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Reducing cellular inflammation and oxidation by supercritical CO_2 extracts from edible-medicinal mushrooms

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ABSTRACT

Extracts from *Hericium erinaceus*, *Ganoderma lucidum* and *Tremella fuciformis* obtained with supercritical CO_2 showed high cellular antioxidant activity (CAA) when tested on Caco-2 cell cultures. Extract obtained in separator 2 (5 MPa) after 3 h extraction at 30 MPa and 40 °C from *G. lucidum* showed a remarkable higher CAA than noticed for the other species. In addition, extracts to THP-1 macrophages reduced secretion of TNF-α and particularly IL-6 (80–90 % applied at 15 μg/mL). The *G. lucidum* extract was also more effective than those from the other two species except for IL-1β. The use of ethanol 5 % (*v*/v) as co-solvent for extractions increased the yield but reduced both activities. Extracts from studied species contained linoleic, oleic, α-linolenic and palmitic acids as the main fatty acids, and ergosterol and ergosterol peroxide (EP) as fungal sterols. Those obtained from *G. lucidum* included ganoderic acids A and D, ganoderol B and lucidenic acid A. When identified compounds were tested, ganoderol B, ganoderic acid A, linoleic acid, α-linolenic acid and EP showed remarkable CAA. The anti-inflammatory activities of α-linolenic and linoleic acids were higher than those noticed for ergosterol and EP, and the latter compounds were more effective than the other tested triterpenoids. These results suggest that the anti-inflammatory effect noticed (particularly for *G. lucidum* extract) might be due to a synergistic activity of the indicated compounds.

1. Introduction

Nowadays, the use of green technologies to extract bioactive compounds for the food industry is encouraged. The implementation of supercritical fluids, *e.g.* as pressurized CO_2, reduces the need for organic solvents, generating safer extracts. When CO_2 is pressurized at mild temperatures, it reaches a supercritical status, allowing the effective extraction of non-polar compounds from inner tissues or food matrices, as it can penetrate easily due to its low viscosity. In addition, when CO_2 is depressurized, no solvent trace remains in the obtained fraction. This advantage, together with the ability to recirculate the utilized CO_2 after the extraction, makes the process environmentally friendly (Morales et al., 2018b).

Although mushrooms contain low fat levels, supercritical fluid extraction (SFE) was successfully applied to extract compounds with beneficial biological properties from edible mushrooms from common genera as *Agaricus sp., Boletus sp., Pleurotus sp*…, and medicinal mushrooms (*Inonotus obliquus, Traemetes versicolour, Fomitopsis betulina* (before *Piptoporus betulinus*)…) (Almeida et al., 2014; Chen et al., 2011; Durak, 2018; Grishin et al., 2016; Huynh et al., 2022; Li et al., 2016; Morales et al., 2018; Vidović et al., 2011). In these studies, supercritical CO_2 extractions were carried out using pressures between 5 and 60 MPa

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(mostly 10–30 MPa), mild temperatures (approx. 30–60 °C), extraction times from 0.5 up to 3 h and co-solvents (mainly 0–15 % ethanol). The obtained yields were always higher if co-solvents were utilized (0.5–3.8 %), although a higher specificity for triterpenoid extraction was achieved with only CO_2 (average 0.5–0.8 %). The main compounds extracted from these basidiomycetes included fatty acids (Kitzberger et al., 2009; Li et al., 2016; Nowak et al., 2022; Vidović et al., 2011), volatile compounds (aroma) (Chen et al., 2018; Durak, 2018), phenolic compounds (Joradon et al., 2022; Krümmel et al., 2022; Mazzutti et al., 2012) and fungal sterols (Morales et al., 2018), as well as particular molecules from certain species such as *Ganoderma lucidum* (ganoderic acids, ganodermanontriol and derivatives) (Zhang et al., 2025; Zhu et al., 2020) and *Hericium erinaceus* (hericenone, hericenes and other triterpenoids) (Joradon et al., 2022). Ergothioneine was also mentioned by Bhattacharya et al. (2014) in *Pleurotus ostreatus* SFE extracts. Many of these molecules were pointed out as potentially responsible for the biological activities noticed for the SFE extracts.

Several biological activities were reported for SFE fractions from different mushrooms, including antimicrobial activities (Dat et al., 2022; Krümmel et al., 2022; Mazzutti et al., 2012; Mishra et al., 2021; Weber et al., 2022); antitumoral activities (Cao et al., 2023; Dat et al., 2022; Zhao et al., 2021; Zhu et al., 2020); physiological enzyme inhibition (Gil-Ramírez et al., 2016; Milovanovic et al., 2021; Milovanovic et al., 2024; Morales et al., 2018); or, to a lesser extent, antiplatelet aggregation and anticoagulant activities (Weber et al., 2022), and protective effects against hypoxic stress on human embryonic kidney cells (Mishra et al., 2021).

The antioxidant capacity stands out as the most evaluated biological activity in SFE fractions obtained from many mushroom species such as *H. erinaceus* (Cao et al., 2023; Joradon et al., 2022; Parada et al., 2015), *Pleurotus eryngii* (Rodríguez-Seoane et al., 2019), *A. blazeii* (Mazzutti et al., 2012), *L. edodes* (Morales et al., 2018), *P. sajor caju* (Krümmel et al., 2022), *P. pulmonarius* (Milovanovic et al., 2021), *G. lucidum* (Gao et al., 2010; Milovanovic et al., 2024), or *Fomes fomentarius* (Brovko et al., 2021). Most of these studies reported interesting antioxidant activities depending on extraction conditions and other parameters. However, only chemical assays were used (DPPH or ABTS scavenging, FRAP, CUPRAC, metal chelating, phosphomolybdenum assay…), thus their actual significance in biological systems are seriously compromised. Opposite to that, the study conducted by Barbosa et al. (2020) is one of the few to use cell cultures. They established the protection exerted by hot water-extracted polysaccharides (PSCs) from *Pleurotus ostreatus* against oxidative damage induced by H_2O_2 in HepG2 cells.

Moreover, some mushroom extracts exhibited immunomodulatory activities, which were associated with the presence of PSCs, particular proteins, or small peptides (Morales et al., 2020; Chakraborty et al., 2021). These compounds are not usually extracted with supercritical CO_2, since this technology is more suited to obtain lipids and other non-polar molecules. Therefore, the effect of CO_2-SFE fractions (with or without ethanol as co-solvent) remains unexplored.

Therefore, in the present work, the anti-inflammatory activity of SFE extracts obtained from three edible-medicinal mushrooms, *Ganoderma lucidum*, *Hericium erinaceus* and *Tremella fuciformis* were studied, together with their cellular antioxidant properties (Caco-2 cells) as a more realistic *in vitro* approach to evaluate their potential antioxidant properties in biological systems. These three species were selected because they are all considered by traditional Asiatic medicine as therapeutical remedies or with interesting bioactive properties, however while Ganorema *sp.* and Hericium erinaceous were subjected to many studies, scare information was reported on Tremella *sp.* Moreover, in these strains most of the studies were focused on their polysaccharides, while studies concerning their sterols or lipid fraction are more limited, particularly for Tremella fuciformis (Wu et al., 2019a). The selection would allow proper comparison concerning their potential.

