Effect of a methanolic extract of *Salvadora oleoides* Decne. on LPS-activated J774 macrophages, its *in vitro* and *in vivo* toxicity study and dereplication of its chemical constituents

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**A B S T R A C T**

*Salvadora oleoides* is used in Pakistani traditional medicine to treat inflammatory conditions, piles, boils, and ulcers. To evaluate the anti-inflammatory potential of *S. oleoides* (a mixture of aerial branches, leaves, and stem bark), we prepared crude extracts in Soxhlet apparatus by successively using different solvents and found the methanolic extract (OLM) to significantly inhibit the LPS-induced expression of pro-inflammatory cytokines and enzymes in J774 macrophages, at 50 µg/mL concentration. We also analysed the chemical constituents of OLM by dereplication, performed by HPLC-MS/MS and molecular networking. The major detected constituents were flavonoids and phenolic acids glycosides, most of them identified for the first time in *S. oleoides*. We also evaluated the toxicity of OLM against five cell lines, namely Caco-2, HepG2, HeLa, J774, and WI-38 by MTT assay. The IC50 was found to be higher than 100 µg/mL against these five cell lines after 72 h treatment. Furthermore, OLM was tested in mice for acute and sub-acute oral toxicity according to the guidelines of the Organisation for Economic Co-operation and Development (OECD). OLM was found non-toxic, except for some fibrosis observed in the spleens of treated mice in the sub-acute oral toxicity test. Our results confirm the anti-inflammatory potential of OLM and that it could be tested in *in vivo* inflammatory models, but its effect on the spleen should be considered before designing the experiments.

1. Introduction

Since the ancient times, humans have been using plants, in different forms, for general wellbeing and to treat various ailments. Because of the effectiveness and easy availability, plant-derived products remain a very effective and free from side effects without any scientific evidence the biggest misconception. Moreover, there is not enough documented data on the safety profile of plant-based medicines. Hence, systemic studies not only about the efficacy but also about the toxicity of natural products-based medicines are required [1,2].

*Salvadora oleoides* Decne. belonging to the family Salvadoraceae is a naturally growing shrub or small tree found in the arid and alkaline regions of Western India and Pakistan. Locally it is called “jal,” “pilu,” or “wan” and has a lot of medicinal uses in traditional medicines. Ripened fruits of *S. oleoides* are considered nutritive and are eaten. Aerial branches of this plant are used as miswak to clean the teeth, maintain oral hygiene, and protect from gum inflammation. Fruits and leaves are used to treat enlarged spleen, fever, and rheumatism. Leaves are also used to treat cough, boils, and ulcers. The leaf paste is used to relieve the inflammation of legs and is also applied topically to open wounds. Its stem bark is useful in rheumatic pain, bronchitis, piles, and tumors.
Besides these traditional uses, *S. oleoides* is reported to possess anti-oxidant (stem and root) [8], anti-inflammatory (leaves) [6], anti-hyperlipidemic (leaves) [9,10], hypo-glycemic (stem and leaves) [7], and anti-bacterial (stem bark) [11] activities. Little data are available about the chemical profile of *S. oleoides* indicating the presence of an alkaloid (stem) [5], some fatty acids and lipidic compounds (aerial parts) [5,6], a few flavonoids (leaves) [10], a glucosinolate (aerial parts, stem, and root) [5], an isocoumarin (whole plant) [12], and a few terpenoid derivatives (aerial parts) [5].

As the leaves, aerial branches, and stem bark of *S. oleoides* are used to treat inflammatory conditions, we decided to evaluate the anti-inflammatory potential of the mixture of these three plant parts. Inflammation is a defensive response of our body during which immune cells, especially macrophages, release pro-inflammatory cytokines and chemokines to neutralize the inflammatory triggers and promote tissue recovery [13]. However, uncontrolled exacerbated inflammatory response may lead to severe disorders such as asthma, inflammatory bowel diseases, neurodegenerative diseases, and rheumatoid arthritis supporting the need for safe anti-inflammatory approaches [14].

Thus, we prepared and tested different non-cytotoxic crude extracts of this plant and found methanolic extract (OLM) to significantly inhibit the expression of pro-inflammatory cytokines (TNF-α, IL-6, and MCP-1) and enzymes, i.e., cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS), in LPS-activated J774 macrophages. In literature we found that there is negligible experimental data on its safety profile and the available data about its chemical constituents are scarce. Therefore, we evaluated the toxicity of OLM in *in vitro* models of different cell lines and in *in vivo* models according to the guidelines of the Organisation for Economic Co-operation and Development (OECD). We also analysed the chemical constituents of OLM by HPLC-MS/MS based dereplication and molecular networking.

### 2. Experimental

#### 2.1. Chemicals and reagents

Dichloromethane (DCM), ethyl acetate (EtOAc), formic acid, hexane (Hex), hydrochloric acid, methanol (MeOH), and Tween 80 were purchased from VWR International (Radnor, PA, USA), Camptothecin, dimethylsulfoxide (DMSO), fetal bovine serum (FBS), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharides (LPS), from *E. coli* serotype O55:B5, and rutin ([9](#) [10]) were purchased from Sigma-Aldrich (Bornem, Belgium). Narcissus ([18](#)) was purchased from Extrasyntese, France. Anesthesia solution (4.75 saline water, 0.25 xylazine, 0.5 ketamine) was kindly provided by Prof. Jean-Paul Dehoux (UCLouvain).

#### 2.2. Collection of the plant material

Leaves, aerial branches, and stem bark of *S. oleoides* were collected from a village named As-haba, District Jhang, Punjab, Pakistan (31°07’08.8”N 72°20’32.1’’E) in May 2018 and 2020. The plant was authenticated by Prof. Zafarullah Zafar, Botany Division, Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan 60800, Pakistan. A voucher number PAK-ZAFAR 001 was assigned and a plant sample was deposited at GNOS/LDRI/UCLouvain, Belgium for future reference. Furthermore, the plant name was confirmed by checking in The World Flora Online (http://www.worldfloraonline.org, accessed on 27 October 2019).

