemicals in the current study were obtained from Merck, USA. *Euphorbia milii* Des Moul. arial parts were gathered from a local garden, Gharbia Governorate, in March 2021. The plant was recognized by Dr. Esraa Ammar, Faculty of Science, Tanta University. A voucher sample (PG-A-EM-W-16) was deposited at the Herbarium of the Plant Ecology Department, Tanta University. The arial parts were dried for 14 days at room temperature at 25°C±2, then for six days in the oven at 40°C. The dried arial parts were then reduced and ground to a fine powder. Thepowder (500 g) was extracted with ethanol (6 L × three times) using the cold maceration method to yield 54.6 g of extract.

1. **LC-ESI-MS/MS profile analysis**

Extract of *E. milii* arial parts wasanalysedadopting procedures previously reported by Attallah et al. (Attallah et al., 2022).

1. **Network pharmacology study**

### Compound targets for Euphorbia milli

### The PubChem Database (http://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2016) was used as an online chemical database to obtain the structure, name, and PubChem IDs for the identified compounds. The 3D molecular structure files were imported into the Binding DB database (https://www.bindingdb.org/bind/index.jsp) as an online tool to identify targets of each structure. We acquired predicted drug targets for each compound. After merging the duplicate data, we chose the target genes with a normalized fit score >0.7 as potential targets for Euphorbia milli-identified compounds.

* 1. **Protein–protein interaction (PPI) data**

The PPI data were obtained from the online databases STRING (Szklarczyk et al., 2016) (https://string db.org/cgi/network?taskId=bIDN4htc9NBY&sessionId=bZWvNlZHMn9h), which have been shown to be reliable for predicting protein-protein interactions. The target proteins were chosen with the human species "Homo sapiens" in mind. STRING was used to identify the proteins that interacted directly or indirectly with the identified targets of Euphorbia milli identified compounds and inflammation.

1. **Anti-inflammatory activity of EMEE** 
   1. Animals

Fifty male Wistar albino rats were got from Veterinary Medicine Faculty, Cairo University, Egypt. Their weights ranged from 190 to 210 g, and they were supplied with food of a standard pellet as well as filtered water. The animals were maintained in 25±2°C temperature. The experiment was approved by the Ethical Committee (Faculty of Pharmacy, Tanta University, Egypt) and the code number was TP/RE/08/22P-0032.

* 1. Carrageenan-induced inﬂammation model

The inflammation was provoked using a subcutaneous (SC) injection of carrageenan solution into the sub-planter right hind paw. We used the left hind paw as a control (Attallah et al., 2022).

* 1. Experimental groups

The animals were sorted into five groups (each contained 10 rats). Group I (or the normal control) administered 0.9% saline orally. The rest of the tested groups were inflamed using carrageenan as previously described and administered the different treatments via oral route. Group II (or the positive control) administered 0.9% saline. Group III administered celecoxib (as a standard treatment). Groups IV and V administered EMEE with doses of 100 and 200 mg/kg, respectively. The rats were anaesthetized and euthanized after four hours. Then, the left and right paws were separated by cutting at the same place and their weights were determined. The average weight of the resulting edema was calculated by determination of the difference between the weights of the right and left paws (Attallah et al., 2022).

* 1. Histological investigation

The tissues of the paws were maintained in formalin solution, then transferred into in paraffin wax. After that thin sections were obtained, stained using hematoxylin and eosin (H&E), and visualized using a light microscope (Alotaibi 2021).

* 1. Immunohistochemical analysis

COX-2 and TNF-α immunostaining of the paw tissues was carried out using ABclonal Technology kits (Massachusetts, USA). Scores were given according to the percentages of the positive staining. Score 0 denotes positive immunostained cells absence. Score 1 denotes positive immunostained cells (1–10%). Score 2 denotes positive immunostained cells (11–50%) and score 3 denotes positive immunostained cells (> 50%) (Negm et al., 2022).

* 1. Molecular investigations

Quantitative real time PCR (qRT-PCR) was utilised to reveal the impact of EMEE on the gene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS). This is in addition to the interleukins IL-5, IL-10, and IL-12. TRIzol reagent (Invitrogen, USA) was used to purify total RNA. Maxima First Strand cDNA synthesis kit with used to synthesise complementary DNA (Thermo Fisher Scientific, USA). Using the Primer 3 PLUS programme (version 0.4.0; available at http://frodo.wi.mit.edu), primer sets for genes were created. The sequences of the primers are shown in Table S1 according to the procedure previously outlined (Freeman et al., 1999) using Applied Biosystem 7500 real-time PCR detection system (Life Technologies, USA) and the SensiFAST SYBR Lo-ROX PCR master mix kit (Bioline, UK). B-actin gene was used a housekeeping gene. In the control group, the relative gene expression for each genes was adjusted to be one (negative control) (Livak and Schmittgen 2001).