Moreover, the composition of the supercritical CO_2 extracts was also analysed. Afterwards, pure standards of the main bioactive compounds identified were also tested as an attempt to further elucidate the chemical nature of the anti-inflammatory and antioxidant compounds from the SFE extracts obtained.

2. Material and methods

2.1. Biological material and samples preparation

Strains used in this investigation were *Hericium erinaceus* (Bull.) Persoon (Lion's mane mushroom), *Ganoderma lucidum* (Curtis) P. Karst (reishi or varnished conk mushroom), and *Tremella fuciformis* (Berk.) (snow fungus or white jelly mushroom). Dried fruiting bodies were purchased from a particular mushroom grower at Seoul Medicine Market (Republic of Korea) following directions of Prof. Sang Hee Shim from Duksung Women's University (College of Pharmacy). Fruiting bodies were ground as described in Gil-Ramírez et al. (2013) and stored at −20 °C until further use.

2.2. Reagents

Methanol (HPLC grade) was obtained from LAB-SCAN (Gliwice, Polland) and ethyl acetate from Labkem (Barcelona, Spain). Ethanol (99.8 %), hexane, sulfuric acid, and glacial acetic acid were purchased from Panreac (Barcelona, Spain). Dimethylsulfoxide (DMSO) and acetonitrile were acquired from Macron Fine Chemicals (Madrid, Spain). Trifluoroacetic acid (TFA), ammonium formate, ergosterol (ERG), oleic (OA), palmitic (PA), linoleic (LA), and α-linolenic (α-LA) acids were purchased from Sigma-Aldrich (Madrid, Spain). Ganoderic acid A (GA-A) and ganoderic acid D (GA-D), ganoderol B, and lucidenic acid A were obtained from Cayman Chemical Company (Ann Arbor, MI, USA).

Ergosterol peroxide (EP) was kindly donated by Prof. Sang Hee Shim. It was extracted and identified by NMR as indicated in Li et al. (2017).

For bioactivity assays, fetal bovine serum (FBS), penicillin, streptomycin, non-essential amino acids, HEPES, and L-glutamine were purchased from Gibco (Paisley, UK). Quercetin, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), and lipopolysaccharide (LPS) from *E. coli* 055:B5 and phorbol 12-myristate 13-acetate (PMA) were acquired from Sigma-Aldrich (Madrid, Spain), whereas DMEM, RPMI, PBS, and HBSS were obtained from Cultek (Madrid, Spain).

2.3. Supercritical fluid extraction (SFE) of mushrooms

The supercritical extraction of *H. erinaceus*, *G. lucidum* and *T. fuciformis* fruiting bodies (500–250 μm particle size) was carried out in a supercritical fluid plant (Thar Technology, Pittsburgh, PA, USA, model SF2000) comprising a 500 cm^3 cylinder extraction vessel and two different separators (S1 and S2, both with an internal volume of 500 cm^3), with independent temperature and pressure control as described in Gil-Ramírez et al. (2013). The extraction unit also included a recirculation system, where CO_2 was condensed and pumped up to the desired extraction pressure. The CO_2 flow was measured using a flow meter from Siemens AIS (Model: Sitrans F C Mass 2100 DI 1.5, Nordborgvej, Denmark). Decompression of the supercritical outlet stream to the recirculation pressure (5 MPa) was accomplished in the separators at 40 °C. Powdered mushrooms (25 g) were mixed with 200–300 g of clean and dried sea sand (Sigma-Aldrich, Madrid, Spain) to increase sample dispersion and placed in the extraction vessel. For all the extractions, the pressure of the extraction vessel, S1 and S2 vessels was 30, 15 and 5 MPa respectively, whereas the extraction temperature was 40 °C for all of them. The pressure of S2 chosen was the CO_2 recirculation pressure of the tank at the ambient temperature when the experiments were performed (approximately 5 MPa). Extracts from S1 and S2 as well as emptying of the extraction vessel were collected once the total decompression of the system took place. The CO_2 flow rate was 60 g/min, and in those fractions extracted with co-solvent, ethanol was pumped at 5 %

of the CO_2 flow rate. The total extraction time was 3 h for those samples that were extracted with only CO_2 and 1.5 h for those that were extracted with co-solvent. In extractions carried out with ethanol, S2 temperature was maintained to 40 °C and emptied every 5–10 min to avoid freezing of the equipment. At the end of each extraction, S1 and S2 vessels were washed with ethanol and then with hexane to collect the obtained extracts separately. The S1 and S2 collected extracts were subsequently dried in a rotary evaporator. Fractions were extracted in duplicate and stored at −18 °C until use.

2.4. Thin layer chromatography

SFE extracts and standard compounds were dissolved in methanol, applied to TLC silica UV254 plates (Scharlab, Barcelona, Spain) and developed using as mobile phase hexane: ethyl acetate: methanol (1:1:0.1 *v*/v/v). Obtained bands were visualized with 10 % sulfuric acid and hot air.

2.5. Sterol quantification by HPLC-DAD

The SFE fractions were injected (20 μL) into a Varian ProStar HPLC (Varian, CA, USA) coupled to a diode array (DAD) detector. Reverse phase chromatographic separation was performed with a Spherisorb ODS2 column (5 μm, 4.6 × 250 mm, Waters, Milford, MA, USA). Solvents utilized as mobile phase were 0.1 % TFA (*v*/v) (A) and methanol (B). The following gradient was used: initial conditions 95 % B, from 95 % to 100 % B in 15 min, and then maintained 100 % B from 15 to 25 min. The flow rate was 0.8 mL/min. The absorbance signals were acquired at 210 and 280 nm to quantify ergosterol and ergosterol peroxide. Both sterols were identified according to their spectra and retention times (9.8 min and 15.3 min, respectively) compared to analytical standards and quantified by calibration curves.

2.6. Triterpenes quantification by HPLC-DAD

Triterpenes analysis of SFE fractions from *Ganoderma lucidum* was performed according to Chen et al. (2008) chromatographic method. A HPLC 1260 Infinity series system with a DAD detector (Agilent Technologies Inc., Santa Clara, CA, USA) and an ACE 5 C18 column (250 mm × 4.6 mm, 5 μm particle size) protected by a guard column filled with the same stationary phase (10 mm × 3 mm) was used. Previously, the SFE fractions were dissolved in DMSO, filtered (0.45 μm PVDF filter) and injected (20 μL). Chromatograms were recorded at 230, 252 and 280 nm. Compounds were tentatively identified by comparison of their retention time and UV − Vis spectrum with those of analytical standards. Quantification was accomplished by using calibration curves of each analytical standard. Results were expressed as mg/g extract.