#### 2.3. Preparation of crude extracts

The collected plant material (leaves, aerial branches, and stem bark) was dried under shade, ground, mixed together, and then extracted in Soxhlet apparatus (8 h) using successively Hex, DCM, EtOAc, and MeOH (50 g or 500 g of the plant powder in 250 mL or 2.2 liters of the solvent). The organic solvents were removed by rotary evaporator at 35 °C and four corresponding crude extracts (OLH, OLD, OLE, and OLM, respectively) were obtained. A decoction (OLW) was also prepared by boiling (15 min) 50 g of the plant powder in one liter of water, filtered, and lyophilized. The extracts were stored at −20 °C until further use.

#### 2.4. Cell cultures

Human lung fibroblast cell line WI-38 (ATCC CCL-75, bought from LGC standards, France), immortal human cell line HeLa (a kind gift from Prof. Marie-Claire de Pauw-Gillet, ULiège), and human liver cancer cell line HepG2 (a kind gift from Prof. Buc-Calderon, UCLouvain) were grown in Dulbecco’s modified eagle medium (DMEM, containing 1 mM pyruvate and 1 g/L glucose, Gibco). Immortalized cell line of human colorectal adenocarcinoma cells Caco-2 (a kind gift from Prof. Ana Beloqui Garcia, LDRI, UCLouvain) were grown in DMEM containing 5% non-essential amino acids and 5% glycine. Murine macrophage cell line J774 was kindly provided by Prof. Françoise Van Banbeke, LDRI, UCLouvain. J774 cells were grown in Roswell Park Memorial Institute medium (RPMI 1640 medium with GlutaMAX, Gibco). All the medium were supplemented with 10% FBS and streptomycin-penicillin (100 UI/mL) (Lonza, Belgium). HeLa, HepG2, WI-38, and J774 cells were kept at 37 °C in a 5% CO2 incubator while Caco-2 cells were kept in a 10% CO2 incubator.

#### 2.4.1. MTT assay

MTT assay was performed on Caco-2, HeLa, HepG2, J774, and WI-38 cells, as described previously [15]. Cells were treated with the crude extracts or camptothecin (as positive control) for 72 h and after that with MTT for 45 min. Final concentrations of crude extracts were 100 μg/mL and 50 μg/mL or 100 μg/mL that was serially diluted to calculate IC50. After 72 h of incubation, MTT was replaced by DMSO and absorbance was measured with a spectrophotometer (SpectraMax-Molecular Devices, UK) at 570 nm. 620 nm was used as a reference wavelength. Based on their solubility, stock solutions of OLD, OLE, and OLW were prepared in DMSO, of OLH in ethanol, and of OLW in EtOH-H2O (25:75). Final % of EtOH, DMSO, and EtOH-H2O (25:75) was 0.5% during the incubation. Non-treated (blank) and vehicle-treated cells were assessed as well. Production of formazan was measured as percentage of blank, considering blank as 100%.

#### 2.4.2. Effect of crude extracts on LPS-induced expression of pro-inflammatory cytokines and enzymes in J774 macrophages

**J774** cells were seeded overnight in a 24-well plate at the density of 2.5 × 10⁵ cells/well in 1 mL per well of RPMI medium. Then the medium was replaced by the crude extracts solutions (50 μg/mL or vehicle (0.25% DMSO or EtOH-H2O (25:75)) with or without LPS (100 ng/mL) [16]). After 8 h incubation, culture medium was collected and stored at −20 °C for enzyme-linked immunosorbent assay (ELISA). Cell culture plates were stored at −80 °C.

#### 2.7. Real-time quantitative PCR (RT-qPCR)

Total RNA was recovered from the cells using TriPure (Roche, Switzerland). The RNA was then quantified and 1 μg of the RNA was used to obtain cDNA using the RT GoScript kit from Promega. RT-qPCR analysis was performed on a QuantStudio 3 instrument (Applied Biosystems), using GoTaq qPCR Master mix from Promega, and by applying the following conditions for amplification: an initial stage at 95 °C (10 min) followed by 45 cycles of denaturation at 95 °C for 3 s, and then annealing at 60 °C for 26 s, and extension at 72 °C for 10 s. At the end of the reaction, melting curves of the products were obtained. The resulting cycle threshold (Ct) values were recorded for each gene and were
Ready-Set-Go! Kit following the manufacturer's instructions. Results are expressed relative to control, using the delta Ct normalised to 60 S ribosomal protein L19 (RPL19) mRNA, used as a reference. Results are expressed relative to control, using the delta Ct method. Primer sequences are given in Table 1 [17].

2.8. Cytokines quantification by ELISA

IL-6 was quantified in the cell culture media by ELISA using the Ready-Set-Go! Kit following the manufacturer’s instructions (Invitrogen, ThermoFisher Scientific) [18].

2.9. HPLC-PDA-HRMS/MS analysis

HPLC-PDA analyses were performed on an HPLC-PDA system, consisting of a Thermo Accela autosampler, Accela pump, and a PDA detector, controlled using a Thermo Scientific ChromQuest software. For HPLC-PDA-HRMS/MS analysis, another similar HPLC-PDA system was connected with a Thermo Scientific LTQ-orbitrap XL mass spectrometer (Thermo Scientific™, Germany), controlled by a Thermo Scientific Xcalibur X software. The chromatographic separation was done on a 5 µm particles Phenomenex Luna C18, 250 × 4.6 mm column. Stock solutions of crude extract and standards were prepared in methanol at 8 mg/mL and 50 µg/mL concentration, respectively and the injection volume was 20 µL. The mobile phase consisted of 0.1% of formic acid in water (solvent A) and acetonitrile (solvent B). Elution of the mobile phase was performed with a flow rate of 0.8 mL/min in gradient mode: 0–5 min (17% B); 5–27 min (17–40% B); 27–28 min (40–100%); 28–38 min (100% B); 38–39 min (100–17% B); and 39–50 min (17% B). Detection wavelengths for chromatograms were set between 200 and 600 nm. HRMS analyses were performed in atmospheric pressure chemical ionization (APCI) positive and negative modes with the following inlet conditions: for positive mode: APCI vaporizer temperature 400 °C; capillary temperature 250 °C; auxiliary gas flow 25 a.u.; sheath gas flow 25 a.u.; sweep gas flow 5 a.u.; capillary voltage 21 V discharge current 5 µA; and tube lens voltage 75 V. For negative mode: the only differences from the positive mode were the capillary voltage (~10 V) and tube lens voltage (~125 V). The MS/MS events were performed on the most abundant ions detected in full MS scans.

