



Attenuating monocytic inflammaging via the miR-146a/TLR4 axis: Characterization and bioactivity of a triterpenoid-rich callus phytocomplex

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ABSTRACT

Inflammaging, driven by monocyte dysregulation, is a hallmark of immunosenescence. Toll-like Receptor 4 (TLR4) pathway hyper-activation accelerates cellular senescence and systemic inflammatory damage, while microRNA-146a (miR-146a) constrains pro-inflammatory cytokine release by targeting IRAK-1. Plant-derived triterpenoids are promising geroprotective agents, but standardized, scalable biotechnological production remains challenging. We established a sustainable in vitro callus culture from Red Sentinel to obtain a standardized triterpenoid-rich extract (RSE). GC-MS/FID analysis quantified its pentacyclic triterpene content (ursolic and oleanolic acids) within a soluble phytocomplex matrix, while X-ray Photoelectron Spectroscopy (XPS), Ultraviolet Photoelectron Spectroscopy (UPS), and thermal analysis (TGA/DSC) characterized surface elemental states, work function, and thermal stability. Biological efficacy was tested in human U937 monocytes, and molecular docking was performed against the TLR4/MD-2 complex. RSE showed high thermal stability and distinct surface chemistry, with specific oxygenated carbon functional states. In U937 monocytes, RSE non-cytotoxically attenuated pro-inflammatory signaling, significantly upregulating miR-146a, down-regulating IRAK-1, and reducing IL-6 secretion. Docking confirmed strong interactions between the pentacyclic triterpenes and the TLR4/MD-2 hydrophobic pocket. These results indicate that plant cell culture technology can generate standardized phytocomplexes capable of modulating the miR-146a/IRAK-1/IL-6 axis, counteracting monocytic inflammaging, and support the value of combining surface physics and molecular biology in anti-aging nutraceutical research.

1. Introduction

Chronic low-grade inflammation, or "inflammaging," is recognized as a central driver of immunosenescence and age-related tissue dysfunction, with monocytes playing a pivotal role in sustaining this pro-inflammatory state (Basu et al., 2025). Among the molecular hubs implicated in this process, the Toll-Like Receptor 4 (TLR4) pathway has emerged as a key upstream driver of inflammaging: age-associated accumulation of endogenous damage-associated molecular patterns (DAMPs) and altered membrane lipid composition progressively sensitize TLR4 signalling (Kim et al., 2023), resulting in chronic low-grade

activation of NF-κB-dependent inflammatory cascades even in the absence of overt infection (Shaw et al., 2010). Identifying natural compounds capable of modulating TLR4-driven molecular circuits therefore represents a priority for geroscience-oriented research. Plant secondary metabolites (SMs), evolved as part of the plant chemical defence system to cope with biotic and environmental stress, have emerged as promising candidates in this context, given their long-recognized relevance for human health, disease prevention, and, more recently, anti-aging applications (Corrêa et al., 2017; Das et al., 2024; Păcuraru-Burada et al., 2024). This is the reason why many studies are being performed to produce SMs with functional properties using in

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in vitro plant callus cultures, also achieving their increased production compared to plants of natural origin (Fazili et al., 2022). Plant callus culture represents an alternative plant material derived from cultivated or wild plants and represent a promising means for obtaining bioactive compounds offering advantages even as stable and sustainable production year-round (Gubitosa et al., 2023; Verardo et al., 2017). For this reason, since 2017, our research group started the *in vitro* production of callus from ripe pulp of apples explants (Gubitosa et al., 2024, 2023; Verardo et al., 2017; Vitiello et al., 2024) like “Red Sentinel apple” (patent <https://air.uniud.it/handle/11390/1228256>). The chemical analysis of this callus showed a concentration of SMs, pentacyclic triterpenic acids, much higher than in the peel and pulp of the fresh fruits. Triterpenic acids have been shown to be beneficial for maintaining human health (Deng et al., 2021; Kashyap et al., 2016) as type 2 diabetes (Ding et al., 2018), liver disease (Liu et al., 2021), cholesterol accumulation (Hao et al., 2020), supporting their candidacy as geroprotective agents.

In this study, we propose a multi-scale characterization framework to bridge the gap between the raw physicochemical properties of natural extracts and their targeted biological outcomes in the context of monocytic inflammaging. Using the Red Sentinel callus Extract (RSE), we aimed to demonstrate how its functional, anti-inflammatory activity is related to its physicochemical identity. The novelty of our approach lies in the integration of different distinct levels of analysis starting from quali-quantitative analysis of the extract bioactive compounds, and the bulk thermal stability and phase transitions of the triterpenic extract were established through Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) ensuring the material's integrity. The surface electronic reactivity was mapped using X-ray Photoelectron Spectroscopy (XPS; Functional Groups, composition) and Ultraviolet Photoelectron Spectroscopy (UPS), providing real-world energy descriptors (Work Function/WF, Valence Band Maximum, HOMO levels) that transcend simple chemical structures. A Fourier Transform Infrared (FTIR) spectroscopy was used to analyze the typical absorption peaks of the extract.

These physical data were integrated with a molecular modelling approach, revealing potential target affinity of the selected candidates for the extracellular TLR4/MD-2 complex, the same receptor axis whose age-related hyper-activation was highlighted above as a central driver of inflammaging (Kim et al., 2023). Lipopolysaccharide (LPS), the major component of the Gram-negative bacterial outer membrane, represents the prototypical and best-characterized TLR4/MD-2 agonist, and is therefore routinely used both as a structural reference for receptor activation and as the experimental stimulus to trigger TLR4-dependent inflammatory signalling in monocyte-based *in vitro* models (Fraternali et al., 2022). Indeed, LPS recognition by TLR4 requires the prior formation of a complex with the MD-2 co-receptor at the cell membrane surface. LPS accommodation within the hydrophobic pocket of MD-2 triggers receptor dimerization and downstream inflammatory signaling pathways (Park et al., 2009); notably, this same dimerization-dependent mechanism can be engaged in a ligand-independent manner by endogenous DAMPs during aging, perpetuating a self-sustaining inflammatory loop.

Triterpenoids have been widely reported to exert anti-inflammatory activity, partly through modulation of TLR4 signalling (Kuzmich et al., 2017) responsible for IL-6 secretion. For instance, Lupeol has been shown to regulate key proteins of the TLR4/MyD88/NF- κ B pathway (Xu et al., 2020), while other derivatives such as celastrol and asiatic acid can interfere with MD-2 binding or attenuate LPS-induced inflammatory responses (Lee et al., 2015). These findings support the potential of triterpenoid scaffolds as modulators of TLR4/MD-2 activation.

Building on this rationale, the biological relevance of this mechanism was validated in human U937 monocytes, a well-established monocytic cell model for studying TLR4/IL-6 inflammatory signaling (Fraternali et al., 2022), whose dysregulation of the miR-146a/IRAK-1 axis has been closely linked to inflammaging and cellular senescence in

monocyte/macrophage lineages (Bhaumik et al., 2009; Ong et al., 2018; Olivieri et al., 2021).

By monitoring the regulation of the signalling adapter IRAK-1, of the epigenetic regulator miR-146a, and of IL-6 production, we demonstrate how the physical and electronic “fingerprint” of the extract translates into a robust suppression of the TLR4-driven NF- κ B inflammatory cascade, offering a mechanistic and translational rationale for RSE as a candidate geroprotective phytocomplex.

2. Materials and methods

2.1. Chemicals and reagents

Gamborg B5 medium (GB5 medium); 6-benzylaminopurine (BA); 1-naphthaleneacetic acid (NAA), extraction and derivatization solvents were of analytical grade and were obtained from Sigma-Aldrich (Milan, Italy). Cholesterol (GC-FID Internal Standard; IS), ursolic, oleanolic, and glycyrrhetic acids, used as standards, Sylon BFT [Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS)], employed as a silylating reagent, were also obtained from Sigma-Aldrich. Milli-Q grade water was produced by the Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK).

2.2. Red sentinel callus extract (RSE) preparation

In vitro callus culture from Red Sentinel (RS; *Malus x robusta* var. Red Sentinel Rosaceae family) pulp explants were produced as described in the patent: <https://air.uniud.it/handle/11390/1228256>. Briefly, mature pulp RS was used as the source of explants for callus induction, the explants were placed on GB5 medium containing 0.2 mg/L BA plus 2.0 mg/L NAA. Subcultures of callus were made after 28 days in the same media. The obtained calli (28 days) were stored at -20°C and then freeze-dried before the extraction and chemical analysis. The RSE was prepared as described in Gubitosa et al. (Gubitosa et al., 2024). Freeze-dried RS pulp callus (260 mg) was extracted in a glass potter with 15 mL of 70% ethanol for 15 min. The homogenate was stirred overnight at 4°C and then centrifuged for 45 min at $4^{\circ}\text{C} \times 13,000\text{g}$. After supernatant recovery, the pellet was extracted a second time with 15 mL of 70% ethanol and stirred at 4°C for 30 min. The supernatant recovered by centrifugation as above, and the two supernatants combined. Ethyl alcohol was completely removed from the extract using a rotavapor (Buchi R 80) at 40°C . The ethanol-free extract was freeze-dried (CHRIST Alpha 1–2 LSC Basic freeze dryer), and the resulting powder was aliquoted into an Eppendorf tube and stored at -20°C until use.

