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# The effects of upstream processing on the chemical profile and immunomodulating activity of *Echinacea purpurea* tinctures

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ABSTRACT

*Echinacea purpurea* (L.) Moench is a medicinal plant that is widely known for its immunomodulating properties. There is a lack of understanding regarding how *E. purpurea* tinctures should be prepared to ensure the most favourable biological activity. To address this gap, a total of 24 tinctures of *E. purpurea* were prepared with fresh or dried plant material, that was chopped (>2000 μm) or shredded (500–2000 μm), and that was 2 or 3-years-old at the time of harvest. Tinctures contained herba or radix or a combination of both. The chemical profile of the tinctures was determined using HPLC coupled with a principal component analysis of $^{1}$H NMR data. This analysis revealed that tinctures prepared with dried plant material (n = 8) had significantly higher concentrations of caffeic acid derivatives than tinctures prepared with fresh plant material, with the exception of 4 tinctures. The immunomodulating ability of tincture extracts, at a concentration of 100 μg/mg, was evaluated *in vitro* using non stimulated and lipopolysaccharide (LPS)-stimulated immortalised bone marrow derived macrophages (iBMDM) and THP-1 cell lines. Tincture extracts containing dried plant material reduced IL-6 production in LPS-stimulated iBMDM and THP-1 cells and increased production of TNF-α in THP-1 cells to a greater extent than tincture extracts prepared with fresh plant material. Despite the absence of caffeic acid derivatives, a number of tincture extracts prepared with fresh and shredded plant material had significant anti-inflammatory activity. The inclusion of herba in the tincture was seen to significantly inhibited IL-6 in both iBMDM and THP-1 cells. This study highlights the importance of selecting appropriate upstream processing methods to ensure the production of quality *E. purpurea* tinctures.

## 1. Introduction

*Echinacea purpurea* (L.) Moench is a medicinal plant which is native to North America and has a long tradition of medicinal use, particularly for upper respiratory tract infections (URIs) (Barnes et al., 2005; Burlou-Nagy et al., 2022; Percival, 2000). This traditional use is supported by preclinical studies which show that Echinacea promotes immune function through modulation of both the innate and adaptive immune responses. Direct and indirect effects on T-cells, macrophages, lymphocytes, cytokines, and natural killer (NK) cell activation are implicated in its activity (Aucoin et al., 2021; Dapas et al., 2014; Li et al., 2017; Park et al., 2018). Many randomised, double-blind, placebo--controlled clinical trials have been published evaluating Echinacea in the treatment or prevention of URIs such as the common cold. Several systematic meta-analyses on the clinical trials investigating these effects have been published. While many studies fail to show a significant efficacy of Echinacea preparations, there is a consistent trend showing a benefit in their prevention of colds (David and Cunningham, 2019).

The metabolites of *E. purpurea* thought to contribute to its biological activity include caffeic acid derivatives, alkylamides (alkamides), polysaccharides and phenolics (Thomsen et al., 2018a). The overall immunomodulating activity appears to depend on the combined effects

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of a number of these constituents (Böhmer et al., 2009). Caffeic acids are of particular interest as the British Pharmacopoeia indicates the caffeic acid derivatives, caftaric and chichoric acids to be marker compounds of the quality of *E. purpurea*.