2.10. MS Data treatment

HRMS data (. RAW) files, run in APCI negative mode, were treated using an open source software MZmine2 version 2.53 [19]. Noise level was set to 7.0 × 10^4 and 1 for mass detection at MS^1 and MS^2 levels, respectively. The ADAP chromatogram builder was used by setting to a minimum group size of 5 scans, group intensity threshold of 7.0 × 10^4, minimum highest intensity of 7.0 × 10^4, and m/z tolerance of 10 ppm. The intensity window S/N as an S/N estimator was used to deconvolute the chromatogram by ADAP wavelets algorithm with a signal to noise ratio set at 200, a minimum feature of 1.0 × 10^4, a peak duration ranging from 0.02 to 0.6 min, a coefficient threshold at 40, and an RT wavelet range of 0.01–0.2 min “Isotope peak grouper” was used to detect isotope pattern forming peaks and to remove additional minor isotopic peaks using an m/z tolerance of 10 ppm, RT tolerance of 0.5 min (absolute), maximum charge set at 1. The most intense isoform was selected as the representative isoform. The resulting mass list was filtered using “duplicate peak filter” and “feature list rows filter” to remove all duplicates and features and keep only MS^2 scans.

2.11. Mass spectral organization and dereplication

MZmine, after MS data treatment, gave a.mgf file which was exported to the GNPS website to construct a molecular network [20]. The precursor ions mass tolerance was set to 2.0 Da with MS/MS fragmentation tolerance of 0.5 Da. A network was created by setting the edges to have a cosine value of 0.72 and more than 5 common peaks. The spectra in the network were then searched against GNPS’s spectral libraries, under the same conditions. Additional putative identification of unmatched peaks was carried out by comparing available MS/MS fragmentation patterns in the literature (Dictionary of Natural Products 30.2 and Reaxys). Data were visualized using Cytoscape 3.8.0 software [21]. Data of peak area was added to the network from.csv file, obtained from MZmine. Size of nodes was set proportionally to the total area of each peak, detected in the analyzed extract.
2.12. Animals and vehicle used for dose administration

The in vivo experiments were all performed on healthy, nulliparous, and non-pregnant female NMRI mice (7–12 weeks old, around 30 g weight), purchased from Janvier Labs, France. Animals were housed in cages, four animals per cage, at a relative humidity of 30–60% and a temperature of 23 °C ± 3 °C and were exposed to a 12:12 h light /dark cycle. The animals had free access to commercial food pellets and acidified water (184 μL of HCl per liter of water) ad libitum throughout the study period. The animals were kept at least for one week to get acclimatized before starting the manipulations. The vehicle used for dose administration (400 μL p.o and 200 μL i.p) consisted of 90% of sterilized water, 7% of tween 80%, and 3% of ethanol. All the in vivo experiments were approved by the Ethical Committee for animals use at the Health Sciences Sector of the Université catholique de Louvain (2017/UCL/MD/017).

2.13. Highest tolerated dose (HTD)

As suggested by the body “Drugs for Neglected Diseases initiative” (DNDi), this study was carried out to determine the highest dose of methanolic extract of S. oleoides (OLM) that can be tolerated and to help us decide the dose for the next tests [22]. Four groups were evaluated (2 mice in each group) with the dose volume of 400 μL and 200 μL for oral and intraperitoneal administration, respectively.

- **Group I**: control group treated with vehicle (p.o);
- **Group II**: control group treated with vehicle (i.p);
- **Group III**: treated with OLM, 200 mg/kg, 300 mg/kg, 500 mg/kg, and 1000 mg/kg (administered every two hours, in total 2000 mg/kg/day) (p.o);
- **Group IV**: treated with OLM, 200 mg/kg, 300 mg/kg, 500 mg/kg, and 500 mg/kg (administered every two hours, in total 1500 mg/kg/day) (i.p).

Group I and II mice were kept in one cage and group III and IV mice were kept in the second cage. Mice were monitored carefully for next two days to detect any signs of illness or death.

2.14. Acute oral toxicity study

Acute oral toxicity of OLM was evaluated according to the OECD Guideline 423, with some modifications [23]. Three mice, kept in the same cage, were treated once with 2000 mg/kg of OLM (p.o). The mice were euthanized using CO₂ after two weeks of observation. Autopsy was done for macroscopic analysis of internal organs (heart, liver, lungs, kidneys, spleen, and stomach). The experiment was repeated two times.

2.15. Sub-acute oral toxicity study

Sub-acute oral toxicity of OLM was evaluated according to the OECD Guideline 407, with some modifications [24]. Twenty mice, divided in two groups, were used (dose volume: 400 μL).

- **Group I**: control group treated with vehicle (p.o);
- **Group II**: treated with OLM, 1000 mg/kg/day, for two weeks (p.o).