2.3. Red Sentinel callus extract (RSE) quali-quantitative triterpenic acids analyses by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detection (GC-FID)

Each dried extract was spiked with internal standard (cholesterol, IS), suspended in water (5 mL) and extracted twice with ethylacetate ($2 \times 30\text{ mL}$). The collected organic phases were washed with H_2O (5 mL), brine (5 mL), then dried (anhydrous Na_2SO_4), filtered, and evaporated *in vacuo* ($T < 35^{\circ}\text{C}$) to a final volume of about 5 mL. An aliquot of this solution was further dried under argon stream, silylated by a mixture (1:1) of pyridine/BSTFA at 80°C for 18 h and analyzed by GC-MS and GC-FID. The qualitative analyses were carried using a Trace GC Ultra gas chromatograph equipped with a capillary column SLB-5 ms (Supelco, Sigma-Aldrich) and coupled to an ion trap mass spectrometer detector Polaris Q (Thermo Scientific). The initial oven temperature was 240°C programmed to 280°C at $2^{\circ}\text{C}/\text{min}$ and kept at 280°C for 16 min and the temperature was then raised to 310°C at a rate of $10^{\circ}\text{C}/\text{min}$ and maintained at this temperature for 21 min. Identification of compounds was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library or with those available in the literature.

The quantitative analysis was carried out using a Fisons GC 8000 series gas chromatograph (Fisons Instruments, Milan, Italy), equipped with a capillary column DB-5MS (Agilent, J&W, Italy) and coupled with a FID detector. The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 25 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 12 min. Quantification of the analytes in the extracts was performed using the internal standard method based on the relative peak area of each analyte to IS (cholesterol) from the average of two replicate measurements. A calibration curve was built for each corresponding standard compound in the extracts. For this purpose, working solutions of each available standard (Ursolic, Oleanolic and Glycyrhethinic acid) were prepared at a concentration ranging from 7 to 160 µg/mL with a constant concentration of the IS (cholesterol, 60.0 µg/mL). The calibration curves obtained had a high level of linearity with a correlation coefficient (r^2) higher than 0.999 for all analytes. When standards were unavailable, the target analyte was quantified using the calibration curve of available standards of similar chemical structures.

2.4. Red sentinel callus extract (RSE) analyses by FTIR, DSC, TGA and UV-Vis

The obtained RSE was analyzed by FTIR (ATR-FTIR, Spectrum Two, PerkinElmer, USA). Transmittance (%) was recorded over the wavelength range 4000–40 cm⁻¹. Each sample underwent 64 scans at a resolution of 1 cm⁻¹, using air as background.

The thermal profile of the extract was investigated using DSC (DSC 6000, PerkinElmer, USA) and TGA (TGA 4000, PerkinElmer, USA). For DSC measurements, approximately 5 mg of sample were placed in aluminum pans and heated from 30 °C to 300 °C at a rate of 10 °C/min. For TGA, the sample was subjected to a heating ramp from 30 °C to 500 °C at 10 °C/min. A nitrogen flow rate of 30 mL/min was maintained throughout the analyses. All measurements were performed in triplicate.

The UV-Vis spectrum of the extract dissolved in 70% v/v ethanol was recorded from 190 to 900 nm using a UV-Vis spectrophotometer (UV-1900i, Shimadzu).

2.5. Red sentinel callus extract (RSE) XPS/UPS analyses

We performed XPS and UPS in an Ultra-High-Vacuum (UHV) system equipped with a VSW HA100 hemispherical analyzer (with a PSP power supply and electronic control). We used a non-monochromatized Mg K α X-ray source ($K\alpha_{Mg} = 1253.6$ eV) and a He-discharge lamp with the He I emission line (21.22 eV) for XPS and UPS analysis, with a final minimum energy resolution of 0.86 and 0.1 eV for XPS and UPS. The binding energy (BE) scale was calibrated using the Au 4f peak at 84.0 eV and the Au Fermi level of a sputtered Au surface. For UPS characterization, we biased the sample at -7.1 V. The core level analysis was performed by Voigt lineshape deconvolution after background subtraction, using a Shirley function. The typical precision for each component's energy position is ± 0.05 eV. The uncertainty for the full width at half-maximum (FWHM) is less than $\pm 2.5\%$, while it is about $\pm 2\%$ for the area evaluation.

Two samples were analyzed. The first is labelled as "Bulk" RS, it is the callus extract ("30aCHter") without any modification and was placed on a clean Si Surface and inserted in UHV. The second, the "Diluted", is a 70% ethanol dilution of the extract. We produced a 6% (w/w) solution from bulk RS at room temperature (RT), then drop-cast it at RT onto a clean Si(100) surface with its native oxide layer, achieving uniform surface coverage.

2.6. Cell culture, viability, and treatment conditions

U937 human myeloid leukaemia cells were cultured in suspension in RPMI-1640 medium (Sigma-Aldrich). Culture media were supplemented with 10% fetal bovine serum (Euroclone, Celbio Biotecnologie, Milan,

Italy), penicillin (100 units/mL), and streptomycin (100 mg/mL) (Euroclone). Cells (5×10^5 /mL) were grown and treated at 37 °C with a gassed atmosphere of 95% air-5% CO₂.

Treatments with RSE were performed in a dose dependent assay (1, 5, 10, 100 and 200 µg/mL) during 7 h to analyse cell death. Viable RSE concentrations were considered to perform LPS pro-inflammatory experiments considering 1 h of RSE pre-treatment and the addition of LPS (1 µg/mL) for another 6 h. Cell viability in U937 cells was assessed using the trypan blue exclusion assay where an aliquot of the cell suspension was diluted 1:2 (v/v) with 0.4% trypan blue, and negative/positive stained cells were counted using a hemocytometer (Strober, 2015).

This test is based on the principle that live cells possess intact cell membranes that exclude trypan blue whereas dead cells do not. With this assay we can obtain three different information: the number of positive cells (i.e. cell death), the number of negative cells (i.e. cell survival), and the total cell number.

2.7. Quantitative real time PCR (RT-qPCR) of mature microRNAs and mRNA

Total RNA isolation kit (Norgen Biotek, Thorold, Canada) was used to extract total RNA from 1×10^6 U937 cells and stored at -80 °C until use. Isolated RNA was reverse transcribed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

For human miR-146a and human RNU44 expressions were quantified using the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA), as previously described (Olivieri et al., 2013). The following primers were used: IL-6 (Forward AGGGCTCTTCGGCAAATGTA/Reverse GAAGGAATGCCCATTAACAACAA), IRAK-1 (Forward CAGACAGGGAAGGGAAACATTTT/Reverse CATGAAACCTGAC TTGCTTCTGAA), and endogenous control GAPDH (Forward GACATG CCGCCTGGAGAAAC/Reverse AGCCCAGGATGCCCTTTAGT). Results were analyzed using the $2^{-\Delta\Delta C_t}$ method and are expressed in the figures as fold change vs CTRL (control; Fraternali et al., 2016).

2.8. Data collection for molecular modelling approaches

First, data for the docking were collected. The crystal structure of the human TLR4 receptor with its LPS ligand (Park et al., 2009; PDB ID: 3FXI; organism: Homo sapiens) was retrieved from the Protein Data Bank (PDB; <https://www.rcsb.org/>, accessed on 021 January 2026) with an optimal resolution of 3.10 Å. The molecules from RS alongside with the positive control Lupeol, were obtained from the PubChem database (Burley et al., 2025), codified as SMILES strings as shown in Table S1 (<https://pubchem.ncbi.nlm.nih.gov/>, accessed on 1 January 2026).

2.9. Molecular docking and interaction analysis with TLR4-MD2

The docking and interaction analysis was performed as described in Ortiz-González et al. (Ortiz-González et al., 2025). Protein preparation, including the selection of the chains of interest, removal of water molecules, and addition of hydrogen atoms, was conducted employing the AutoDock Tool (MGLTOOLS v.1.5.7; Forli et al., 2016). The SMILES of the anti-inflammatory molecules were used to generate the structural coordinates with RDKit (v.2022.03.5) (RDKit: Open-Source Cheminformatics. <https://www.rdkit.org/>).

The Autodock Vina software (Trott and Olson, 2010) was used for performing the docking of candidate molecules with the TLR4 receptor. The structural information from the LPS binding site was then used to determine the docking grid of the potential binding of the molecules with the receptor. The selected coordinates were (X_center = 29.472, Y_center = -10.500, Z_center = 11.806), and the docking box size was

(36,75, 41,25, 33,75) Å. The exhaustiveness level was set to 256, and the twenty-five best binding modes, ranked by their binding energy, were saved. This procedure was performed for five replicates for each ligand, each replicate with a different seed.

The docking poses for each ligand were analysed with in-house Python (<https://www.python.org/>) scripts, utilising MDAnalysis (v.2.7.0; Gowers et al., 2016), ProLIF (v.2.0.3; Bouysset and Fiorucci, 2021), and RDKit (v.2022.03.5). MDAnalysis was utilised to load and prepare structures for interaction calculation and visualization of 2D molecule plots, performed with ProLIF. The results were then visualised using VMD (v.1.9.3; Humphrey et al., 1996) and matplotlib (v.3.4.2; Hunter, 2007). All compounds displaying values comparable to that of Lupeol (-8.25 ± 0.44 kcal/mol) a known anti-inflammatory compound that acts on the TLR4/MD-2 pathway (Xu et al., 2020).

2.10. Statistical analysis

Data on U937 cells are expressed as mean $\pm$ standard deviation (SD) from at least three independent experiments. Statistical comparisons between two groups were performed using a two-tailed Student's *t*-test, while differences among multiple groups were evaluated via one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Statistical significance was defined as $P < 0.05$. All analyses were conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, USA).