2.7. Lipid identification by UHPSFC-MS

SFE extracts were analysed according Tejedor-Calvo et al. (2023) using an ultrahigh performance supercritical fluid chromatography system (UHPSFC; ACQUITY UPC2, Waters, Milford, MA, USA) with an ACQUITY UPC2 Torus DIOL column (130 Å, 1.7 μm, 100 mm × 3 mm, Waters) equipped with a Torus DIOL (130 Å, 1.7 μm, 5 mm × 2.1 mm, Waters) guard column. The column temperature and the active back pressure regulator (ABPR) were set at 50 °C and 11 MPa, respectively. The injection volume was 1 μL and the flow rate was set at 1.6 mL/min. Methanol 100 % and methanol containing 20 mM ammonium formate were used as mobile phase using the following gradient: 0 min, 1 % modified methanol; 18 min, 50 %; 19 min, 50 %; 19.5 min 1 %; 21 min 1 %.

The UHPSFC was connected to a Xevo 2G QTOF-MS (Waters). Two T-pieces (Waters) were used to control the back pressure at the column outlet and to infuse methanol (0.25 mL/min) as a makeup liquid. The MS was operated in negative electrospray ionization (ESI) mode with a scan range of *m/z* 80–1200. The capillary voltage was 2.6 kV, the sampling cone voltage 42 V, the source temperature 120 °C, the drying gas temperature 390 °C, the cone gas flow 40 L/h, and the drying gas flow 740 L/h. A collision energy ramp between 15 and 55 eV was used to acquire data independent, targeted, and data dependent MS/MS data. Data were acquired using MassLynx v4.1 (Waters). Raw data were processed using the open-source software package MZmine 3.2.8 (http://mzmine.sourceforge.net/). Data were generated by targeted peak detection with a *m/z* tolerance of 0.5 mDa or 5 ppm and a retention time tolerance of 0.2 min.

Tentative compound identification was carried out by comparing experimental with theoretical MS data using the exact mass of the $[M-H]^-$ ion (http://www.lipidmaps.org). Moreover, MS/MS data were compared with spectra in Massbank (https://massbank.eu/MassBank/), CRC, Wiley and reference literature. The data presented for tentatively identified compounds include the name of the compounds, common abbreviation, molecular formula, retention time, and theorical and experimental m/z data (Table 2). Fatty acids percentage was calculated on the total number of fatty acids detected.

2.8. Cellular antioxidant activity

Human colorectal adenocarcinoma cells (Caco-2) (ATCC, Manassas, VA, USA) were growth in DMEM medium supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine and maintained in humidified air containing 5 % CO_2 at 37 °C. Cellular antioxidant activity (CAA) of SFE samples and standard compounds was evaluated following the method described in Siles-Sánchez et al. (2024). Briefly, Caco-2 cells were seeded at a density of 2×10^5 cells/mL on a 96-well plate and allowed to reach confluency. The day of the experiment, cells were incubated for 1 h at 37 °C with 50 μL of subtoxic sample concentrations. Afterwards, 50 μL of 25 μM DCFH-DA were added to each well. Later on, medium was removed, and cells were washed with PBS (×3). Then, 600 μM of a free radical generator (AAPH) was added to each well and fluorescence was immediately recorded in a microplate reader (Cytation 5, Agilent Biotek, Santa Clara, CA, USA). Fluorescence reduction was monitored every 5 min for 1 h (13 readings) at excitation/emission wavelengths of 485/538 nm. The inhibition percentage of DCFH oxidation was calculated for each concentration against the blank as:

$$\text{Inhibition } (\%) = (1 - \text{AUC sample}/\text{AUC blank}) \text{ x100}$$

Once the percentage of inhibition was determined, the equation was calculated, and 50 % inhibition was set at IC_{50} value.

2.9. Anti-inflammatory activity

Human THP-1 monocytes (ATCC, Manassas, VA, USA) were growth in RPMI medium with the same supplements and maintenance as indicated for Caco-2 cells. The anti-inflammatory activity of SFE samples and standards was assessed following the protocol detailed in Gómez de Cedrón et al. (2024). In this case, human monocytes (THP-1) were plated (5×10^5 cells/well) in 24-wells plate, being differentiated into macrophages using 100 ng/mL PMA for 48 h. Prior to the assessment of the anti-inflammatory activity, the toxic effect of the extracts was evaluated following the MTT protocol (Mosmann, 1983).

For immunomodulatory test, subtoxic concentration of the samples was added to the macrophages and incubated for 24 h at 37 °C in presence of LPS (0.05 μg/mL). Afterwards, supernatants were taken and stored at −80 °C. Finally, the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) was quantified in the collected supernatants using an ELISA kit (BD Biosciences, Aalst, Belgium), in accordance with the manufacturer's instructions.