The mice were observed daily, thoroughly. After the last dose, four mice (two from each group) were randomly taken for satellite control and kept for one further week under observation. From the remaining mice, blood samples were taken for hematocrit analysis from the tails in HIRSCHMANN® sodium heparinized disposable microhematocrit capillary tubes. The animals were anesthetized (100 μL of anesthesia per 10 g body weight of mice, i.p) and blood samples were collected for
biochemical analysis, by cardiac puncture, in VACUETTE® LH lithium tubes coated with lithium heparin on the inside. Afterwards, the mice were euthanized by cervical dislocation and internal organs (heart, liver, lungs, kidneys, spleen, and stomach) were removed. After weighing and macroscopic analysis, the organs were washed with PBS and fixed in a 4% paraformaldehyde solution (PFA). Satellite mice were treated the same way after keeping them under observation for one further week.

2.16. Biochemical and hematocrit analysis

The tubes (microhematocrit capillary and LH lithium) were centrifuged at 4000 g for 10 min. Hematocrit was calculated by measuring the ratio of the packed cells to the total volume. Plasma was collected after centrifugation, from LH lithium tubes and biochemical parameters were measured by using Fujifilm® DRI-CHEM NX500i biochemistry analyzer according to the manufacturer’s recommendations. Calibration of all the assays was realized with the test-specific calibrator, provided by the manufacturer. 10 μL plasma samples were used for each parameter. Biochemical parameters assayed were albumin (Alb), gamma-glutamyl transferase (GGT), creatine (CRE), direct bilirubin (DBIL), total albumin (TBIL), aspartate aminotransferase (GOT or AST), and alanine aminotransferase (GPT or ALT).

2.17. Histological analysis

Four animals from treated group and three animals from control group were randomly chosen for histological analysis, done at “IREC Imaging Platform” of UCLouvain. Stomachs, spleens, livers, and kidneys, fixed in 4% PFA, were embedded in paraffin. Three sections of 5 μm thickness were cut, for each organ, fixed on glass slides, and stained with hematoxylin and eosin (H&E) and Sirius red by using the standard techniques. Slides were examined and images of the tissues were captured by LEICA SCN400 slide scanner and then analyzed by Cytomine software. Collagen content in the cells was quantified by an open-source software QuPath.

2.18. Statistical analysis

All statistical analyses were done by the GraphPad Prism 8.0.1 software. The statistical test used to analyze the data of each experiment figure is not included in the text.
Table 2
Putative identification of major compounds present in OLM, detected by HPLC-DAD-HRMS/MS (APCI negative mode) analysis.

| Code | t_R (min) | UV(λmax nm) | m/z | MS major ion (s) | MS/MS fragments [m/z] | Molecular formula | Δρpm | ΔΔDa | Putative identification | Ref. |
|------|-----------|-------------|-----|-----------------|----------------------|-------------------|------|------|------------------------|------|
| 1    | 3.28      | n.d.        | 387.1123 | [M – H + FA]^- | 215.0319; 179.0556 | C_{12}H_{20}O_{11} | -3.77 | -1.29 | Sucrose                |      |
| 2    | 4.20      | n.d.        | 341.1071 | [M – H]^2-     | 153.0189              | C_{12}H_{20}O_{2} | 1.56 | 0.49 | Protocatechic acid hexoxide |      |
| 3    | 4.97      | n.d.        | 342.1240 | [M – H + FA]^- | 383.1176              | C_{12}H_{24}O_{2} | -3.53 | -1.35 | n.i.                |      |
| 4    | 5.10      | 254         | 579.1921 | [M – H + FA]^- | 323.0970              | C_{12}H_{18}O_{4} | -3.25 | -1.73 | Sinapoyl dihexose     |      |
| 5    | 9.95      | n.d.        | 243.1835 | [M – H]^2-     | 299.0849              | C_{12}H_{20}O_{3} | 0.51 | 0.21 | n.i.                |      |
| 6    | 10.64     | n.d.        | 417.1399 | [M – H]^2-     | 131.0711; 173.0813 | C_{12}H_{18}O_{3} | -1.85 | -0.54 | n.i.                |      |
| 7    | 11.57     | 275         | 325.0914 | [M – H]^2-     | 163.0395              | C_{12}H_{16}O_{3} | -2.90 | -0.94 | Hexose coumaric acid isomer |      |
| 8    | 12.88     | n.d.        | 327.1076 | [M – H + FA]^- | 147.0444; 165.0552 | C_{12}H_{20}O_{3} | -1.20 | -0.39 | n.i.                |      |
| 9    | 13.35     | n.d.        | 499.1654 | [M – H + FA]^- | 453.1590              | C_{12}O_{16}H_{20}O_{3} | -4.01 | -1.82 | n.i.                |      |
| 10   | 13.38     | 255, 350    | 755.2021 | [M – H]^2-     | 300.0261; 310.0340; 591.1326; 489.1010 | C_{12}H_{20}O_{25} | -1.81 | -1.37 | Quercetin-trihexose     |      |
| 11   | 15.35     | 255, 351    | 739.2076 | [M – H]^2-     | 284.0308; 285.0386; 575.1574; 473.1065 | C_{12}H_{20}O_{19} | -1.29 | -0.95 | Kaempferol-trihexose^b | [29] |
| 12   | 15.68     | n.d.        | 371.0981 | [M – H]^2-     | 249.0603; 231.0501 | C_{12}H_{20}O_{18} | -2.48 | -0.92 | Hydroxyferuloyl hexose |      |
| 13   | 15.90     | 258, 350    | 769.2170 | [M – H]^2-     | 315.0490; 314.0414; 605.1476 | C_{12}H_{20}O_{25} | -2.75 | -1.2 | Isorhamnetin trihexose |      |
| 14   | 16.04     | n.d.        | 525.3065 | [M – H + FA]^- | 479.2998 | C_{12}H_{20}O_{24} | -2.25 | -1.08 | n.i.                |      |
| 15   | 16.96     | 255, 353    | 609.1442 | [M – H]^-     | 301.0340; 300.0260 | C_{12}H_{20}O_{16} | -2.23 | -1.36 | Rutin^b^b^b | [30, 31] |
| 16   | 17.57     | n.d.        | 305.0813 | [M – H]^2-     | 263.0749 | C_{12}H_{20}O_{16} | -3.04 | -1.17 | Sinapoyl-hexose |      |
| 17   | 18.15     | 254, 348    | 463.0876 | [M – H]^2-     | 301.0340; 300.0260 | C_{12}H_{20}O_{12} | -0.11 | -0.05 | Isorhamnetin hexose^b | [31] |
| 18   | 18.36     | 254, 352    | 623.1600 | [M – H]^2-     | 315.0497; 300.0260 | C_{12}H_{20}O_{16} | -1.94 | -1.21 | Narcisin^b | [29] |
| 19   | 19.25     | 288         | 340.0841 | [M – H]^2-     | 178.0325 | C_{12}H_{18}O_{6} | 5.85 | 1.99 | Avenanthramide S peptide isomer |      |
| 20   | 19.36     | 260, 348    | 447.0923 | [M – H + FA]^- | 284.0311; 289.0389; 325.0496 | C_{12}H_{20}O_{11} | -0.98 | -0.44 | Kaempferol hexose |      |
| 21   | 19.50     | 253, 334    | 477.1019 | [M – H]^2-     | 314.0419; 315.0490; 357.0600 | C_{12}H_{20}O_{12} | -2.94 | -1.40 | Isorhamnetin hexide |      |
| 22   | 21.20     | n.d.        | 431.1901 | [M – H + FA]^- | 205.1225 | C_{12}H_{20}O_{6} | -3.75 | -1.44 | Roseoside or isomer derivative |      |
| 23   | 21.63     | n.d.        | 385.1848 | [M – H]^2-     | 179.0556 | C_{12}H_{20}O_{4} | -1.25 | -0.23 | Azelaic acid |      |
| 24   | 26.77     | n.d.        | 421.1859 | [M – H + FA]^- | 125.0970 | C_{12}H_{20}O_{4} | -3.63 | -1.36 | n.i.                |      |