3. Results and discussion

3.1. GC-MS and GC-FID quali-quantitative analysis of RSE

GC-MS and GC-FID analyses of RSE allowed the identification and quantification of β -Sitosterol and eight pentacyclic triterpenoids among which Tormentic acid accounts for about 40% of the total, followed by Corosolic, Ursolic, Maslinic, Annurcoic, Oleanic, Pomolic acids and Uvaol (Table 1).

3.2. Physicochemical fingerprinting of RSE: FTIR, DSC, TGA, UV-Vis and UPS/XPS analyses

The multi-technique characterization provided a comprehensive "chemical identity" of the RSE.

XPS analysis was performed to elucidate the surface elemental composition and functional groups of the callus extract in both bulk and diluted forms. The bulk RSE exhibited a composition of C 60.1% and O 36.5%, with traces of Na (0.6%) and K (2.8%). Upon dilution in ethanol, a significant decrease in oxygen content (to 25.9%) was observed, accompanied by an increase in carbon (71.8%). High-resolution C1s spectra deconvolution identified four main contributions (Fig. 1A and B): aliphatic/aromatic carbons (285.0 eV), hydroxyl/ether groups (C-O,

286.4–286.6 eV), carbonyls (C=O, 287.8–288.0 eV), and carboxylic/ester groups (COO, 289.0 eV).

The reduction in the C-O/C=O ratio from the bulk to the diluted sample suggests the removal of residual solvent/alcohols entrapped within the callus matrix (Jia and Li, 2018; Peruzzi et al., 2021). Both samples displayed a marked insulating character, evidenced by a significant BE shift requiring calibration to the C1s reference at 285.0 eV (Beamson and Briggs, 1993; Chiarella et al., 2014).

Electronic properties were further investigated via UPS (Fig. 1C). Despite the insulating nature of the extract limiting the detection of specific organic sigma or pi bands, the Work Function (WF) was calculated at 2.1 eV. The valence band offset relative to the Fermi level indicates a wide band gap of at least 5 eV, confirming the extract's character as a wide-gap organic insulator.

This electronic profile is consistent with the presence of saturated triterpenic structures identified via GC-MS, FTIR, and UV-Vis (Table 1 and Table S2).

The FTIR spectrum of the natural extract (Fig. 2A) shows the characteristic features of a mixture enriched in pentacyclic triterpenic acids and sterols. A broad decrease in transmittance between approximately 3600 and 3000 cm^{-1} is observed, attributable to strongly hydrogen-bonded O-H stretching modes arising from carboxylic acid groups and secondary alcohol functions. This broad O-H envelope is consistent with the presence of triterpenic acids (Oleanolic, Ursolic, Maslinic, Corosolic, Pomolic, Annurcoic, and Tormentic acids) and the diol Uvaol, which were independently identified by GC-MS analysis.

The aliphatic region displays the typical pattern of C-H stretching vibrations of long, saturated hydrocarbon chains. Bands in the 2955–2920 cm^{-1} and 2870–2850 cm^{-1} ranges correspond to asymmetric and symmetric CH_3/CH_2 stretching vibrations, respectively, reflecting the highly aliphatic skeletons of pentacyclic triterpenes and β -Sitosterol (Altunayar-Unsalan, 2024). A pronounced absorption is detected in the carbonyl region, with a marked decrease in transmittance around 1705–1690 cm^{-1} , characteristic of the C=O stretching of hydrogen-bonded carboxylic acids (Gómez-Pulido et al., 2021). This feature confirms the predominance of triterpenic acids previously identified by GC-MS.

In the fingerprint region, bands between 1450 and 1375 cm^{-1} can be ascribed to CH_2/CH_3 bending modes, while the complex pattern observed between 1260 and 1000 cm^{-1} is consistent with C-O stretching vibrations of carboxylic acids and alcohols. Additional bands at lower wavenumbers (900–700 cm^{-1}) correspond to out-of-plane C-H bending of substituted ring systems, as expected for pentacyclic triterpenes and sterols. Overall, the FTIR data corroborate the molecular profile determined by GC-MS. Due to the high structural similarity and overlapping functional groups of these compounds, FTIR serves as a confirmatory rather than discriminatory technique for individual triterpenic constituents revealed by GC-MS.

The UV-Vis spectrum (Fig. 2B) showed the absence of conjugated chromophores. A strong peak around 206 nm was observed. This deep-UV absorption is characteristic of $\sigma \rightarrow \sigma^*$ and $n \rightarrow \sigma^*$ transitions in saturated pentacyclic triterpenes and sterols. The spectral profile is fully consistent with the triterpenic acids, Uvaol, and β -Sitosterol identified by GC-MS and confirmed by FTIR and serves as complementary evidence rather than a discriminatory technique for individual constituents.

The thermal behaviour of the extract was evaluated through DSC and TGA to assess its stability. The DSC thermogram (Fig. 2C) displayed a stable baseline from 30 to approximately 90 °C, indicating the absence of low-melting impurities or residual solvents. A broad exothermic event was observed between 100 and 130 °C, with a maximum around 110–115 °C. This transition corresponds to structural relaxation, enthalpic recovery, or crystallization of semi-ordered domains rather than melting. Such diffuse exothermic rearrangements are characteristic of pentacyclic triterpenes and sterols when present as amorphous or partially crystalline mixtures. Similar behaviour has been reported for α - and β -amyrin, which exhibit broad exothermic transitions associated

Table 1

Identification and quantification ($\mu\text{g}/\text{mg dw} \pm$ standard deviation, coefficient of variation and percentage) of β -Sitosterol and triterpene compounds in the RSE by GC-MS and GC-FID.

No	Compound	($\mu\text{g}/\text{mg} \pm \text{SD}$)	CV %	%
1	β -Sitosterol	2.82 ± 0.08	2.90	2.4
2	Uvaol	2.02 ± 0.07	3.53	1.7
3	Oleanolic acid	4.61 ± 0.07	1.47	3.8
4	Ursolic acid	17.21 ± 0.10	0.57	14.3
5	Maslinic acid	15.71 ± 0.14	0.88	13.1
6	Corosolic acid	17.75 ± 0.23	1.30	14.8
7	Pomolic acid	0.83 ± 0.12	14.04	0.7
8	Annurcoic acid	12.04 ± 0.28	2.35	10.0
9	Tormentic acid	46.99 ± 0.59	1.26	39.2
	Total	119.98 ± 0.11	0.09	

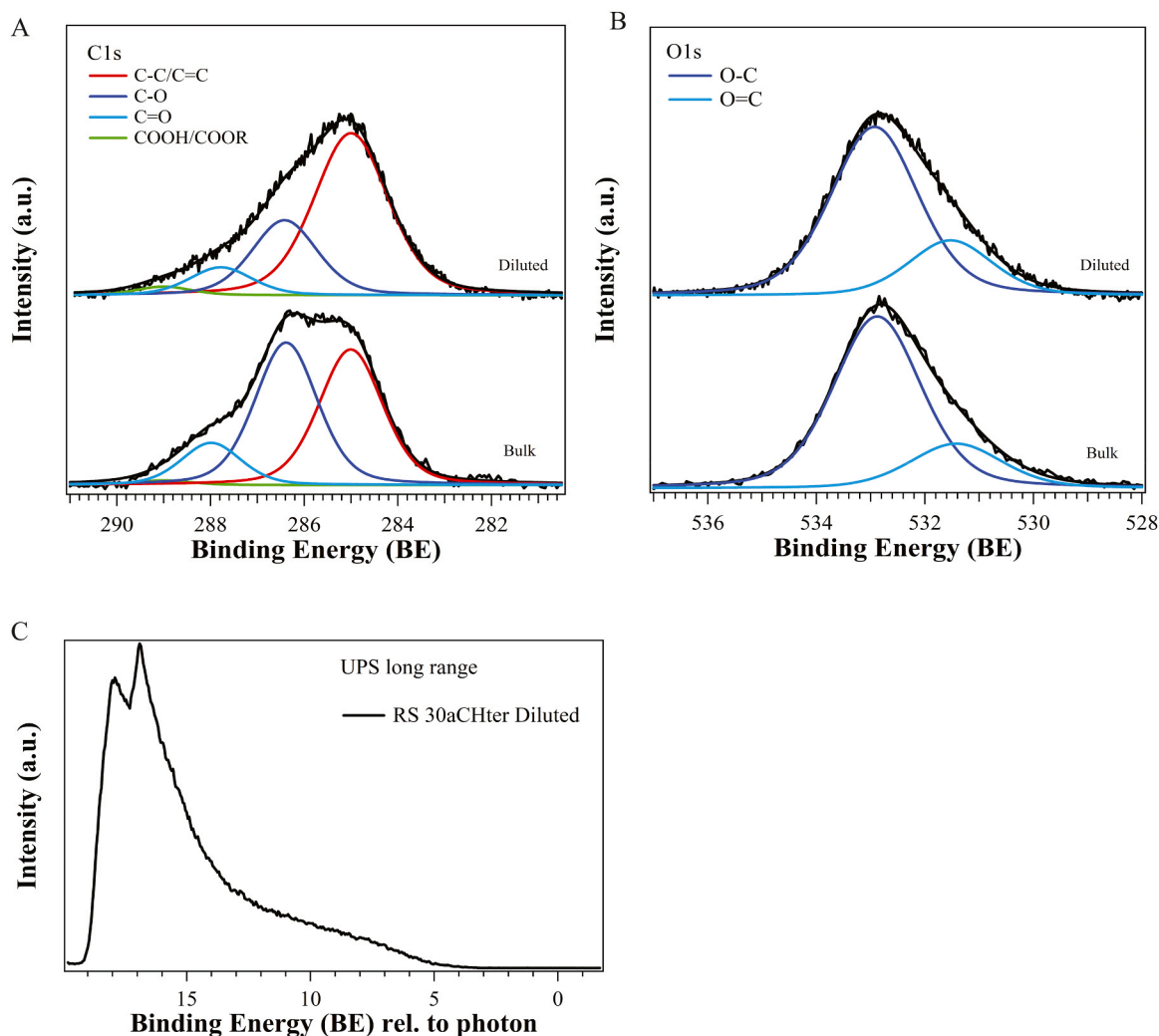


Fig. 1. Surface spectroscopic characterization of RSE in Bulk and Diluted configurations. High-resolution C1s (A) and O1s (B) core-level XPS spectra. C) UPS long-range spectrum.

with structural reorganization rather than sharp melting points due to their pentacyclic skeleton and limited crystallinity (Da Silva Júnior et al., 2017).