Many factors affect the quality, safety, and efficacy of traditional herbal medicinal products. This study focuses on factors associated with post-harvest storage and processing which can affect the phytochemical profile of herbal medicines and thus their biological effects (Zhang et al., 2022; Zhou et al., 2022). The purpose of post-harvest processing is to modify the nature of crude herbal materials, which results in an enhancement of their therapeutic effects, as well as reducing their toxicity. This may cause the concentrations of some compounds to increase, and others may decrease or even disappear on processing (Sun et al., 2019). The use of fresh or dried plant material in the tincture is one such post-harvest parameter that can have an effect on the chemical composition of *E. purpurea* (L.) Moench plant material. The caffeic acid derivatives, caftaric acid and chichoric acid have shown sensitivity to drying temperature, with their concentrations decreasing with an increase in temperature (Stuart and Wills, 2003 ; Kim et al., 2000a). The particle size of the plant material used in extraction is another factor that can have a significant influence on the chemical composition of herbal tinctures. (Makanjuola, 2017). A study by Stuart et al. showed that decreasing the particle size of dried *E. purpurea* radix resulted in an increased yield of both chichoric acid and alkylamides (Stuart and Wills, 2000b). A third factor which has an impact on the phytochemical profile of *E. purpurea* tinctures is the age of the plant material at the time of harvest. Gray et al. found that *E. purpurea* roots which were dried and three years old at the time of harvest had higher phenolic concentrations than roots that were two years old at the time of harvest (Gray et al., 2003). The part of the plant (herba or radix or a combination) used in the tincture can also impact the phytochemical profile of *E. purpurea tinctures*. This is highlighted in a study by Vieira et al., who found that ethanolic extracts of *E. purpurea* flowers had higher quantities of phenols than ethanolic extracts of radix or leaves (Vieira et al., 2022). Gastrointestinal digestion is another factor that can have a significant effect on the phytochemical profile of herbal medicines. Enzymatic breakdown of constituents and metabolism of the chemical components by gut microbiota has a significant effect on the therapeutic effects of an herbal medicine, as these processes can alter the nature of the metabolites absorbed and thus biological effects of the medicine. Although not a focus of this study, it is important to note that gastrointestinal digestion can have a significant impact on the phenolic composition of *E. purpurea* extracts (Ávila-Gálvez et al., 2024).

It is also interesting to consider the relationship between the influence that upstream processing has on the chemical profile of tinctures and the subsequent biological activity of those tinctures. IL-6, TNFα and IFN-β, are routinely used as markers of the immune response when studying the immunomodulating and anti-inflammatory activities of *E. purpurea* in THP-1 cells (Randolph et al., 2003; Vieira et al., 2022). IL-6 is a member of the proinflammatory cytokine family and is considered an excellent indicator of the inflammatory status (Message and Johnston, 2003; Uciechowski and Dempke, 2020). TNF-α is also a pro-inflammatory cytokine (Dapas et al., 2014). The biological role of TNF-α is varied but it's somewhat complex mechanism of action clearly points to an important role in conferring immunity (Idriss and Naismith, 2000). IFN-β plays an important role in the modulation of both the adaptive and innate immune response and plays an integral role in the immune response to viral and other infections of the lung (Brierley and Fish, 2002). RANTES is a chemokine and acts as a potent chemoattractant for cells which play an important role in the immune response, such as monocytes, NK cells, eosinophils and basophils (Schrum et al., 1996).

The impact of upstream processing on the biological activity of *E. purpurea* tinctures has not been previously investigated. The current work aims to illustrate the impact that upstream processing has on the metabolomic fingerprint of *E. purpurea* tinctures and the subsequent effect this has on the tinctures' bioactivity. The study considered tinctures that were prepared with dried and with fresh plant material. Dried plant material was allowed to dry under ambient conditions until the moisture levels stabilised and was subsequently tinctured. Fresh plant material was tinctured directly after harvesting. The size of the size-reduced plant material used in extraction is another factor that can have a significant influence on the chemical composition of herbal tinctures (Makanjuola, 2017). In this study plant material was either chopped (> 2000 μm, as estimated visually) or shredded (500–2000 μm, as estimated visually) and the size of the size-reduced plant material used in tincture preparation was investigated. The age of the plant at the time of harvest as well as the part of the plant (herba, radix or a combination) used in the tincturing process was also considered. The chemical profile of caffeic acids in the tinctures was investigated using HPLC. An NMR-based metabolomic study was performed in order to provide a macroscopic view of how the metabolic fingerprint, covering all metabolites, of *E. purpurea* tinctures, is affected by upstream processing. Lipopolysaccharide (LPS)-stimulated murine macrophages and subsequently LPS-stimulated and non-LPS stimulated THP1 cells were used to assess the impact that upstream processing has on the ability of *E. purpurea* tinctures to modulate the production of IL-6, TNF-α, IFN-β and RANTES.