*a* Previously identified in *Salvadora oleoides*; ^b* Previously identified in species of genus *Salvadora*; ^d* Fragments obtained from [M – H]^2- adduct; n.d. UV signal not detected; n.i. non-identified compound; ^c* compounds identified by standard comparison. Retention times correspond to those recorded on the HPLC-PDA-HRMS system used in this study.

is mentioned in the legend of the relevant figure and table. For all tests, the significance level was p < 0.05.

3. Results and discussion

3.1. Initial screening of the crude extracts by MTT assay

As one of the frequently used extraction schemes in our lab, we prepared four crude extracts (OLH, OLE, OLD, and OLM) from *S. oleoides* by Soxhlet extraction using successively four solvents of increasing polarity, namely Hex, DCM, EtOAc, and MeOH, respectively. Each of the four obtained extracts contain different chemical constituents depending on the polarity of the extraction solvents, from OLH to OLM, non-polar to polar compounds. A decoction (OLW) was also prepared to mimic the traditional use of the plant.

MTT assay (MTT reduction into formazan by mitochondrial dehydrogenase) was performed on two cell lines, namely J774 and WI-38, to evaluate the effect of these extracts on cellular metabolic activity, as an indicator of cell viability. J774 cells were selected because they will be used in the next assay and WI-38 cells were used to evaluate if a potential effect on J774 cells was cell-specific or not. Production of formazan was measured as percentage of non-treated cells (blank), considering blank as 100%. Formazan production by vehicle-treated cells was more than 97% (Fig. S1). As shown in Fig. 1, OLH, OLE, and OLM treated cells showed decreased formazan production while OLM and OLW treated cells were quite active in producing formazan and hence OLM and OLW were considered non-cytotoxic. IC_{50} values obtained for camptothecin (positive control) were 7.1 ± 0.6 ng/mL and 34.2 ± 4.9 ng/mL for J774 and WI-38 cells, respectively, as reported previously [15].
incubating the cells with the crude extracts (50 ng/mL) for 8 h, as previously reported [15]. As expected for this model, LPS incubation increased the expression of cytokines (i.e., IL-6, TNF-α, and MCP-1) and enzymes (iNOS and COX-2) that are associated with inflammatory processes (Figs. S2,3).

Given the cytotoxicity of OLH, OLD, and OLE on J774 cells, the effect of only OLM and OLW was analyzed on the expression of pro-inflammatory cytokines and enzymes in LPS-activated J744 cells by incubating the cells with the crude extracts (50 ng/mL) and LPS (100 ng/mL) for 8 h, as previously reported [15]. As expected for this model, LPS incubation increased the expression of cytokines (i.e., IL-6, TNF-α, and MCP-1) and enzymes (iNOS and COX-2) that are associated with inflammatory processes (Figs. S2,3).

First, we analyzed the mRNA levels of IL-6 by RT-qPCR. OLW did not decrease the LPS-induced expression of IL-6 mRNA (data not shown here), while OLM significantly inhibited the IL-6 mRNA expression (Fig. 2A). Then, the effect of OLM was further analyzed on TNF-α and MCP-1 expression. OLM significantly inhibited TNF-α and MCP-1 mRNA expression (Fig. 2B, C). Likewise, OLM also decreased significantly the mRNA expression of COX-2 and iNOS (Fig. 2D, E), two important enzymes involved in the inflammatory process [25,26]. To confirm the effect of OLM at protein level, IL-6, TNF-α, and MCP-1 levels in the culture medium were quantified. OLM decreased LPS-induced secretion of these cytokines by J774 cells, further supporting its inhibitory effect on inflammation.