Above 130 °C, the DSC signal showed a gradual exothermic drift up to 300 °C without discrete peaks, suggesting slow structural rearrangements and pre-decomposition relaxation rather than oxidative degradation. This behaviour aligns with the high thermal stability expected for pentacyclic triterpenoids and sterols.

The TGA profile (Fig. 2D) further supported this interpretation. A small mass loss ($\approx 3\%$) occurred between 30 and 120 °C, corresponding to the release of adsorbed water and light volatiles. A more pronounced mass loss was observed between 120 and 200 °C, consistent with early degradation of minor components and dehydration reactions. The main decomposition event occurred between 200 and 260 °C, which matches the known thermal behavior of triterpenic acids such as oleanolic and ursolic acids, whose decarboxylation and fragmentation processes occur within this temperature range. Above 260 °C, the mass loss progressed more gradually, reflecting the higher thermal stability of β -Sitosterol and the pentacyclic triterpene backbone.

Overall, the combined DSC and TGA data corroborate the presence of a thermally stable matrix dominated by pentacyclic triterpenic acids, Uvaol, and β -Sitosterol. The absence of sharp melting transitions, the presence of broad exothermic relaxation events, and the high residual mass at elevated temperatures are fully consistent with the molecular

profile identified by GC-MS and the functional groups confirmed by FTIR.

Surface analysis via XPS revealed the elemental composition of the extract. Bulk RSE samples showed a higher oxygen content (36.5%) compared to diluted samples (25.9%), suggesting the removal of residual solvents upon dilution. Critically, the C1s core level deconvolution identified a peak at 289.0 eV, assigned to carboxylic groups (COO). This experimental evidence directly supports the presence of free C-28 acidic groups in the triterpenes and was considered to computationally analyse RSE.

UPS analysis characterized the extract as an organic insulator with a measured work function of 2.1 eV and a wide band gap estimated at ≥ 5 eV, consistent with the saturated chemical nature of the target triterpenoids (Table 2 and Fig. S1).

3.3. Molecular docking calculations

It is well established that the association of TLR4 with MD-2 is an indispensable requirement for activating or inhibiting signaling (Park et al., 2009). To study and achieve a better understanding of the potential mechanism of action of these candidate compounds, a molecular modelling approach was performed. In this instance, the receptor complex TLR4/MD-2 was selected to perform a directed molecular docking calculation approach.

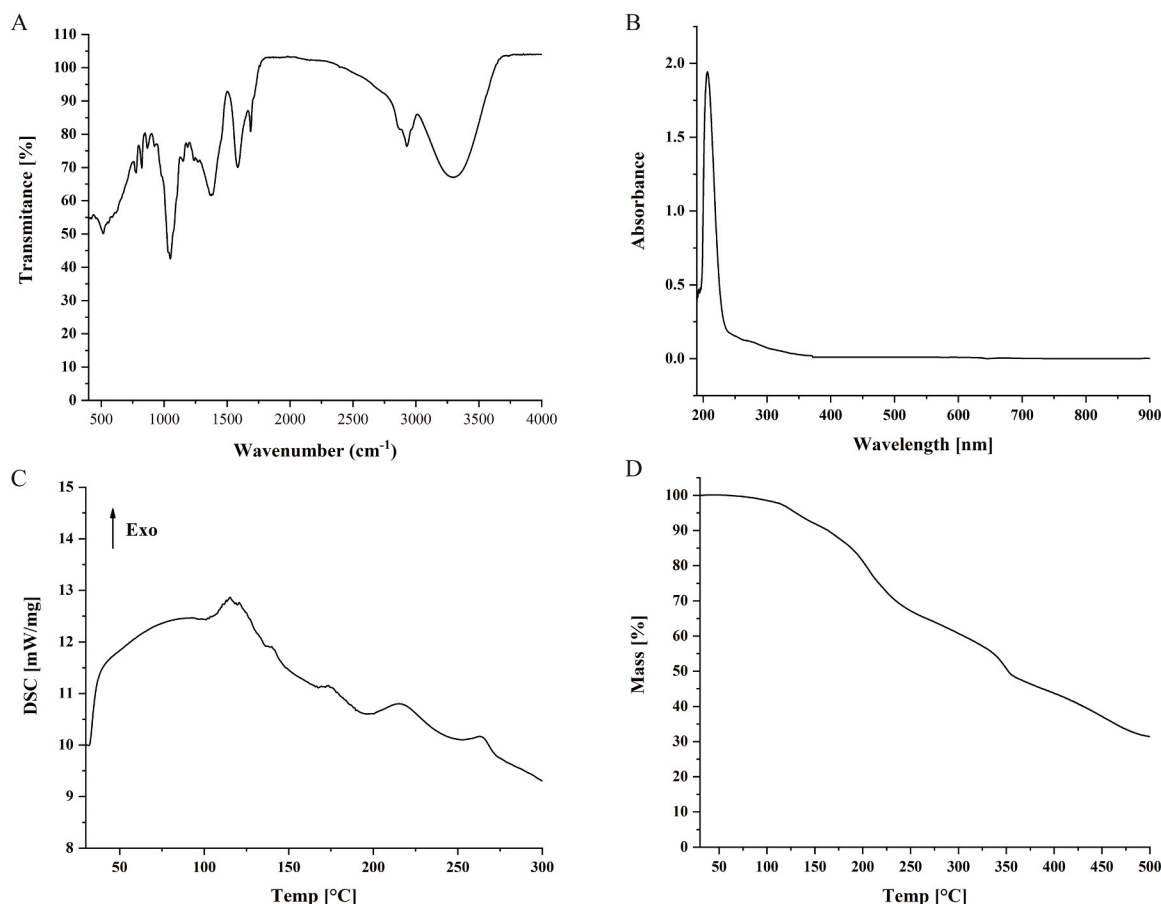


Fig. 2. FTIR analysis represents key triterpene functional groups corroborated by the UV-Vis absorption, thermal stability and phase transitions are defined by DSC and TGA. A) FTIR spectrum; B) UV-VIS spectrum; C) DSC thermogram; D) TGA profile.

Docking calculations performed on the TLR4/MD-2 complex revealed favourable binding profiles for all evaluated ligands when considering the 25 top-ranked poses and their corresponding replicas. Binding energies ranged from -7.7 to -11.22 kcal/mol, although some variability in docking scores was observed among ligands, the overall distributions remained within an energetically favourable range, suggesting a consistent binding potential across the series (Fig. 3).

To evaluate the computational reliability of the docking results, score distributions from independent replicates of the generated poses were compared for each SMs ligand (Fig. S2). The observed similarity among replicate distributions suggests consistent prediction trends and reproducible binding mode identification with the docking approach. Detailed inspection of the docking binding poses revealed the presence of multiple ligand accommodation regions within the MD-2 pocket. Among the evaluated compounds, β -Sitosterol, consistent with its highest predicted affinity, was observed to penetrate deeply into the hydrophobic core of MD-2 (Fig. 4).

To further rationalize these positional differences, ligand-protein interaction analyses were conducted. This approach enabled the characterization of the main stabilizing contacts and provided insight into the structural determinants underlying the observed variations in docking energies.

Our results indicate that hydrophobic interactions predominate over van der Waals interactions (Fig. 4 and Fig. S2-S4). The interaction patterns identified in this study can be interpreted considering the structural organization of the MD-2 binding cavity. Docking pose inspection and interaction fingerprint analysis revealed that all evaluated ligands established contacts within Site I, where aromatic residues, particularly Phe151, play a key role in ligand stabilization through non-polar interactions. This observation is consistent with previous structural

reports describing Site I as an aromatic-rich accommodation region (Lami et al., 2026).

Greater variability was observed within Site II, which comprises the central hydrophobic core of the MD-2 pocket. β -Sitosterol showed the highest interaction frequency among Site II residues, with 100% contact. This binding profile correlates with its deeper insertion into the pocket, as described in the docking pose analysis, and is likely facilitated by its extended aliphatic chain that enables extensive hydrophobic contacts with residues such as Leu61, Ile63, Phe76, Ile94, Phe104, and Ile117. A comparable, although less pronounced, interaction pattern was observed for Ursolic acid.

In contrast, the remaining ligands exhibited more limited engagement within Site II, primarily interacting with Leu61 and other peripheral hydrophobic residues. This behaviour is consistent with their reduced aliphatic extension and more external binding orientations within the pocket.

Notably, no significant contacts were detected for any ligand within Site III, a region characterized by a higher contribution of polar residues located toward the outer pocket interface. This absence of interaction further supports the preferential localization of the studied triterpenoid scaffolds within the hydrophobic regions of MD-2.