## 2. Materials and methods

### 2.1. General experimental procedure

Chromatograms were obtained using a Waters 1525 Binary HPLC system equipped with a Waters 2487 Dual • Absorbance Detector (Waters, Milford, MA, USA). $^{1}$H NMR data were acquired using a Brüker Advance III 400 MHz NMR spectrometer (Brüker, Coventry, England). iBMDM culture supernatants were assayed for the presence of IFN-β, RANTES, IL-6 and TNF-α with ELISA kits (R&D Systems, Minneapolis, MN, USA). THP-1 culture supernatants were assayed for the presence of TNF-α, IL-6, IL-1β and RANTES with ELISA kits (R&D Systems, Minneapolis, MN, USA).

### 2.2. Sample collection and identification

*Echinacea purpurea* plants (aerial and root; herba and radix) that were two and three years old at the time of harvest were sourced from Shanbally Herb Dispensary Co. Tipperary, Ireland. Plants were identified by horticulturist Heiko Klee, and voucher samples are housed in the Trinity College Dublin herbarium.

### 2.3. Echinacea purpurea tincture preparation

*E. purpurea* plant material that was grown for 2 or 3 years prior to harvesting was cleaned and divided into two batches (Fig. 1). Batch 1, for each age, was air dried for 7 weeks before tincturing. Batch 2 was used fresh in the tincturing process.

Determination of moisture level: The moisture content of the plant material was measured before further processing using a MA35 moisture balance (Sartorius, Goettingen, Germany). 5 g of plant material was ground in a blender (Shardor, London, United Kingdom) and heated in the MA35 moisture balance to 105 °C for 20 min. The plant material was weighed after heating and the mass lost was taken to be the moisture content.

Processing of *E. purpurea*: A second division of the plant material that had been separated into fresh or dried material was carried out by chopping or shredding the plant material to different sizes (Fig. 1). Plant material was chopped using hand-held secateurs, or was shredded using a Parkside garden shredder (Lidl, Dublin, Ireland), to yield chopped (> 2000 μm, as estimated visually) and shredded (500–2000 μm, as estimated visually) material, respectively (Fig. 1).