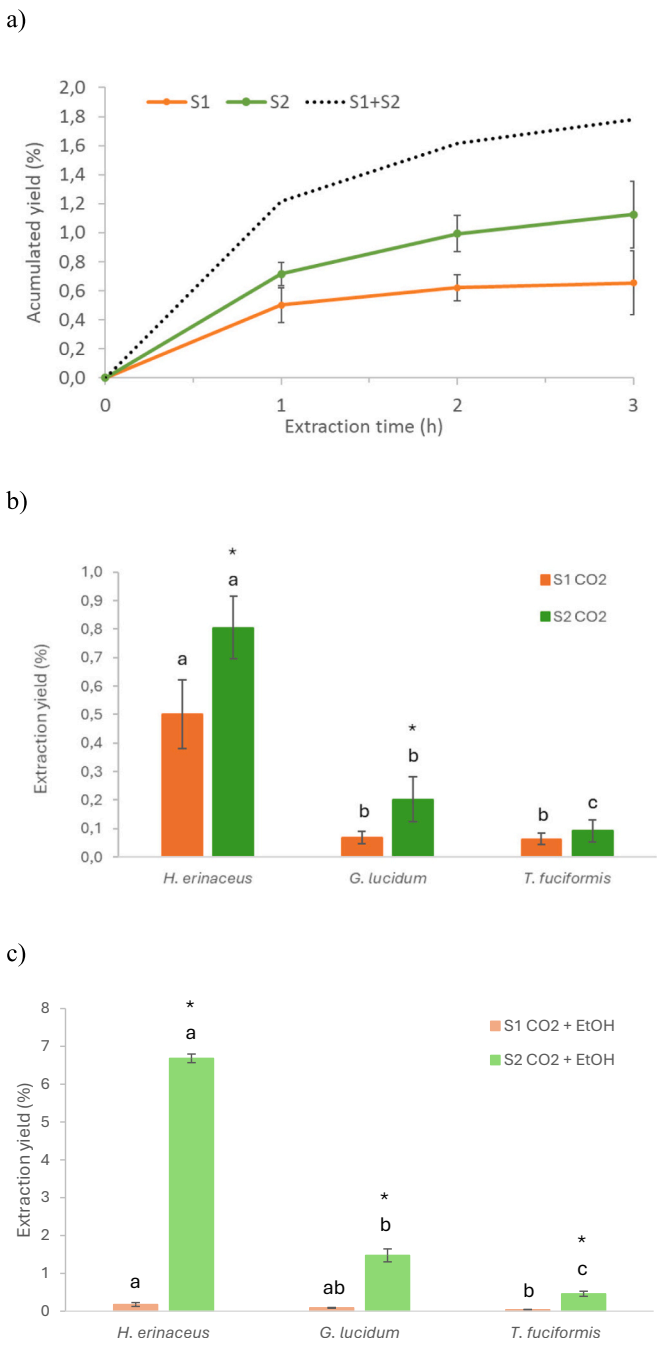

Fig. 1. a) Accumulated extraction yield in separators 1 (S1, CO_2 depressurisation at 15 MPa and 40 °C) and 2 (S2, CO_2 depressurisation at 5 MPa and 40 °C) during the SFE extraction (CO_2 at 30 MPa and 40 °C) of *H. erinaceus*. b) Extraction yields obtained in both separators after 3 h extraction from *H. erinaceus, G. lucidum* and *T. fuciformis* using only CO_2. *An asterisk denotes significant statistical differences ($p < 0.05$) between S1 and S2 separator for each mushroom. [a,b,…]Different letters denote significant statistical differences ($p < 0.05$) between extracts obtained from different mushroom species. c) Extraction yields obtained in both separators after 3 h extraction from *H. erinaceus, G. lucidum* and *T. fuciformis* using CO_2 with 5 % ethanol. *An asterisk denotes significant statistical differences ($p < 0.05$) between S1 and S2 separator for each mushroom. [a,b,…]Different letters denote significant statistical differences ($p < 0.05$) between extracts obtained from different mushroom species.

2.10. Statistical analysis

Statistical analyses were performed using one-way ANOVA and Tukey's b *post hoc* test at $p < 0.05$ (Statistics 26 Core System, SPSS Inc., IBM Company, New York, NY, USA).

3. Results and discussion

3.1. Kinetics of supercritical CO_2 extractions

According to previous studies carried out using the same SFE extraction device, the use of ethanol (up to 10 % *v*/v) as co-solvent influenced more the extraction yield than temperature, and even more than pressure (Gil-Ramírez et al., 2013). Thus, extractions from *Hericium erinaceus* were carried out using 30 MPa and 40 °C to compare them with those obtained from *A. bisporus* (Gil-Ramirez et al., 2013). After 3 h extraction, yields obtained from *A. bisporus* were 0.7 % using only CO_2 as extraction solvent and 2.2 % with the modifier. Yields were calculated by combining the content of both separators (S1 and S2). However, the separators content was independently evaluated in the extractions conducted using *H. erinaceus* to figure out if some compounds were differently accumulated in one or the other separator. Firstly, the extraction was followed during 3 h to visualize the extraction kinetics using only CO_2, as the use of modifier was certainly going to increase the yield.

Results indicated that higher yields were obtained with the increase of extraction time following a hyperbolic regression curve (Fig. 1a) being 3 h extraction at the beginning of the saturated zone, suggesting that after 3 h, larger extraction times might result only in a slightly higher yield increase. Nevertheless, the obtained yield (S1 + S2, 1.78 %) was higher than the one noticed for *A. bisporus*, mainly from extracted material accumulation in S2.

Other reports noticed a similar cumulative yield curve, but a slightly higher extraction yield from *H. erinaceus* at 30 MPa, 40 °C (1.63 %) than the 1.22 % noticed after 1 h extraction (Joradon et al., 2024). The optimal extraction conditions pointed by Cao et al. (2023) were 30 MPa, 35 °C and 80 min, where 2.51 % yield was achieved, although, in this case, ethanol was used as co-solvent. Therefore, in the present study extractions were carried out with and without ethanol and not only from *H. erinaceus*, but also from *Ganoderma lucidum* and *Tremella fuciformis*.

Supercritical CO_2 extracted more compounds from *H. erinaceus* than the other two mushroom species (Fig. 1b). Most of them were accumulated in S2, except for *T. fuciformis* that showed similar yields due to the significant low yield obtained in S2 separator. Thus, in general, when ethanol was added as co-solvent the extraction yields were increased for the evaluated mushrooms (Fig. 1c) particularly in *H. erinaceus* where the total yield (S1 + S2, 6.86 %) was more than 5 folds higher than using only CO_2 and higher than previous studies (Cao et al., 2023). Yields obtained from *G. lucidum* were lower than other reports (Milovanovic et al., 2024; Zhu et al., 2020), but in those cases higher pressures, temperatures or extraction times were used. No previous data were found on SFE extractions from *T. fuciformis*. The yields obtained when the co-solvent was used increased only in the S2 of the extractions carried out from *G. lucidum* and *T. fuciformis*.

3.2. Evaluation of fungal sterols

The triterpene ergosterol is the main sterol of fungal membranes (Gil-Ramírez et al., 2013). It is present in all mushroom species and in larger amounts than other sterol or triterpenoid derivatives. Thus, ERG contents were firstly evaluated in the SFE obtained fractions to follow the extraction effect on these types of compounds since sterols and triterpenoids were pointed as responsible for many of the biological activities described for fungal lipophilic fractions (Dat et al., 2022;