3.2. Effect of OLM and OLW on LPS-induced expression of pro-inflammatory cytokines and enzymes in J774 macrophages

Table 3

| Organs       | Control group (n = 8) | Treated group (n = 8) | Satellite control group (n = 2) | Satellite treated group (n = 2) |
|--------------|-----------------------|-----------------------|--------------------------------|--------------------------------|
| Heart        | 0.16 ± 0.01           | 0.16 ± 0.01           | 0.15 ± 0.01                     | 0.15 ± 0.01                     |
| (µg)         | 0.50 ± 0.52           | 0.52 ± 0.52           | 0.43 ± 0.01                     | 0.45 ± 0.01                     |
| (µg)         | 0.02 ± 0.02           | 0.02 ± 0.02           | 0.01 ± 0.01                     | 0.01 ± 0.01                     |
| Kidneys      | 0.45 ± 0.47           | 0.47 ± 0.02           | 0.48 ± 0.02                     | 0.48 ± 0.02                     |
| (µg)         | 0.02 ± 0.01           | 0.01 ± 0.01           | 1.41 ± 0.03                     | 1.39 ± 0.06                     |
| (µg)         | 0.02 ± 0.02           | 0.01 ± 0.01           | 1.48 ± 0.04                     | 1.48 ± 0.04                     |
| Liver        | 1.86 ± 0.09           | 2.01 ± 0.09           | 1.78 ± 0.13                     | 2.08 ± 0.11                     |
| (µg)         | 5.85 ± 6.33           | 6.23 ± 6.33           | 5.28 ± 0.32                     | 6.01 ± 0.38                     |
| (µg)         | 0.24 ± 0.27           | 0.30 ± 0.27           | 0.20 ± 0.01                     | 0.20 ± 0.01                     |
| Lungs        | 0.23 ± 0.23           | 0.23 ± 0.23           | 0.27 ± 0.03                     | 0.20 ± 0.01                     |
| (µg)         | 0.02 ± 0.02           | 0.02 ± 0.02           | 0.00 ± 0.01                     | 0.00 ± 0.01                     |
| (µg)         | 0.13 ± 0.13           | 0.13 ± 0.13           | 0.16 ± 0.01                     | 0.13 ± 0.03                     |
| Spleen       | 0.14 ± 0.01           | 0.10 ± 0.01           | 0.47 ± 0.03                     | 0.37 ± 0.08                     |
| (µg)         | 0.01 ± 0.01           | 0.01 ± 0.01           | 0.07 ± 0.03                     | 0.07 ± 0.08                     |
| (µg)         | 0.38 ± 0.38           | 0.38 ± 0.38           | 0.38 ± 0.38                     | 0.37 ± 0.37                     |
| Stomach      | 0.83 ± 0.79           | 0.79 ± 0.79           | 0.98 ± 0.03                     | 0.91 ± 0.07                     |
| (µg)         | 0.15 ± 0.08           | 0.15 ± 0.08           | 2.92 ± 0.14                     | 2.63 ± 0.19                     |
| (µg)         | 2.63 ± 2.51           | 2.63 ± 2.51           | 2.63 ± 2.51                     | 2.63 ± 2.51                     |
| Body weight  | 31.68 ± 31.71         | 31.71 ± 31.71         | 33.70 ± 30.40                   | 34.70 ± 30.30                   |
| (g)          | 0.64 ± 0.36           | 0.64 ± 0.36           | 0.64 ± 0.36                     | 0.64 ± 0.36                     |

Relative weight calculated as organ-to-body weight (%) = (organ weight ÷ body weight) × 100. Data are expressed as mean ± SEM. n: number of animals in the group. A: absolute weight; R: relative weight. The data were analyzed by the Student’s t test for comparisons between the control and treated groups. No significant difference was found. Significantly different: p < 0.05.
compounds that occur in plants as free aglycones (flavonoids and phenolic acids glycosides. Flavonoids are polyphenolic an extensity used technique to identify already known compounds in ural Product Social Molecular Networking (GNPS). Dereplication is now identify its major chemical constituents by carrying out a dereplication study of OLM.

Data are expressed as mean ± SEM. n: number of animals in the group. The data were analyzed by the Student’s t test for comparisons between the control and treated groups. No significant difference was found. Significantly different: p < 0.05.

| Parameter | Control (n = 5) | Treated (n = 7) | Treated satellite (n = 2) | Treated satellite (n = 1) |
|-----------|----------------|----------------|--------------------------|--------------------------|
| Alb (g/dL) | 2.10 ± 0.05    | 2.18 ± 0.07    | 1.85 ± 0.05              | 2.00                     |
| GGT (U/L)  | < 10           | < 10           | < 10                     | < 10                     |
| DBIL (mg/ dl) | 0.12 ± 0.02  | 0.18 ± 0.03    | 0.15 ± 0.05              | 0.10                     |
| TBIL (mg/ dl) | 0.5 ± 0.02   | 0.63 ± 0.05    | 0.60 ± 0.10              | 0.50                     |
| CRE (mg/ dl) | < 0.2         | < 0.2          | < 0.2                    | < 0.2                    |
| GOT (U/L)  | 74.80 ± 3.72   | 72.71 ± 4.3    | 65.50 ± 3.50             | 62.0                     |
| GPT (U/L)  | 26.40 ± 2.61   | 24.86 ± 1.68   | 23.50 ± 0.50             | 25.0                     |

Data are expressed as mean ± SEM. n: number of animals in the group. The data were analyzed by the Student’s t test for comparisons between the control and treated groups. No significant difference was found. Significantly different: p < 0.05.

on pro-inflammatory cytokines (Fig. 2F-H).

3.3. Chemical composition of OLM

For a better understanding of the in vitro activity (we observed in LPS-activated J774 cells model) and the toxicity of OLM, we tempted to identify its major chemical constituents by carrying out a dereplication strategy based on HPLC-HRMS/MS analysis along with the Global Natural Product Social Molecular Networking (GNPS). Dereplication is now an extensity used technique to identify already known compounds in crude extracts [27]. HPLC-MS/MS analyses were realized in APCI positive ionization and hence negative mode data were used for molecular networking. MS/MS spectra of the detected compounds are given in supplementary information (Fig. S4-S27). Based on the LC-UV-MS/MS chromatographic analysis of OLM, the major detected constituents are flavonoids and phenolic acids glycosides. Flavonoids are polyphenolic compounds that occur in plants as free aglycones (e.g., quercetin) or glycosidic forms (bound to sugar moieties) (e.g., rutin). In the molecular network of OLM (Fig. 3), the main clusters (A and C) correspond to phenolic derivatives, organized by their fragmentation spectra similarities.