The integration of experimental and computational data offers a robust validation of the RSE mechanism of action. The identification of the COO component at 289.0 eV by XPS is a key finding; this data support the presence and preservation of the chemical groups that, according to our docking calculations, is potentially involved in the interaction with the TLR4/MD-2 complex.

The biological efficacy of the RSE is not merely the sum of its parts but arises from a combined action of triterpenes and β -sitosterol present in the crude extract that suggests a potential cooperative effect. This

Table 2

Comprehensive physicochemical, thermal, and surface electronic characterization of RSE. The table summarizes the experimental parameters for both the raw material (Bulk RSE) and the ethanol-diluted sample (Diluted RSE). FTIR analysis identifies key triterpenic functional groups, specifically the carbonyl stretching at 1687 cm^{-1} , which is corroborated by the UV-Vis absorption maximum at 206.5 nm. Thermal stability and phase transitions are defined by DSC and TGA, pinpointing a major degradation threshold at $314\text{ }^{\circ}\text{C}$. XPS data provides the surface elemental atomic composition and the deconvolution of the C1s core level (C-C, C-O, C=O, and COO), highlighting a significant reduction in oxygen content upon dilution. Finally, UPS analysis characterizes the extract as a wide-gap organic insulator, defined by a WF of 2.1 eV and an estimated band gap at least E_g 5 eV.

Technique	Parameter	Bulk RSE	Diluted RSE (in EtOH)
XPS	Elemental Composition (at%)	C: 60.1%, O: 36.5%, Na: 0.6%, K: 2.8%	C: 71.8%, O: 25.9%, K: 2.9%
	C1s Components (Binding Energy)	285.0 eV (C-C/C=C)	285.0 eV
		286.4 eV (C-O)	(C-C/C=C)
		287.8 eV (C=O)	286.6 eV (C-O)
		289.0 eV (COO)	288.0 eV (C=O)
	O1s Components (Binding Energy)	532.9 eV (O-C)	532.9 eV (O-C)
		531.4 eV (O=C)	531.4 eV (O=C)
	Surface Property	Insulating (High BE shift)	Insulating (High BE shift)
UPS	Work Function (WF)	-	2.1 eV
	Estimated Band Gap	-	At least E_g 5 eV
FTIR	Key Functional Groups (cm^{-1})	1687 (C=O), 3295 (-OH), 1584 (COO-)	-
TGA	Thermal Stability	120 $^{\circ}\text{C}$	Mass loss at 600 $^{\circ}\text{C}$: 69.6%
UV-Vis	Absorption Max	206.5 nm	-

synergy was experimentally observed in U937 monocytes, where the total phytocomplex induced a more significant downregulation of inflammatory markers compared to literature values for isolated compounds. The coexistence of pentacyclic triterpenes and phytosterols likely facilitates a multi-target modulation of the NF- κ B pathway, ensuring a robust bioactivity even at low concentrations (Table 3).

3.4. Cytotoxicity and anti-inflammatory effects of Red Sentinel Extract (RSE)

To validate the multi-target activity predicted by docking, we investigated the expression of key inflammatory markers in LPS-stimulated U937 cells.

To evaluate the anti-inflammatory activity of the RSE, we employed LPS-stimulated U937 cells as a monocyte-based *in vitro* model. To identify the optimal concentration range for assessing the putative anti-inflammatory activity of RSE, a preliminary dose-response assay was conducted by quantifying cell death after 7 h of exposure (Fig. 5A). Concentrations of callus extract exceeding $10\text{ }\mu\text{g/mL}$ resulted in a significant increment of cell death, whereas RSE concentrations up to $10\text{ }\mu\text{g/mL}$ did not affect cell viability. Based on these observations, subsequent experiments with LPS were performed using RSE concentrations within the range of $1\text{--}10\text{ }\mu\text{g/mL}$, revealing no cytotoxic effects in unstimulated and stimulated cells (Fig. 5A).

Anti-inflammatory activity has been investigated considering TLR4 signalling pathway through miR-146a, IRAK-1 and IL-6 expressions. The activation of the TLR4 by LPS triggers a signalling cascade that sequentially recruits and activates IRAK-1, leading to the nuclear translocation of NF- κ B that drives the transcription of pro-inflammatory

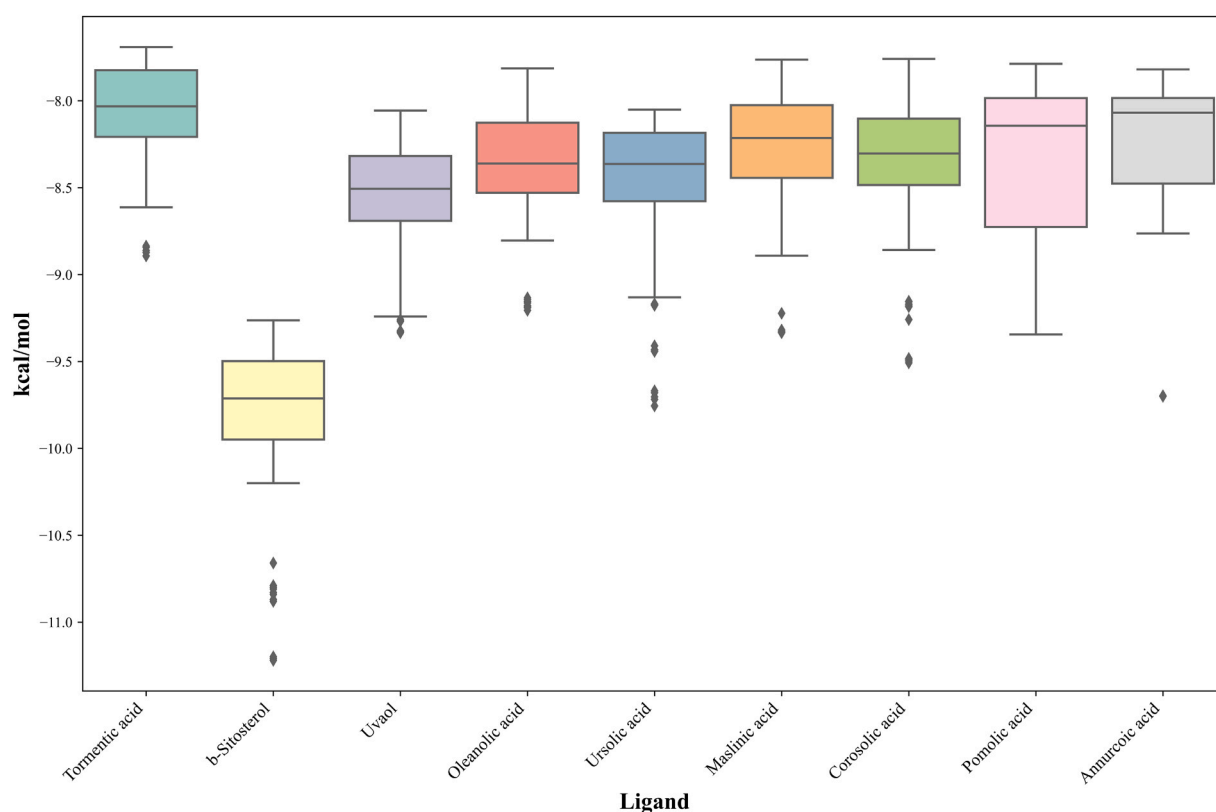


Fig. 3. Binding affinity (kcal/mol) distribution of Secondary Metabolites (SMs) ligands present in RES to complex TLR4/MD-2.