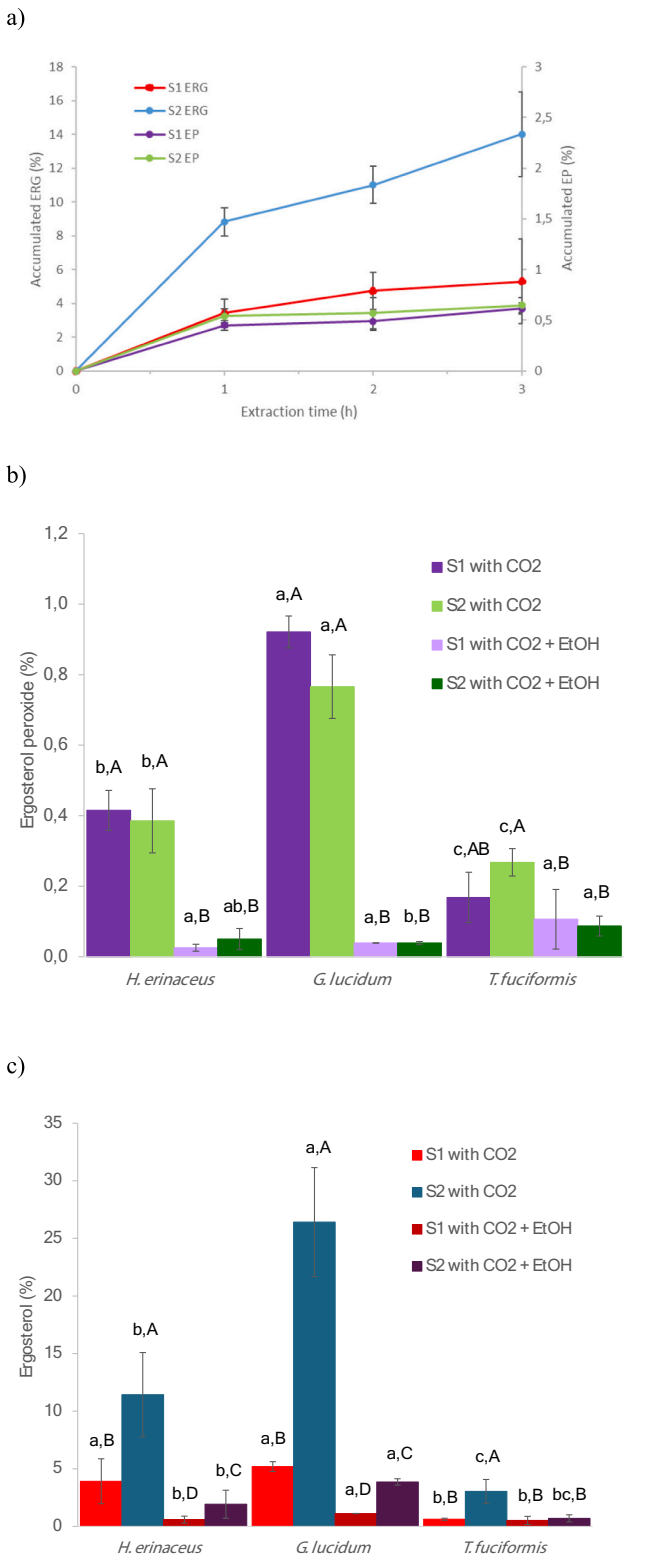

Fig. 2. a) Accumulated ergosterol (ERG) and ergosterol peroxide (EP) concentrations in separators 1 (S1, CO_2 depressurisation at 15 MPa and 40 °C) and 2 (S2, CO_2 depressurisation at 5 MPa and 40 °C) during the SFE extraction (CO_2 at 30 MPa and 40 °C) of *H. erinaceus*. b) Ergosterol (ERG) and c) ergosterol peroxide (EP) levels in both separators after 3 h extraction from *H. erinaceus, G. lucidum* and *T. fuciformis* using CO_2 and CO_2 with ethanol. Different letters denote significant statistical differences ($p < 0.05$) between extracts obtained from different mushroom species using the same extraction condition (a-e) and obtained from the same mushroom species at different extraction conditions (A-D).

Table 1
Triterpenoid composition of *G. lucidum* SFE extracts obtained in separator 2 (S2, depressurisation at 5 MPa and 40 °C) using CO_2 or CO_2 with ethanol (mg/g extract). Rt (retention time).

Compound	Rt (min)	λ_{max} (nm)	CO_2	CO_2 + EtOH
Ganoderic acid A	38.16	256	51.00 ± 1.60*	31.14 ± 0.90
Lucidenic acid A	46.89	256	0.25 ± 0.02	1.23 ± 0.11*
Ganoderic acid D	53.06	256	3.91 ± 0.05	4.78 ± 0.21*
Ganoderol B	88.25	238sh, 244, 252sh	10.66 ± 0.57	25.22 ± 0.53*

* An asterisk denotes significant statistical differences ($p < 0.05$) between extraction conditions for the same compound.

Joradon et al., 2022; Milovanovic et al., 2021; Milovanovic et al., 2024). Moreover, when SFE extracts were developed by TLC as preliminary determination of their composition, the more intense band migrated at the same Retention factor (Rf) than this compound (Rf = 0.76) (data not shown). However, a second band of slightly lower intensity and lower Rf was also noticed to migrate at the same Rf than ergosterol peroxide (Rf = 0.65). Since this compound also showed interesting biological properties (Nowak et al., 2022), it was also quantified in the obtained extracts.

Results indicated that ERG was present in the extracts obtained from *H. erinaceus* in higher concentration than EP (Fig. 2a). The longer the extraction time the higher concentration of ERG was obtained, mainly in S2. The kinetic curve in this separator showed a not so hyperbolical shape as noticed for the yield (Fig. 1a) suggesting that the fractions obtained at 3 h were more enriched in this compound than those obtained with shorter extraction times. EP concentrations were similar in both separators, and it seemed not to increase with longer extraction times. Discrepancies concerning the latter compound could be noticed within different authors since some of them indicated that EP could be an artefact generated during sample processing (Adam et al., 1967) as it is usually not present in mushroom. Other reports determine EP in fruiting bodies (Jeong and Parl, 2020; Nowak et al., 2022) and describe their interesting biological activities as antiviral (HDV, HBV) (Chiou et al., 2024; Huang et al., 2021), as antitumoral agent (Nam et al., 2001), as anti-inflammatory compound (Gao et al., 2007), *etc.* When a freshly prepared methanol extract of *H. erinaceus* was developed by TLC a light band with Rf of 0.65 was also noticed. This band was more intense in SFE extracts, but it could also be thicker due to other triterpenoids co-migrating with EP. Nevertheless, EP was detected (by HPLC) in the SFE extracts in higher amounts than in fruiting bodies (data not shown). It might be partially because it was concentrated by SFE extraction, and partially because the slightly acidic character of the CO_2 during extraction might enhance partial sterols transformation into their peroxide derivatives. The fact that this compound was not increasing with extraction time suggested that all the EP was already separated with only 1 h extraction and therefore, it might not be an artefact. Nevertheless, if it was generated, SFE could be a proper methodology to generate fractions with higher levels of this bioactive compound. Moreover, in the present study EP was already concentrated since according to previous reports, concentrations in several mushroom species ranged from 0.01 to 0.03 % (Jeong and Parl, 2020) or 0.3–0.6 % (Nowak et al., 2022) and the total EP for *H. erinaceus* reached 0.8 % as the sum of S1 and S2 contents (Fig. 2b).

When content of these sterols was determined in the SFE extracts from the other mushrooms, differences in their composition could be noticed being CO_2 a more selective solvent to obtain ERG and EP enriched fractions than using CO_2 with ethanol (Fig. 2b & c). With both solvents, the ERG concentrations were higher in S2 than S1. *G. lucidum* SFE extracts obtained in S2 with CO_2 contained higher ERG concentration than the other two species (Fig. 2c). *T. fuciformis* showed very low

Table 2
Lipid compounds identified in the SFE extracts obtained from *H. erinaceus, G. lucidum* and *T. fuciformis* in separator 2 (S2, depressurisation at 5 MPa and 40 °C)) using CO_2 or CO_2 with ethanol. Experimental and theoretical data for in negative $[M\text{-}H]^-$ are shown. RT correspond to retention time.