**Table S1.** Sequences of the primers

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene** | **Primer Sequences (5´-3´)** | **Reference Sequences** | **PCR product size (bp)** |
| **B-Actin** | F: TGGCTCCTAGCACCATGAAG  R: AACGCAGCTCAGTAACAGTCC | [NM\_007393.5](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=930945786) | 193 |
| **Granulocyte-macrophage colony-stimulating factor (GM-CSF)** | F: CTCACTGGCCCCATGTATAGC  R: TCCTCCTCAGGACCTTAGCC | [NM\_009969.4](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=145301581) | 174 |
| **monocyte chemoattractantprotein-1 (MCP-1)** | F:ACCACCATCAGTCCTCAGGTA  R: CACTGGCTGCTTGTGATTCTC | [NM\_011331.3](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=1450319423) | 134 |
| **iNOS** | F: GGTGAAGGGACTGAGCTGTTA  R: TGAAGAGAAACTTCCAGGGGC | [NM\_010927.4](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=927028884) | 163 |
| **IL-12** | F:CATCTGGCGTCTACACTGCT  R: GCGTGATTGACACATGCTGG | [NM\_001159424.3](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=-2011367563) | 137 |
| **IL-10** | F: AAGCTCCAAGACCAAGGTGTC  R: TTCCGTTAGCTAAGATCCCTGG | [NM\_010548.2](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=291575143) | 183 |
| **IL-5** | F: AGAAGAAGCTCACTGGACAGG  R: CATGGTAGCCGAATGCTGGAA | [NM\_008370.2](https://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&id=118130832) | 179 |

**Table S2.** Top 20 biological KEGG pathways of genes related to inflammations among the studied data set

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Pathway | no of Genes | Fold Enrichment | Genes |
| 1 | Pathways in cancer | 17 | 14.62385 | ESR1 MMP9 CDK6 MET IL2 PPARD VEGFA PGF FLT3 CDK2 PIM1 IGF1R EGFR KIT TERT AR F2 |
| 2 | PI3K-Akt signaling pathway | 13 | 16.74282 | CDK6 PIK3CG MET IL2 VEGFA PGF FLT3 CDK2 KDR IGF1R EGFR NTRK2 KIT |
| 3 | Chemical carcinogenesis | 10 | 23.14315 | ESR1 AHR VEGFA CYP1B1 CYP1A1 CYP1A2 EGFR CYP3A4 AR PPARA |
| 4 | MAPK signaling pathway | 10 | 15.50748 | MET VEGFA PGF FLT3 KDR IGF1R EGFR NTRK2 KIT TNF |
| 5 | Ras signaling pathway | 9 | 17.76312 | MET VEGFA PGF FLT3 KDR IGF1R EGFR NTRK2 KIT |
| 6 | Proteoglycans in cancer | 8 | 18.05624 | ESR1 MMP9 MET VEGFA KDR IGF1R EGFR TNF |
| 7 | MicroRNAs in cancer | 7 | 19.82261 | MMP9 CDK6 MET VEGFA PIM1 CYP1B1 EGFR |
| 8 | Rap1 signaling pathway | 7 | 15.19733 | MET VEGFA PGF KDR IGF1R EGFR KIT |
| 9 | EGFR tyrosine kinase inhibitor resistance | 6 | 34.62684 | MET VEGFA KDR IGF1R EGFR AXL |
| 10 | Focal adhesion | 6 | 13.6776 | MET VEGFA PGF KDR IGF1R EGFR |
| 11 | Human papillomavirus infection | 6 | 8.264411 | CDK6 VEGFA CDK2 EGFR TERT TNF |
| 12 | Metabolism of xenobiotics by cytochrome P450 | 5 | 30.80541 | CYP1B1 CYP1A1 CYP1A2 CYP3A4 CYP2D6 |
| 13 | PPAR signaling pathway | 5 | 30.39467 | SCD PPARD FABP5 FABP4 PPARA |
| 14 | Endocrine resistance | 5 | 23.99579 | ESR1 MMP9 IGF1R EGFR CYP2D6 |
| 15 | Prostate cancer | 5 | 23.50103 | MMP9 CDK2 IGF1R EGFR AR |
| 16 | Breast cancer | 5 | 15.50748 | ESR1 CDK6 IGF1R EGFR KIT |
| 17 | Hepatitis C | 5 | 14.51975 | CDK6 CDK2 EGFR PPARA TNF |
| 18 | Hepatocellular carcinoma | 5 | 13.6503 | CDK6 MET IGF1R EGFR TERT |
| 19 | Transcriptional misregulation in cancer | 5 | 11.87292 | MMP9 MET FLT3 CDK9 IGF1R |
| 20 | Human T-cell leukemia virus 1 infection | 5 | 10.26847 | IL2 CDK2 TERT LCK TNF |

**Figure S1.** The total ion chromatogram of detected metabolites of *E. milii* ethanol extract in negative ion mode

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