The 1st eluted flavonoid glycoside was putatively identified as a quercetin trihexoside derivative (10) which gave a deprotonated molecular ion [M − H]− at m/z 755 and fragmented to the corresponding aglycone, which gave a signal at m/z 300/301, with a loss of one or two proton as previously reported for flavonoid glycosides in negative mode (Table 2) [28]. Same fragmentation signals were observed for compounds 15 and 17 with deprotonated molecular ions [M − H]− at m/z 609 and 463, identified as quercetin-3-0-rutinoside (rutin) (15) and quercetin hexoside, respectively [28]. A kaempferol trihexoside derivative (11) was also putatively identified with a deprotonated molecular ion [M − H]− at m/z 739 and fragmented to the corresponding aglycone, which gave a signal at m/z 284/285 [28]. With the same fragmentation pattern was found compound 20, putatively identified as a kaempferol hexoside. An isorhamnetin trihexoside (13) was also tentatively identified with a deprotonated molecular ion [M − H]− at m/z 769 and fragmented to the aglycone with signal at m/z 314/315, corresponding to the loss of sugars moiety. The same fragmentation pattern was observed for compounds 18 and 21, corresponding to narcissin and an isorhamnetin hexoside, respectively.

In addition, three phenylpropanoid glycoside derivatives were also detected: sinapoyl dihexoside isomer (4, [M − H]− at m/z 533 and a fragment corresponding to the loss of sinapoyl aglycone at m/z 323), coumaric acid hexoside (7, [M − H]− at m/z 325 and a fragment corresponding to the coumaric acid aglycone at m/z 163), hydroxyferuloyl hexoside (12, [M − H]− at m/z 371 and a fragment at m/z 249) and sinapoyl hexoside (16, [M − H]− at m/z 385 and a fragment at m/z 263).

The presence of rutin (15) and narcissin (18) was further supported by co-injection with commercial standards (Fig. 4). To the best of our knowledge, these compounds (except rutin) are reported in S. oleoides for the first time.

We consume flavonoids in our daily diet (fruits, vegetables, nuts, herbal teas, red wine, etc.) in varying amounts. This is the reason why flavonoids are generally considered nontoxic and safe, unless somebody consumes very high amounts of purified flavonoids [32]. Flavonoids are potent antioxidants, have the potential to inhibit the enzymes and transcription factors which are important in inflammation. They have shown anti-inflammatory properties in in vitro and in vivo experimental models [33]. In our crude extract, we observed rutin (15) as a major flavonoid (Fig. 3). Rutin, a dietary flavonoid, was found to dose dependently (1, 10, and 100 μM) decrease the secretion of IL-6, TNF-α, and IL-1β in LPS-activated rat peritoneal macrophages [34]. It also dose dependently (25, 50, and 100 μM) suppressed the activation of NF-κB and production of TNF-α in LPS-activated primary human umbilical vein endothelial cells (HUVECs) [35]. When tested at 5 μM, we previously found that rutin decreased IL-6 secretion in the same model used here [15]. Narcissin, another flavonoid, at 200 μM significantly decreased the production of IL-6, IL-1β, TNF-α, and COX-2 in LPS-activated M1 macrophages [36]. Three kaempferol glycosides isolated from the leaves of Cinnamomum osmophloeum, tested at 15–40 μM, significantly inhibited the secretion of TNF-α and IL-12 in LPS+IFN-γ activated mouse peritoneal macrophages [37].

Another major compound of the HPLC-DAD chromatogram is compound 4, a sinapoyl dihexoside. Sinapoyl alcohol glycosides were also found to be anti-inflammatory in in vivo and in vitro experimental models. For example, syringin (sinapoyl alcohol-O-glucoside), significantly inhibited the secretion of TNF-α in LPS-activated human monocytes/macrophages at the concentration of 50 μM [38]. In another study, syringin, at the concentration of 12.5 μg/mL (33.56 μM) and 25 μg/mL (67.13 μM), significantly decreased the production of IL-6, IL-1β, TNF-α, iNOS, and COX-2 in LPS-stimulated primary human umbilical vein endothelial cells (IEC6 cells) [39]. At the same concentration, syringin significantly decreased the production of IL-6, IL-1β, TNF-α, and MCP-1 in LPS-activated RAW264.7 macrophages [40].

1-p-coumaroyl-β-D-glucose, a glucose ester of coumaric acid, isolated from the bark of Salix alba, attenuated the production of NO and PGE2 and inhibited the protein expressions of iNOS, COX-2, IL-1β, and TNF-α in LPS-stimulated RAW264.7 cells (tested at 100, 200, and 400 μM) in [41].

We also putatively identified other compounds as avenanthramide, a rososide derivative, and azelaic acid (Table 2) which may also contribute to the anti-inflammatory properties of the extract [42,43].

All these data support the hypothesis that OLM contains several anti-inflammatory compounds which may act additively or synergistically to explain the activity observed.
3.4. In vitro toxicity of OLM