Compound identity			Negative ESI $[M\text{-}H]^-$			Samples					
Common name	Other information	Molecular formula (neutral)	RT (min)	m/z experimental	m/z theoretical	*H. erinaceus* (CO_2)	*G. lucidum* (CO_2)	*T. fuciformis* (CO_2)	*H. erinaceus* (CO_2 + EtOH)	*G. lucidum* (CO_2 + EtOH)	*T. fuciformis* (CO_2 + EtOH)
Caprylic acid	C8:0	$C_8H_{16}O_2$	2.22	143.1078	143.0692	1.06	0.49	0.47	0.33	0.18	0.68
Capric acid	C10:0	$C_{10}H_{20}O_2$	2.22	171.1391	171.6391	0.00	0.00	0.10	0.00	0.08	0.09
Lauric acid	C12:0	$C_{12}H_{24}O_2$	2.22	199.1704	199.1311	0.49	0.56	0.26	0.55	0.11	0.19
Myristic acid	C14:0	$C_{14}H_{28}O_2$	2.22	227.2017	227.1297	0.50	0.26	0.37	0.19	0.46	0.44
Palmitic acid	C16:0	$C_{16}H_{32}O_2$	2.08	255.2311	255.2304	7.10	7.65	8.07	9.41	8.45	9.56
cis-9-palmitoleic acid	C16:1	$C_{16}H_{30}O_2$	2.11	253.2173	253.2159	1.32	0.36	0.44	0.75	0.17	0.35
Margaric acid	C17:0	$C_{17}H_{34}O_2$	2.11	269.2486	269.2483	0.00	1.24	1.65	1.21	0.63	1.04
Stearic acid	C18:0	$C_{18}H_{36}O_2$	2.11	283.2643	283.2607	1.59	0.64	0.91	2.43	0.66	1.19
Oleic acid	C18:1n9	$C_{18}H_{34}O_2$	2.15	281.2486	281.2494	28.23	26.67	33.96	26.50	28.10	28.70
cis-vaccenic acid	C18:1n11	$C_{18}H_{34}O_2$	2.60	281.2486	281.2476	1.54	0.45	0.81	0.49	0.24	0.84
Linoleic acid	C18:2n6	$C_{18}H_{32}O_2$	2.22	279.2313	279.2321	32.89	43.09	34.19	35.53	42.08	37.45
α-Linolenic acid	C18:3n3	$C_{18}H_{30}O_2$	2.57	277.2173	277.2169	10.24	8.83	9.67	12.27	9.45	10.98
14Z-eicosenoic acid	C20:1	$C_{20}H_{38}O_2$	2.22	309.2799	309.2797	3.44	1.44	2.61	3.53	1.57	1.37
11,14-eicosadienoic acid	C20:2	$C_{20}H_{36}O_2$	2.29	307.2643	307.2645	2.80	2.57	1.38	2.43	0.79	1.44
5,8,11-eicosatrienoic acid	C20:3n3	$C_{20}H_{34}O_2$	2.32	305.2486	305.2498	1.38	0.22	0.29	0.57	0.18	0.51
Heneicosylic acid	C21:0	$C_{21}H_{42}O_2$	2.74	325.3112	325.2372	1.91	1.33	0.99	1.06	0.34	0.59
EPA	C20:5n3	$C_{20}H_{30}O_2$	2.39	301.2173	301.2170	0.90	0.56	0.26	0.57	0.12	0.24
Behenic acid	C22:0	$C_{22}H_{44}O_2$	2.26	339.3269	339.3265	0.64	0.64	0.19	0.95	1.91	0.38
cis-erucic acid	C22:1n9	$C_{22}H_{42}O_2$	2.29	337.3112	337.3117	0.79	0.84	0.74	0.20	0.10	0.52
DHA	C22:6n3	$C_{22}H_{32}O_2$	2.22	327.2330	327.2158	1.30	1.83	1.91	1.53	2.02	1.83
Lignoceric acid	C24:0	$C_{24}H_{48}O_2$	2.29	367.3582	367.3579	1.59	0.66	0.55	0.38	1.91	1.26
Nervonic acid	C24:1	$C_{24}H_{46}O_2$	2.32	365.3425	365.8224	0.31	0.16	0.18	0.12	0.40	0.08

Table 3
Cellular antioxidant activity (CAA) of SFE extracts obtained from *H. erinaceus, G. lucidum* and *T. fuciformis* (separator 2 (S2, depressurisation at 5 MPa and 40 °C)) using CO_2 or CO_2 with ethanol and their main contributors in extracts.

Sample		IC_{50} (μg/mL)
H. erinaceus	CO_2	419.77 ± 36.85^{b}
	CO_2 + EtOH	$872.96 \pm 44.13^{a,*}$
G. lucidum	CO_2	258.90 ± 11.20^{c}
	CO_2 + EtOH	$418.14 \pm 21.04^{b,*}$
T. fuciformis	CO_2	512.54 ± 20.59^{a}
	CO_2 + EtOH	$902.77 \pm 78.99^{a,*}$
Quercetin	–	12.69 ± 1.03
Ergosterol Peroxide (EP)	–	91.42 ± 2.23
Ergosterol (ERG)	–	198.65 ± 8.29
Oleic Acid (OA)	–	128.71 ± 9.69
Linoleic acid (LA)	–	75.95 ± 3.54
α-Linolenic acid (α-LA)	–	84.84 ± 6.17
Ganoderic acid A (GA-A)	–	82.19 ± 5.69
Ganoderol B (GO-B)	–	42.81 ± 5.21

Different lower-case letters (a,b…) indicate significant statistical differences ($p < 0.05$) between extracts obtained from different mushroom species using the same extraction condition. An Asterisk denotes significant differences ($p < 0.05$) between extracts obtained from the same mushroom species using different extraction conditions.

ERG levels, but ERG is used as fungal growth biomarker because their levels increase with the mycelial growth. When the mushroom is growing in optimal conditions, ERG concentrations are optimal and when the substrates are lacking nutrients lower concentrations are found. Thus, in this case, perhaps the *T. fuciformis* fruiting bodies utilized for this study were harvested when the cultivation substrate was almost exhausted. *Ganoderma lucidum* extracts also showed higher EP levels than fractions from the other mushroom species when extractions were carried out only with CO_2 (Fig. 2b) and for this metabolite, no large differences were noticed between S1 and S2 obtained extracts.

3.3. Evaluation of other triterpenoids

The concentration of other triterpenoids pointed as antioxidant (using chemical methods) and anti-inflammatory compounds (Jia et al., 2021; Milovanovic et al., 2021) was also evaluated in the S2 of the three species (where a higher yield was obtained). Ganoderic acids A (GA-A) and D (GA-D), ganoderol B (GO-B) and lucidenic acid A were only detected in *G. lucidum* extracts as they seem to be specie specific (Table 1). Ganoderic acid A was the major contributor of the determined triterpenoids within the SFE extracts obtained with supercritical CO_2 representing 5.1 % of the SFE extract, while the extract obtained with CO_2 and ethanol showed lower GA-A but higher ganoderol B levels (2.5 %) than the obtained only with CO_2. Both extracts showed similar GA-D concentrations and low levels of lucidenic acid A. Other publications also noticed GA-A in CO_2-SFE extracts but in lower concentrations (0.21 or 0.08 % in respect of cultivated and wild *G. lucidum* strains) (Milovanovic et al., 2024). They were obtained at 35 MPa, 50 °C and 1.4 h extraction time, suggesting that probably longer extractions are needed to reach the yields noticed in this study. GO-B and C were also reported in that work but in lower concentrations than GA-A. Zhu et al. (2020) also detected GA-A and ganodermanontriol in SFE extracts obtained at 65, 85 and 105 MPa (50 °C, 4 h extraction with CO_2) but their amounts were not quantified (Zhu et al., 2020).