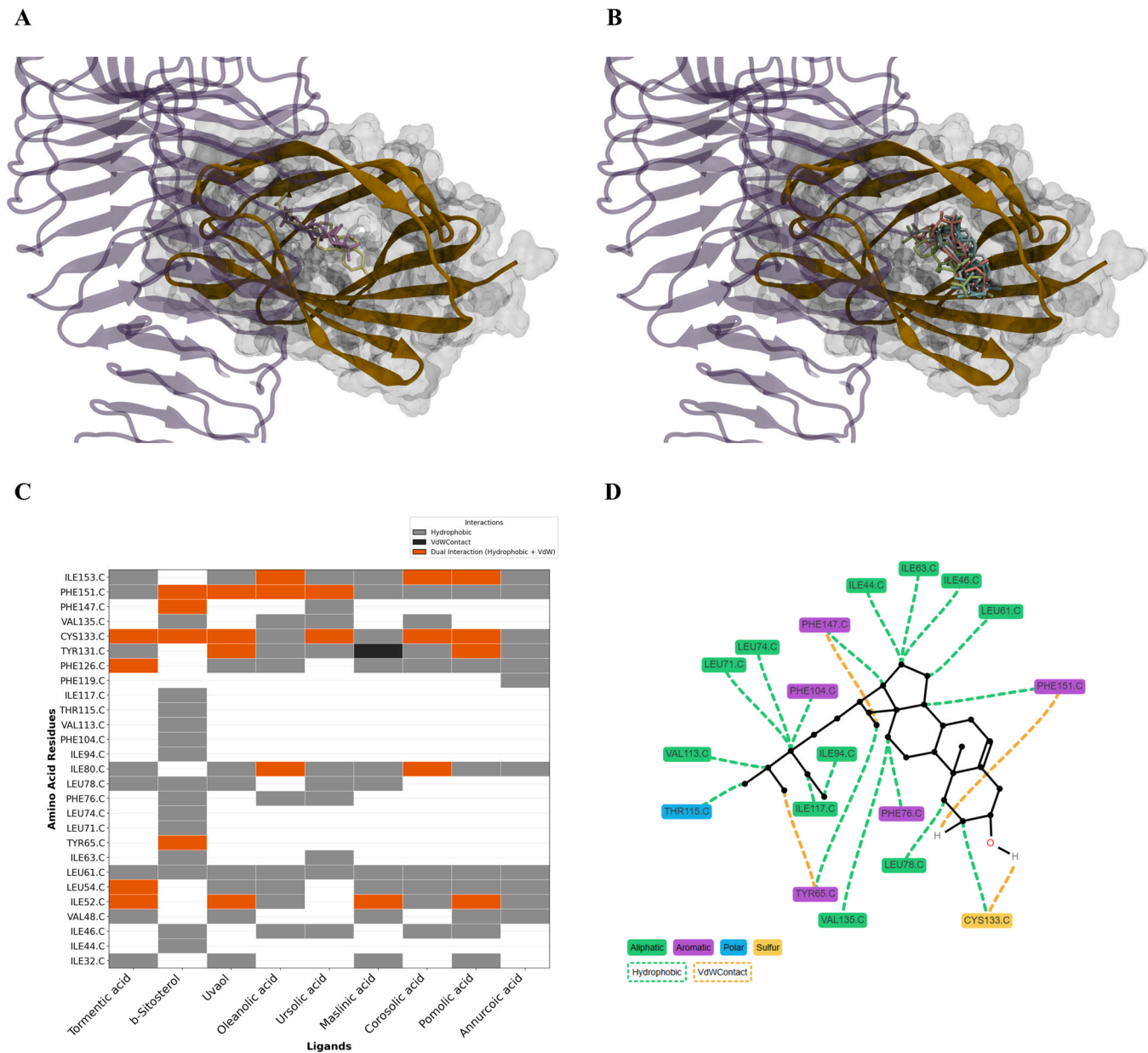


Fig. 4. Detailed inspection of the docking binding poses identified multiple ligand accommodation regions within the MD-2 pocket. A) Molecular docking results showing β -Sitosterol (purple) and Lupeol (yellow) located in the MD-2 binding pocket (yellow, surf cartoon) of the TLR4/MD-2 system (purple). Hydrogens have been omitted for clarity. B) Molecular docking results showing the rest of the molecules of the study located in the MD-2 binding pocket of the TLR4/MD-2 system (using the same colour scheme). C) Heatmap showing the distribution of interactions between amino acid residues of the MD-2 protein (Chain C on the PDB) and Red Sentinel compounds. Hydrophobic interactions are marked in grey, van der Waals contact in black and residues with both types of interactions in orange. D) β -Sitosterol calculated interactions.

Table 3
Annurcoic acid, Ursolic acid and β -Sitosterol have the necessary scaffold for high-affinity binding to the TLR4/MD-2 complex corroborated by the carboxylic (COO-) signal identified via XPS.

Compound	Class	Experimental Evidence (XPS)	Docking Affinity (kcal/mol)
Annurcoic acid	Triterpene	C-28 Carboxylic group (289.0 eV)	-8.8
Ursolic acid	Triterpene	Structural scaffold validation	Qualitative match
β -Sitosterol	Phytosterol	Aliphatic chain confirmation	Synergistic role

cytokines such as IL-6. The miRNA-146a acts as an epigenetic target of IRAK1 by silencing its expression.

As indicated in Fig. 5B, miR-146a is down-regulated during LPS pro-inflammatory stimulus while the expression of its target, IRAK-1, and IL-6, are up-regulated. On the other hand, RSE treatments during an acute LPS pro-inflammatory stimulus (RS + LPS) up-regulated miR-146a and down-regulated IRAK-1 and IL-6 expression levels.

The treatment with the triterpenic extract significantly modulated the TLR4-mediated signaling cascade. Specifically, a marked down-regulation of IL-6 and IRAK-1 was observed when exposed to LPS treatment, correlating with the competitive binding predicted for the SMs present in the extract at the TLR4/MD-2 site (Fig. 5C). In addition, RSE influenced the expression of miR-146a, a critical epigenetic regulator of the TLR4 inflammatory response.

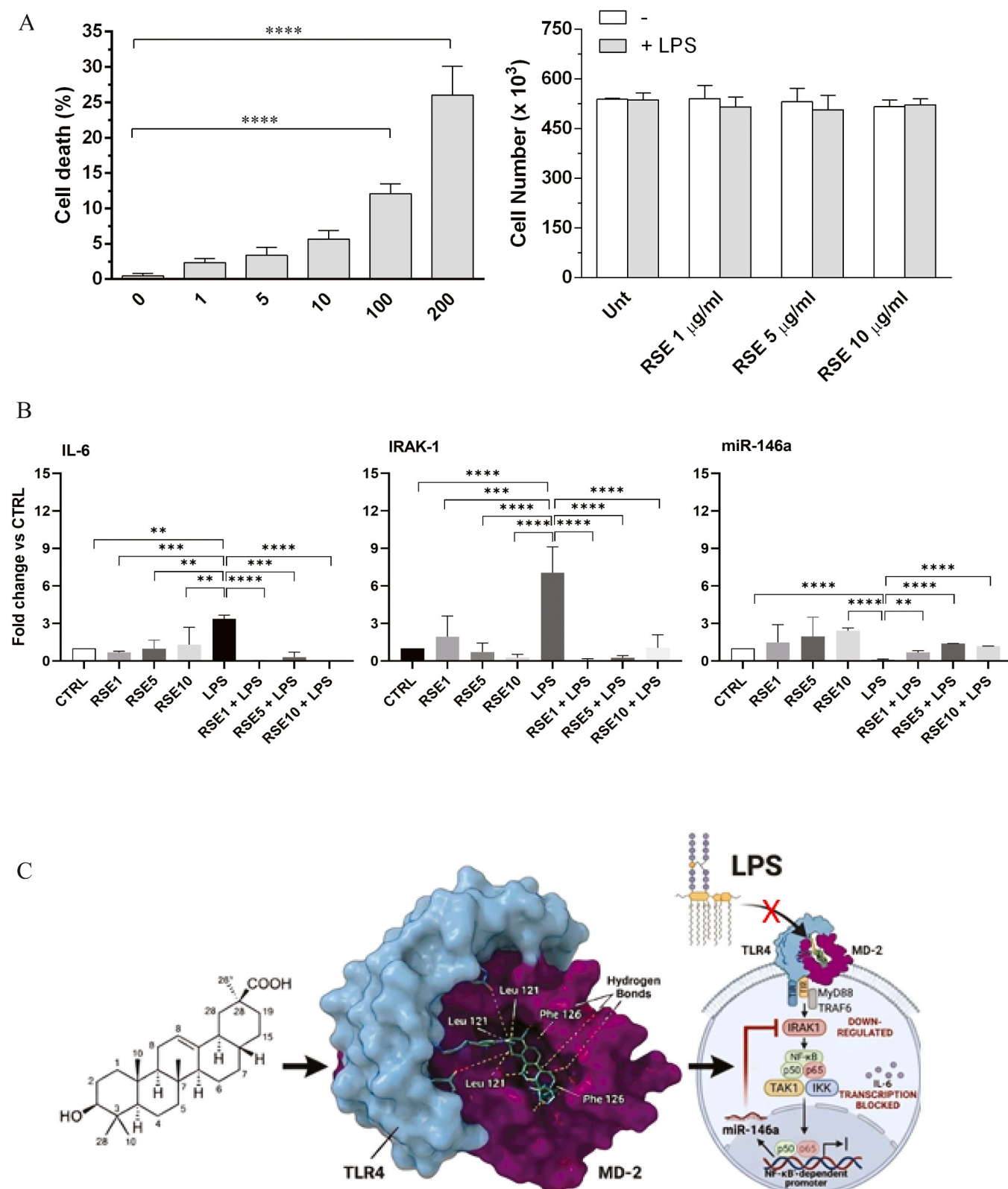


Fig. 5. Biological Validation of TLR4 Pathway Modulation in RSE U937 cells treatment. A) U937 cells were preliminary exposed to increasing concentrations of RS extract for 7 h. LPS (1 $\mu\text{g}/\text{mL}$) treatments were performed for 6 h after a RSE (1, 5, 10 $\mu\text{g}/\text{mL}$) pre-treatment of 1 h. After the treatments U937 cells were processed for cell death/number evaluation. B) RT-qPCR analysis of IL-6, IRAK-1 and miR-146a expression levels. Values are expressed as mean $\pm$ SD of three independent experiments. Differences among multiple groups were evaluated via ANOVA followed by Tukey's post-hoc test. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Previous studies in monocytic models have shown that prolonged LPS exposure induces miR-146a as a late negative-feedback mechanism, contributing to the attenuation of NF- κ B signaling through post-transcriptional targeting of IRAK-1 (Taganov et al., 2006). In contrast, in our acute setting, LPS exposure for 6 h resulted in a reduction of miR-146a expression. Biologically, this early suppression of the epigenetic brake allows the cell to rapidly execute a complete initial immune response via TLR4 signaling, leading to the observed accumulation of IRAK-1 and the release of downstream pro-inflammatory cytokines like IL-6.

4. Conclusion

In this study, a multi-analytical approach was employed to characterize a triterpenoid-rich extract obtained from plant callus cultures.

Cellular data provide experimental confirmation that the electronic and vibrational features identified via UPS/XPS and FTIR/DSC, TGA and UV-Vis translate into a potent anti-inflammatory pharmacological effect. The surface characterization of RSE through XPS support the molecular docking calculations conducted on TLR4/MD-2 by verifying the elemental and functional integrity of the extract's bioactive constituents. Specifically, the deconvolution of the C1s core level identified a distinct component at 289.0 eV, which is assigned to carboxylic/ester groups (COO). This finding is consistent with the importance of maintaining the free C-28 carboxylic acid group in the triterpenic scaffold, a structural feature observed in our computational analysis.