Before exploring further, the anti-inflammatory properties of OLM, it is important to assess its potential toxicity. Therefore, we decided to explore this element in vitro and then in vivo. As the in vivo experiments would require large quantities of OLM, we collected more quantities (about 2.6 kg of dry powder) of *S. oleoides* and prepared the OLM extract, named “OLM (batch 2)”, as discussed in point 2.3. We obtained 360 g of OLM with a similar effect on LPS-activated J774 cells as previous OLM, named “OLM (batch 1)” (Fig. S28), and has a similar chromatographic profile. All the next experiments were performed on “OLM (batch 2)” and is named as “OLM” in the manuscript. To support further this absence of toxicity, we also tested OLM on three other cell lines, namely HeLa, Caco-2, and HepG2, by MTT assay. HeLa is the first immortal human cell line, obtained from human cervical cancer cells, and has been extensively used for various studies including toxicity evaluation of the substances and have shown a significant correlation with in vivo results [44]. Caco-2 is an immortalized cell line of human colorectal adenocarcinoma cells and it mimics intestinal epithelium. HepG2 is a human liver cancer cell line, we used it as an in vitro model to evaluate the toxic effect of OLM on liver. We repeated the experiments three times in triplicates and calculated the IC$_{50}$ value that was higher than 100 µg/mL for HeLa, Caco-2, and HepG2 cells. For crude extracts to be tested in vitro, 100 µg/mL is quite high concentration and OLM seems to be safe and non-cytotoxic. Camptothecin was used as positive control. The IC$_{50}$ values of camptothecin were 27.9 ± 1.6, 23.2 ± 0.9, and 131.3 ± 4.9 100 ng/mL for HeLa, HepG2, and Caco-2 cells, respectively.

3.5. In vivo toxicity of OLM

3.5.1. Highest tolerated dose (HTD)

This study was conducted to determine the maximum dose that can be tolerated. In control and treated mice (2000 mg/kg p.o and 1500 mg/kg i.p), we observed neither any death nor significant changes in general appearance and behavior in the two days following dose administration.

3.5.2. Acute oral toxicity study

In HTD test, 2000 mg/kg oral dose was found non-toxic, so the same
dose was used to evaluate the acute oral toxicity. Oral route of dose administration was used because it is closer to most of the traditional uses of the plant. After extract administration (2000 mg/kg) to a group of 3 mice, they were kept under observation for two weeks. The experiment was repeated two times and in both the repetitions none of the animals neither died nor showed any sign of illness, behavior change, or significant loss in body weight. After euthanizing the animals, the principal organs affected by the metabolic reactions of toxicants (i.e., stomach, liver, spleen, heart, kidneys, and lungs) [45] were observed macroscopically and were found in good shape and condition (Fig. S29).

3.5.3. Sub-acute oral toxicity study

For sub-acute oral toxicity study (1000 mg/kg/day, for two weeks), twenty mice divided in two groups (control and treated) were used. During the first week of the experiment, two mice from the control group had a great weight loss and showed signs of severe pain and were hence euthanized. The mice were from the same cage and the possible reason can be the gavage. None of the other animals from all the four groups (control, treated, satellite control, and satellite treated) showed any behavior change, signs of illness, or significant weight loss during the whole study period (Fig. 5). The alterations of body weight, absolute internal organs weight, and relative internal organs weight of test animals after exposure to the toxic substances are among the prominent signs of toxicity [46,47]. Here, internal organs (heart, liver, lungs, kidneys, spleen, and stomach) were in good shape, texture, color, and appearance (Fig. S30) and no significant difference in the absolute and relative weight of internal organs compared to the control animals was observed (Table 3). Hematocrit values were also in normal range and there was no significant difference between the values of control and test animals (Table 4). We also analyzed key biochemical parameters (albumin, TBIL, DBIL, AST, ALT, GGT, and creatinine) in the blood samples. Three blood samples (one from control group, one from treated group, and one from satellite treated group) were hemolyzed during collection and processing and hence were discarded. In remaining samples, there was no significant difference in biochemical parameters (albumin, TBIL, DBIL, AST, ALT, GGT, and creatinine) of control and treated mice (Table 5).

For histological analysis of the kidneys, liver, spleen, and stomach, 50% animals (four from treated group and three from control group) were randomly chosen. Sections of the organs were stained with Sirius red.
hematoxylin and eosin (H&E) and we did not find any pathological change in the tissues and organs (Fig. 6).

To detect potential signs of fibrosis, we stained the slides with Sirius red, a dye that gives red color to collagen fibers (Fig. 7). Percentage of Sirius red positive area in the functional cell areas (eliminating main vessels and destroyed tissues) of kidneys, liver, and stomach was 6.1 ± 1.7 and 7.8 ± 1.3, 1.2 ± 0.2 and 1.3 ± 0.2, and 35.8 ± 1.5 and 33.8 ± 2.7, for treated and control mice, respectively. Collagen content in the spleens of treated mice (18.3 ± 1.6%) was significantly higher (p < 0.05), compared to control group (11.5 ± 1.4%), suggesting the presence of fibrosis in the spleens of the treated mice. Fibrosis can occur as a result of injury, inflammation, or neoplasia [48]. Another reason of fibrosis can be erythrocyte toxicity. As a secondary effect of increased hemolysis, excess hemosiderin is deposited in macrophages. Destruction of macrophages in spleen causes release of iron which can cause oxidative stress leading to tissue injury and fibrosis [49,50]. In our experiments, it does not seem to be the cause of fibrosis, as the hematocrit values were not affected. We also did not find any difference in weight of spleen and other internal organs (liver, kidneys, lungs, and heart), between control and treated mice. Further studies are required to search the reasons for the fibrosis of spleen observed in treated mice.

4. Conclusions

We report that methanol extract of *S. oleoides* (OLM) significantly inhibited the expression of pro-inflammatory cytokines (TNF-α, IL-6, and MCP-1) in LPS-activated J774 macrophages at 50 μg/mL concentration. We also found that, in the MTT assay, IC50 value of OLM was higher than 100 μg/mL when tested against HeLa, Caco-2, HepG2, WI-38, and J774 cells. In in vivo HTD study, 2000 mg/kg oral and 1500 mg/kg intraperitoneal dose seemed non-toxic. 2000 mg/kg oral dose was found non-toxic in in vivo acute toxicity study. 1000 mg/kg oral dose caused fibrosis in spleen in in vivo sub-acute toxicity study. Using a dereplication strategy and molecular networking, 17 compounds were putatively identified, most of them were flavonoid and phenolic acids glycosides. Taken together, our results will contribute to a better understanding of the anti-inflammatory potential, chemical composition, and toxicity of *S. oleoides*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.09.004.

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