3.4. Evaluation of fatty acids

Supercritical CO_2 extractions are used to extract non-polar compounds, such as unsaponifiable (*i.e.* triterpenoids) lipids but also saponifiable such as fatty acids. Therefore, the fatty acid profile of the obtained SFE extracts (S2) was also evaluated. UHPSFC-MS analysis indicated that linoleic acid was the mayor contributor to the lipid profile in all samples (particularly in both *G. lucidum* extracts) closely followed by oleic acid (Table 2). Other large contributors to the lipid profile were α-linolenic and palmitic acids. Supplementation with ethanol for SFE extraction slightly enhanced α-LA and PA percentages compared to extractions using only CO_2. Within species, extracts obtained from the three selected mushrooms showed similar PA percentages, but those obtained from *H. erinaceus* showed a slightly higher α-LA contribution than *T. fuciformis*, and this one higher than *G. lucidum*. These compounds were also previously pointed as main fatty acids in mushrooms lipid profile (Barros et al., 2008; Gałgowska and Pietrzak-Fiecko, 2022). Other fatty acids were identified such as some C20 and C22 derivatives (including DHA), but in relatively lower percentages and with almost no remarkable differences between extracting conditions or studied mushroom specie.

3.5. Cellular antioxidant activity of SFE extracts

The antioxidant properties of SFE fractions obtained in S2 were evaluated using Caco-2 cells. The extracts obtained with only CO_2 were compared to those obtained with CO_2 and ethanol because they showed significantly different extraction yields indicating that they contained different compounds or concentrations.

In the three evaluated mushroom species, extracts obtained with only CO_2 showed higher cellular antioxidant activity than those with the use of co-solvent (Table 3). These results suggested that more lipophilic compounds might be more involved in the noticed antioxidant activity than other more easily extracted with more polar solvents such as ethanol. These results differed from many reports indicated that the antioxidant properties of these mushrooms (Li et al., 2023; Ahmad et al., 2024; Qiu et al., 2024) were due to their PSC content. However, most of those reports used spectrophotometric determinations using DPPH or other radical scavenging *in vitro* tests to suggest their antioxidative abilities. Moreover, these large molecules will have difficulties to reach cell cytoplasm as their entrance is impaired by lipid cell membranes. Barbosa et al. (2020) suggested that PSC might stimulate release of signalling compounds by attaching themselves to specific membrane receptors that might trigger antioxidant and other biological responses. Only a couple of recent studies suggested the potential contribution of other bioactive compounds such as sterols, triterpenoids or fatty acids to the antioxidant properties of *Ganoderma sp.* lipophilic fractions (Guo et al., 2022; Kou et al., 2022).

Both *G. lucidum* extracts showed higher activities than *H. erinaceus* and *T. fuciformis* extracts obtained with or without ethanol. Moreover, the IC_{50} value recorded for the CO_2 extract (258.90 μg/mL) was close to plant extracts considered as powerful antioxidants such as an UAE marjoram extract (180.67 μg/mL) including large amounts of phenolic compounds (Siles-Sánchez et al., 2024).

In order to correlate the antioxidant activity found in the extracts with their main sterols (ERG and EP), triterpenoids (GA-A and GO-B) and fatty acids (OA, LA and α-LA), the cellular antioxidant activity of these compounds was individually evaluated using the same methodology (Table 3). Quercetin was also included as it is usually utilized as reference standard (Martinelli et al., 2021). Results indicated that within fatty acids LA and α-LA showed the highest cellular antioxidant activity, while OA activity was lower. Regarding sterols, the EP antioxidant activity was higher than ERG and in the range of LA and α-LA. Ganoderic acid A IC_{50} value was also similar to those reported for LA and α-LA but GO-B value was remarkable being only slightly 3-fold less than quercetin.

Although the fatty acids differences within both extracts from the three species were not very pronounced *G. lucidum* SFE extracts showed higher LA percentage than the other two mushroom extracts. Moreover, this specie is the only one including ganoderic acids and its CO_2 extract was the one with significantly higher EP concentration. Therefore, this compound combination might explain the higher cellular antioxidant activity noticed for this mushroom compared to the other two and