In our docking studies, this carboxylic moiety was shown to engage in hydrogen bonding interaction with specific amino acid residues in the MD-2 pocket, suggesting a potential stabilization of the complex in an antagonistic conformation. The experimental detection of these functional groups by XPS, alongside the FTIR peak at 1687 cm^{-1} , confirms that the extraction process preserves the bioactive acidic nature of the compounds.

Furthermore, the wide band gap of at least E_g 5 eV and the insulating character revealed by UPS are consistent with the saturated pentacyclic structure of the triterpenes, which lack extended π -conjugation. This electronic profile supports the hypothesis that the biological activity of the extract is governed by specific stereochemical and electrostatic recognition (lock-and-key mechanism) rather than non-specific electronic transfer, further reinforcing the reliability of our *in silico* predictions in describing the anti-inflammatory potential of the RSE.

The integrated multidisciplinary framework combining experimental and computational approaches successfully correlated the thermal stability (major mass loss at 260°C) and spectroscopic electronic levels of the extracts with their multi-target biological activity.

Furthermore, RSE biological activity cannot be attributed solely to a single constituent, as the combined action of triterpenes and β -sitosterol present in the crude extract suggests a potential cooperative effect. While Ursolic acid and Annurcoic acid provide high-affinity binding to the TLR4/MD-2 complex (confirmed by the carboxylic signal at 289.0 eV in XPS), the presence of β -Sitosterol contributes to membrane stabilization and potential modulation of co-receptor signalling. This RSE phytocomplex synergy resulted in a more robust inhibition of the inflammatory cascade in U937 monocytes compared to isolated compounds, highlighting the advantage of using a standardized whole-plant extract for functional food and pharmaceutical applications. These findings demonstrate that plant cell culture technology can yield standardized phytocomplexes capable of modulating the miR-146a/IRAK-1/IL-6 axis to counter monocytic inflammaging, highlighting the utility of integrated surface physics and molecular biology in anti-aging nutraceutical development.

CRedit authorship contribution statement

Annalisa Aluigi: Writing – review & editing, Investigation. **Luca Casettari:** Writing – original draft, Validation, Supervision,

Investigation, Data curation. **Enrique Llobet-Serra:** Investigation, Data curation, Conceptualization. **Carpio Laureano:** Investigation, Data curation, Conceptualization. **Liana Cerioni:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Garcia-Meseguer Rafael:** Writing – review & editing, Supervision, Conceptualization. **Mattia Tiboni:** Writing – original draft, Validation, Methodology. **Daniele Fraternali:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Noemi Pappagallo:** Methodology, Formal analysis, Data curation. **Serena Benedetti:** Methodology, Formal analysis, Data curation. **Maria Cristina Albertini:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Roberto Verucchi:** Validation, Supervision, Methodology, Investigation. **Lucrezia Aversa:** Validation, Methodology, Investigation, Data curation. **Andrea Gorassini:** Writing – review & editing, Methodology.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, to better integrate the relationship between RSE physicochemical properties and its biological effects, the authors used AI Gemini (Google) to investigate existing knowledge gaps. The authors provided all the scientific data and technical information, which were subsequently processed and organized with some suggestions received from the AI tool. After using this AI tool, the authors meticulously reviewed, edited, and validated the appropriate outputs to ensure scientific accuracy and take full responsibility for the final content of the publication.

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Declaration of Competing Interest

Since this is an academic study based on natural extracts: 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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.mad.2026.112251](https://doi.org/10.1016/j.mad.2026.112251).

Data availability

Data will be made available on request.

References

- Altunayar-Unsalan, C., 2024. DSC and FTIR study on the interaction between pentacyclic triterpenoid lupeol and DPPC membrane. *J. Bioenerg. Biomembr.* 56, 553–561. <https://doi.org/10.1007/s10863-024-10030-1>.
- Basu, S., Ulbricht, Y., Rossol, M., 2025. Healthy and premature aging of monocytes and macrophages. *Front. Immunol.* 16, 1–16. <https://doi.org/10.3389/fimmu.2025.1506165>.
- Beamson, G., Briggs, D., 1993. High Resolution XPS of Organic Polymers: The Scienta ESCA300 Database. A25 *J. Chem. Educ.* 70. <https://doi.org/10.1021/ed070pA25.5>.
- Bhaumik, D., Scott, G.K., Schokrpur, S., Patil, C.K., Orjalo, A.V., Rodier, F., Lithgow, G.J., Campisi, J., 2009. MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. *Aging (Albany NY)* 1, 402–411. <https://doi.org/10.18632/aging.100042>.
- Bouysset, C., Fiorucci, S., 2021. ProLIF: a library to encode molecular interactions as fingerprints. *J. Cheminform.* 13, 72–81. <https://doi.org/10.1186/s13321-021-00548-6>.

- Burley, S.K., Bhatt, R., Bhikadiya, C., Bi, C., Biester, A., Biswas, P., Bittrich, S., Blaumann, S., Brown, R., Chao, H., Chithari, V.R., Craig, P.A., Crichlow, G.V., Duarte, J.M., Dutta, S., Feng, Z., Flatt, J.W., Ghosh, S., Goodsell, D.S., Zardecki, C., 2025. Updated resources for exploring experimentally-determined PDB structures and Computed Structure Models at the RCSB Protein Data Bank. *Nucleic Acids Res.* 53, D564–D574. <https://doi.org/10.1093/nar/gkaf1091>.
- Chiarella, F., Toccoli, T., Barra, M., Aversa, L., Ciccullo, F., Tatti, R., Verucchi, R., Iannotta, S., Cassinese, A., 2014. High mobility *n*-type organic thin-film transistors deposited at room temperature by supersonic molecular beam deposition, 143302/1-143302/5 *Appl. Phys. Lett.* 104. <https://doi.org/10.1063/1.4870991>.
- Correia, R.C.G., Peralta, R.M., Haminiuk, C.W.I., Maciel, G.M., Bracht, A., Ferreira, I.C.F. R., 2017. New phytochemicals as potential human anti-aging compounds: Reality, promise, and challenges. *Crit. Rev. Food Sci. Nutr.* 942–957. <https://doi.org/10.1080/10408398.2016.1233860>.
- Da Silva Júnior, W.F., Pinheiro, J.G.D.O., Moreira, C.D.L.D.F.A., Rüdiger, A.L., Barbosa, E.G., Lima, E.S., Da Veiga Júnior, V.F., Da Silva Júnior, A.A., Aragão, C.F.S., De Lima, Á.A.N., 2017. Thermal behavior and thermal degradation kinetic parameters of triterpene α , β amyrin. *J. Therm. Anal. Calorim.* 127, 1757–1766. <https://doi.org/10.1007/s10973-016-6046-x>.
- Das, G., Kameswaran, S., Ramesh, B., Bangeppagari, M., Nath, R., Talukdar, A.D., Shin, H.S., Patra, J.K., 2024. Anti-aging effect of traditional plant-based food: an overview. *Foods* 13, 3785–3822. <https://doi.org/10.3390/foods13233785>.
- Deng, J., Wang, H., Mu, X., He, X., Zhao, F., Meng, Q., 2021. Advances in research on the preparation and biological activity of maslinic acid. *Mini-Rev. Med. Chem.* 21, 79–89. <https://doi.org/10.2174/1389557520666200722134208>.
- Ding, H., Hu, X., Xu, X., Zhang, G., Gong, D., 2018. Inhibitory mechanism of two allosteric inhibitors, oleanolic acid and ursolic acid on α -glucosidase. *Int. J. Biol. Macromol.* 107, 1844–1855. <https://doi.org/10.1016/j.ijbiomac.2017.10.040>.
- Fazili, M.A., Bashir, I., Ahmad, M., Yaqoob, U., Geelani, S.N., 2022. *in vitro* strategies for the enhancement of secondary metabolite production in plants: a review. *Bull. Natl. Res. Cent.* 46, 35–47. <https://doi.org/10.1186/s42269-022-00717-z>.
- Forli, S., Huey, R., Pique, M.E., Sanner, M.F., Goodsell, D.S., Olson, A.J., 2016. Computational protein–ligand docking and virtual drug screening with the AutoDock suite. *Nat. Protoc.* 11, 905–919. <https://doi.org/10.1038/nprot.2016.051>.
- Fraternal, D., Rudov, A., Prattichizzo, F., Olivieri, F., Ricci, D., Giacomini, E., Carloni, S., Azzolini, C., Gordillo, B., Jara-Palacios, M.J., Verardo, G., Albertini, M. C., 2016. Chemical composition and “*in vitro*” anti-inflammatory activity of *Vitis vinifera* L. (var. Sangiovese) tendrils extract. *J. Funct. Foods* 20, 291–302. <https://doi.org/10.1016/j.jff.2015.11.013>.
- Fraternal, D., Dufat, H., Albertini, M.C., Bouzidi, C., D’Adderio, R., Coppari, S., Di Giacomo, B., Melandri, D., Ramakrishna, S., Colomba, M., 2022. Chemical composition, antioxidant and anti-inflammatory properties of *Monarda didyma* L. essential oil. *PeerJ* 21, 1–22. <https://doi.org/10.7717/peerj.14433>.
- Gómez-Pulido, L.D.M., González-Cano, R.C., Domínguez, E., Heredia, A., 2021. Structure determination of oleanolic and ursolic acids: a combined density functional theory/vibrational spectroscopy methodology. *R. Soc. Open. Sci.* 8, 210162–210172. <https://doi.org/10.1098/rsos.210162>.
- Gowers, R., Linke, M., Barnoud, J., Reddy, T., Melo, M., Seyler, S., Domański, J., Dotson, D., Buchoux, S., Kenney, I., Beckstein, O., 2016. In: Benthall, Sebastian, Rostrup, Scott (Eds.), MDAnalysis: A Python Package for the Rapid Analysis of Molecular Dynamics Simulations. In: Proceedings of the 15th Python in Science Conference, Austin, TX, pp. 98–105. <https://doi.org/10.25080/Majora-629e541a-00e>.
- Gubitosa, F., Fraternal, D., De Bellis, R., Gorassini, A., Benayada, L., Chiarantini, L., Albertini, M.C., Potenza, L., 2023. *Cydonia oblonga* Mill. Pulp Callus Inhibits Oxidative Stress and Inflammation in Injured Cells. *Antioxidants* 12, 1076–1087. <https://doi.org/10.3390/antiox12051076>.
- Gubitosa, F., Fraternal, D., Benayada, L., De Bellis, R., Gorassini, A., Saltarelli, R., Donati Zeppa, S., Potenza, L., 2024. Anti-Inflammatory, Antioxidant, and Genoprotective Effects of Callus Cultures Obtained from the Pulp of *Malus pumila* cv Miller (Annurca Campana Apple). *Foods* 13, 2036–2052. <https://doi.org/10.3390/foods13132036>.
- Hao, W., Kwek, E., He, Z., Zhu, H., Liu, J., Zhao, Y., Ma, K.Y., He, W.-S., Chen, Z.-Y., 2020. Ursolic acid alleviates hypercholesterolemia and modulates the gut microbiota in hamsters. *Food Funct.* 11, 6091–6103. <https://doi.org/10.1039/D0FO000829J>.
- Humphrey, W., Dalke, A., Schulten, K., 1996. VMD: Visual molecular dynamics. *J. Mol. Graph.* 14, 33–38. [https://doi.org/10.1016/0263-7855\(96\)00018-5](https://doi.org/10.1016/0263-7855(96)00018-5).
- Hunter, J.D., 2007. Matplotlib: A 2D Graphics Environment. *Comput. Sci. Eng.* 9, 90–95. <https://doi.org/10.1109/MCSE.2007.55>.
- Jia, Y., Li, F., 2018. Studies of Functional Nucleic Acids Modified Light Addressable Potentiometric Sensors: X-ray Photoelectron Spectroscopy, Biochemical Assay, and Simulation. *Anal. Chem.* 90, 5153–5161. <https://doi.org/10.1021/acs.analchem.7b05261>.
- Kashyap, D., Sharma, A., Tuli, H.S., Punia, S., Sharma, A.K., 2016. Ursolic Acid and Oleanolic Acid: Pentacyclic Terpenoids with Promising Anti-Inflammatory Activities. *Recent. Pat. Inflamm. Allergy Drug. Discov.* 10, 21–33. <https://doi.org/10.2174/1872213X10066160711143904>.
- Kim, H.J., Kim, H., Lee, J.H., Hwangbo, C., 2023. Toll-like receptor 4 (TLR4): new insight immune and aging. *Immun. Ageing* 24, 67–78. <https://doi.org/10.1186/s12979-023-00383-3>.
- Kuzmich, N., Sivak, K., Chubarev, V., Porozov, Y., Savateeva-Lyubimova, T., Peri, F., 2017. TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines* 5, 34–59. <https://doi.org/10.3390/vaccines5040034>.
- Lami, F., Romerio, A., Valle-Gómez, L., Martín-Cámara, O., Martín-Santamaría, S., Peri, F., 2026. Validating natural compounds as Toll-Like Receptor 4 (TLR4) antagonists: experimental challenges and therapeutic perspectives. *J. Med. Chem.* 69, 72–86. <https://doi.org/10.1021/acs.jmedchem.5c02252>.
- Lee, J.Y., Lee, B.H., Kim, N.D., Lee, J.Y., 2015. Celastrol blocks binding of lipopolysaccharides to a Toll-like receptor4/myeloid differentiation factor2 complex in a thiol-dependent manner. *J. Ethnopharmacol.* 172, 254–260. <https://doi.org/10.1016/j.jep.2015.06.028>.
- Liu, G., Cui, Z., Gao, X., Liu, H., Wang, L., Gong, J., Wang, A., Zhang, J., Ma, Q., Huang, Y., Piao, G., Yuan, H., 2021. Corosolic acid ameliorates non-alcoholic steatohepatitis induced by high-fat diet and carbon tetrachloride by regulating TGF- β 1/Smad2, NF- κ B, and AMPK signaling pathways. *Phytother. Res.* 35, 5214–5226. <https://doi.org/10.1002/ptr.7195>.
- Olivieri, F., Lazzarini, R., Babini, L., Prattichizzo, F., Rippon, M.R., Tiano, L., Di Nuzzo, S., Graciotti, L., Festa, R., Bruge, F., Orlando, P., Silvestri, S., Capri, M., Palma, L., Magnani, M., Franceschi, C., Littarru, G.P., Procopio, A.D., 2013. Anti-inflammatory effect of ubiquinol-10 on young and senescent endothelial cells via miR-146a modulation. *Free. Radic. Biol. Med.* 63, 410–420. <https://doi.org/10.1016/j.freeradbiomed.2013.05.033>.
- Olivieri, F., Prattichizzo, F., Giuliani, A., Maccachione, G., Rippon, M.R., Sabbatinelli, J., Bonafè, M., 2021. miR-21 and miR-146a: The microRNAs of inflammaging and age-related diseases. *101374- Ageing Res. Rev.* 70, 101393. <https://doi.org/10.1016/j.arr.2021.101374>.
- Ong, S.M., Hadadi, E., Dang, T.M., Yeap, W.H., Tan, C.T.Y., Ng, T.P., Larbi, A., Wong, S. C., 2018. The pro-inflammatory phenotype of the human non-classical monocyte subset is attributed to senescence. *Cell. Death & Dis.* 9, 266–278. <https://doi.org/10.1038/s41419-018-0327-1>.
- Ortiz-González, C., Casar, B., Gozalbes, R., Serrano-Candelas, E., Crespo, P., Carpio, L.E., 2025. Identification and Characterization of ERK2 Dimerization Inhibitors by Integrated In Silico and In Vitro Screening. *Int. J. Mol. Sci.* 26, 11481–11498. <https://doi.org/10.3390/ijms262311481>.
- Păcularu-Burada, B., Cîrîc, A.I., Begea, M., 2024. Anti-Aging Effects of Flavonoids from Plant Extracts. *Foods* 13, 2441–2471. <https://doi.org/10.3390/foods13152441>.
- Park, B.S., Song, D.H., Kim, H.M., Choi, B.-S., Lee, H., Lee, J.-O., 2009. The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex. *Nature* 458, 1191–1195. <https://doi.org/10.1038/nature07830>.
- Peruzzi, C., Battistoni, S., Montesarchio, D., Cocuzza, M., Marasso, S.L., Verna, A., Pasquardini, L., Verucchi, R., Aversa, L., Erokhin, V., D’Angelo, P., Iannotta, S., 2021. Interfacing aptamers, nanoparticles and graphene in a hierarchical structure for highly selective detection of biomolecules in OECT devices. *Sci. Rep.* 11, 9380–9391. <https://doi.org/10.1038/s41598-021-88546-4>.
- Shaw, A.C., Joshi, S., Greenwood, H., Panda, A., Lord, J.M., 2010. Aging of the innate immune system. *Curr. Opin. Immunol.* 22, 507–513. <https://doi.org/10.1016/j.coi.2010.05.003>.
- Strober, W., 2015. Trypan Blue Exclusion Test of Cell Viability. *A3.B.1 -A3.B.3 Curr. Protoc. Immunol.* 111. <https://doi.org/10.1002/0471142735.ima03bs111>.
- Taganov, K.D., Boldin, M.P., Chang, K.J., Baltimore, D., 2006. NF- κ B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *PNAS* 103, 12481–12486. <https://doi.org/10.1073/pnas.0605298103>.
- Trott, O., Olson, A.J., 2010. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* 31, 455–461. <https://doi.org/10.1002/jcc.21334>.
- Verardo, G., Gorassini, A., Ricci, D., Fraternal, D., 2017. High Triterpenic Acids Production in Callus Cultures from Fruit Pulp of Two Apple Varieties. *Phytochem. Anal.* 28, 5–15. <https://doi.org/10.1002/pca.2638>.
- Vitiello, M., Braca, A., De Leo, M., Fraternal, D., 2024. Profiling specialized metabolites of two *Malus domestica* Borkh. varieties: In vitro pulp callus culture vs fruit peel and pulp. *Sci. Hortic.* 338, 113821–113831. <https://doi.org/10.1016/j.scienta.2024.113821>.
- Xu, M., Li, X., Song, L., Tao, C., Fang, J., Tao, L., 2020. Lupeol alleviates coxsackievirus B3-induced viral myocarditis in mice via downregulating toll-like receptor 4. *J. Int. Med. Res.* 48, 1–11. <https://doi.org/10.1177/0300060520910908>.

Web References

- RDKit, Open-Source Cheminformatics: <https://www.rdkit.org>.
 Protein Data Bank: <https://www.rcsb.org/>, accessed on 21 January 2026.
 PubChem database: <https://pubchem.ncbi.nlm.nih.gov/>, accessed on 1 January 2026.
 Python: <https://www.python.org>.