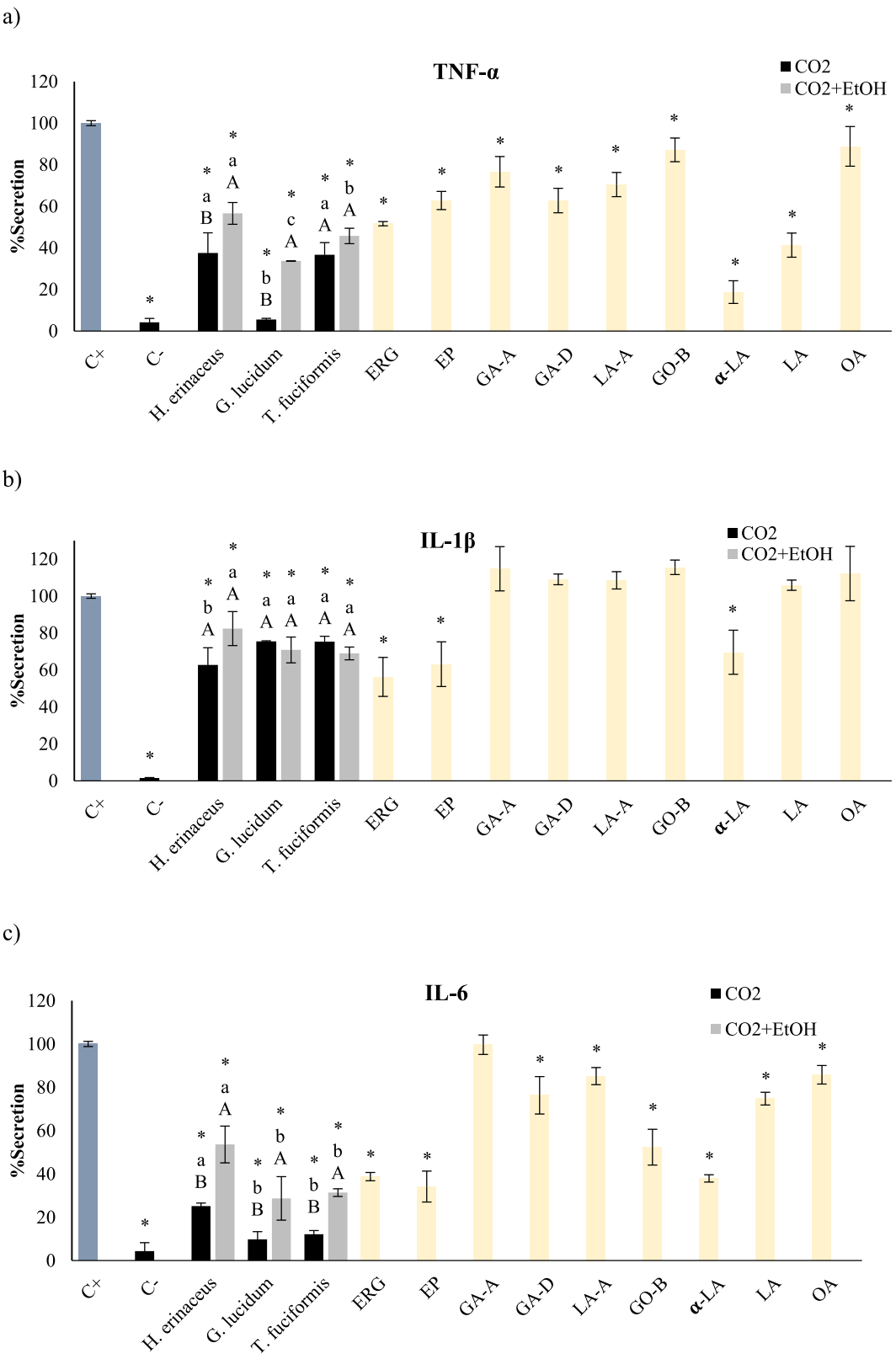

Fig. 3. Anti-inflammatory activity of SFE extracts (15 μg/mL) obtained in separator 2 (S2, depressurisation at 5 MPa and 40 °C) from *H. erinaceus, G. lucidum* and *T. fuciformis* using CO_2 and CO_2 with ethanol as co-solvent and their main contributors on a) TNF-α b) IL-1β and c) IL-6. ERG: ergosterol, EP: ergosterol peroxide, GA-A: ganoderic acid A, GA-D: ganoderic acid D, LA-A: lucidenic acid A, GO-B: ganoderol B, α-LA: α-Linolenic acid, LA: linoleic acid, OA: oleic acid. The positive control (C+) corresponds to cells stimulated with LPS, while the negative control (C-) corresponds to cells grown only in medium, without LPS stimulation. An asterisk (*) denotes significant statistical differences ($p < 0.05$) between the samples with respect to C+. Different lower-case letters (a-c) indicate significant statistical differences ($p < 0.05$) between extracts obtained from different mushroom species using the same extraction condition. Upper-case letters (A-B) denote significant differences ($p < 0.05$) between extracts obtained from the same mushroom species using different extraction conditions.

particularly for its CO_2 extracted fraction. Nevertheless, the contribution of other compounds detected in lower concentrations and/or stablishing symbiotic/antagonist relations cannot be ruled out. The obtained results also suggested that SFE extracts could penetrate Caco-2 cell membrane and inhibit ROS generation.

3.6. Anti-inflammatory activity of SFE extracts

The anti-inflammatory properties of the SFE fractions (S2) were also evaluated on macrophages. Results showed that extracts obtained using only CO_2 were more effective preventing the LPS-induced cytokine release than those extracted including ethanol as co-solvent. When tested at 15 μg/mL, all extracts successfully reduced TNF-α secretion more than 50 % (Fig. 3a) being the CO_2 extract obtained from *G. lucidum* the one that showed a remarkable inhibition with a value close to the negative control. In contrast, IL-1β secretion was less reduced (20–40 %) by the SFE extracts, showing similar inhibitory effects regardless the mushroom specie or type of solvent utilized for their extraction (Fig. 3b). Moreover, all the studied extracts drastically reduced IL-6 release, being more effective those extracted with only CO_2 as notice for TNF-α. In this case, the supercritical CO_2 extracts from *G. lucidum* and *T. fuciformis* were slightly more effective than those from *H. erinaceus* (Fig. 3c).

Interestingly, while most of the studies have mainly associated the anti-inflammatory effects of fungal fractions with their PSC content (Chakraborty et al., 2021) as they are the compounds known to activate immune responses, recent findings have highlighted other compounds. Tada et al. (2022) found out that an ethanolic extract from *H. erinaceus* containing ERG attenuated the production of IL-1β, IL-6 and IL-8 in human macrophages. Similarly, Wu et al. (2019b) reported that a triterpenoid from *G. lucidum* could inhibit IL-6 and IL-1β production in LPS stimulated RAW264.7 macrophage cultures. Thus, an attempt to further identify anti-inflammatory compounds present in the SFE fractions was carried out. The major compounds identified in the SFE extracts were also individually assessed at the same concentration as SFE extracts on macrophages. The fungal sterols ERG and EP reduced respectively 50 % and 40 % the release of TNF-α (Fig. 3a). They also inhibited the secretion of IL-1β (Fig. 3b) and particularly IL-6 by more than 70 % (Fig. 3c). Therefore, they might contribute to the anti-inflammatory activities recorded for the SFE extracts (*i.e. G. lucidum* CO_2 extract, contained the highest ERG and EP concentrations and achieved remarkable reductions) but they were not the exclusive responsible compounds. When the other *G. lucidum* triterpenoids were tested, only a slight inhibitory activity was noticed (inhibition of 20 % TNF-α secretion by GA-D and 50 % IL-6 by GO-B (Fig. 3a & c)) and *H. erinaceus* and *T. fuciformis* lacked these compounds but still they showed interesting anti-inflammatory responses. However, the main fatty acids found in the extracts (tested at 7.5 μg/mL as at 15 μg/mL showed certain cytotoxicity) showed interesting anti-inflammatory activities, particularly α-LA, inhibiting the secretion of the three pro-inflammatory cytokines. LA followed by OA also reduced cytokine release but to a lesser extent. These data suggested that the anti-inflammatory activities of SFE fungal extracts could be related to a synergistic combination of mainly fatty acids and fungal sterols although other lipid constituents (*i.e.* other triterpenoids) might be also involved.

4. Conclusions

Supercritical CO_2 extractions are environmentally friendly methods to obtain extracts from edible-medicinal mushrooms such as *G. lucidum, H. erinaceus* and *T. fuciformis* with biological activities potentially beneficial for human health such as antioxidant and anti-inflammatory properties. The extraction process showed very low yields (due to their low-fat content) but high selectivity for sterols and other triterpenoids. In general, higher extraction yields and ergosterol concentrations were achieved at lower depressurisation conditions (5 MPa for separator 2) after supercritical CO_2 extractions. The slightly acid character of the supercritical CO_2 might induce partial transformation of ERG into EP increasing the concentration of this bioactive metabolite. Although the use of ethanol as co-solvent resulted in an enhanced extraction yield, it was less effective extracting the compounds involved in their high antioxidant and anti-inflammatory activities. These metabolites were more concentrated with increasing extraction times and when collected under S2 conditions rather than S1. Extracts constituents such as LA, α-LA, EP, GA-A and particularly GO-B (in *G. lucidum*) were more involved in the cellular antioxidant activity noticed, while α-LA, EP and ERG induced a higher anti-inflammatory response. However, they were not fully responsible for the interesting activities noticed suggesting that a combination of all these (or other minor compounds) is needed to induce both biological activities. Thus, further studies are at the present being carried out to isolate other specific molecules and to test their individual bioactivities. Results indicated that SFE extracts obtained from these mushroom species showed an interesting potential as bioactive ingredients for the design of novel functional food.

CRediT authorship contribution statement

María de las Nieves Siles-Sánchez: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Paula García-Ponsoda:** Writing – original draft, Investigation, Formal analysis, Data curation. **Eva Tejedor-Calvo:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **Susana Santoyo:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Alejandro Ruiz-Rodríguez:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Data curation. **Rebeca Lavega:** Writing – original draft, Resources, Methodology, Investigation. **Laura Jaime:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Formal analysis, Conceptualization. **Cristina Soler-Rivas:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

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Data availability

Data will be made available on request.

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