Preparation of tinctures: Tinctures were prepared from chopped and

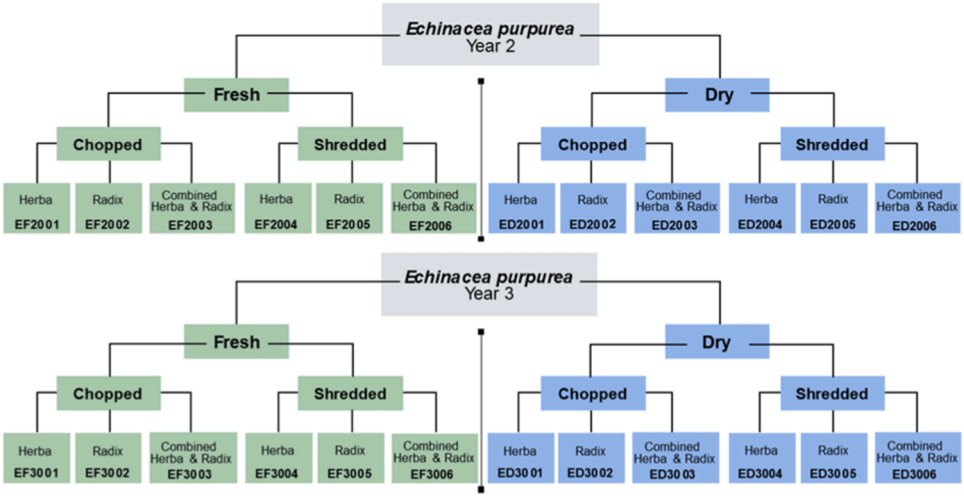


**Fig. 1.** Flowchart of herba, radix and herba radix combination tincture preparation showing tincture labels.

shredded plant material containing herba only, radix only and a combination of herba and radix (95 % w/w herba) (Fig. 1). The extraction solvent was 65 % v/v ethanol (Corcoran Chemicals, Dublin, Ireland)/ 35 % v/v deionised water and a "drug" (plant mass) extraction ratio (DER) of 1:11 w/w was used. The DER was based on the dry plant weight. As per the Shanbally Herb Dispensary tincture preparation method, the plant material was allowed to steep in the extraction solvent for six weeks and the solution was then stirred. Ten weeks later the tincture was again stirred. After 16 weeks of steeping the plant material was removed from the extraction solvent. A 6 L stainless steel press (VidaXL, London, United Kingdom) was filled with the plant material and tightened until a light resistance was met. Pressure was applied until no further residual solvent was collected from the plant material. The collected tinctures were then bottled in 1 L dark plastic bottles and stored at 0–4 °C.

### 2.4. HPLC analysis

Reverse phase HPLC was performed for quantitative and qualitative analysis of caffeic acid derivatives, using a Waters 1525 Binary HPLC system equipped with a Waters 2487 Dual α Absorbance Detector (Waters, Milford, MA, USA) (Brown et al., 2011). Separation was performed on a GromSil C18 column (250 mm length, 4 mm diameter and 120 Å pore size) (Thermoscientific, Vilnius, Lithuania) with UV detection at 330 nm. The column temperature was kept at 25°C throughout. The aqueous mobile phase (mobile phase A) was 0.1 % ortho-phosphoric acid (VWR Chemicals, Leicestershire, England) in HPLC grade water, and the organic mobile phase (mobile phase B) was acetonitrile (Sigma-Aldrich, Isère, France). Both mobile phases were degassed by sonication for 20 min before use. A flow rate of 1.5 mL/min and a gradient elution was used (as detailed in Table 1). The *E. purpurea* tinctures were injected neat with an injection volume of 5 µL. Calibration curves for the caffeic acid derivatives; chlorogenic acid (CliniSciences, Dublin, Ireland), caftaric acid (CliniSciences, Dublin, Ireland), chichoric (CliniSciences, Dublin, Ireland) and cynarin (CliniSciences, Dublin, Ireland) were prepared. The calibration curves ranged in concentration from 0.6 µg/mL to 600 µg/mL. Empower software (Waters, Milford, MA, USA) was used for peak evaluation.

**Table 1**
HPLC gradient elution profile.

| Time (minutes) | % Mobile phase A (aqueous) | % Mobile phase B (organic) |
|---|---|---|
| 0 | 90 | 10 |
| 13 | 78 | 22 |
| 14 | 60 | 40 |
| 30 | 90 | 10 |

### 2.5. $^{1}H$ NMR

The aqueous-ethanolic tinctures were subject to rotary evaporation (Rotavapor R-100, Büchi, Flawil, Switzerland) at 60 °C and 120 mbar for 20 min. Following this the samples were freeze dried (Lyovapor L-200, BUCHI, Switzerland) at −58°C and 2 mbar over 24 h. The samples were subsequently dried in an oven (WTE binder, Tuttlingen, Germany) at 30 °C for 48 h. The dried samples were dissolved in DMSO-d6 (Sigma-Aldrich, St Louis, MO, USA) (1.5 % w/v) with agitation in a shaking water bath (Reciprocal Shaking bath model 25, Precision Scientific, Chennai, India) at 65 rpm for 24 h at 25 °C. Solutions were then centrifuged (Heraeus Fresco 17 centrifuge, Thermo Scientific, Waltham, MA, USA) for 10 min at 13 rpm to remove the insoluble fraction. The supernatant was then transferred into NMR tubes. This was repeated once such that two $^{1}H$ NMR measurements were made for each sample. Quality control (QC) samples were prepared for each of the comparisons made in subsequent metabolomic analysis, as detailed in Table 2. QC samples were prepared by combing equal volumes and concentrations of the indicated samples. A vortex mixer (Whirlimixer, Glasgow, United Kingdom) was used to create a homogeneous sample and this was then aliquoted to an NMR tube for analysis. $^{1}H$ NMR spectra were collected using a Brüker Advance III 400 MHz NMR spectrometer (Brüker, Coventry, England) operating at a proton NMR frequency of 400.23 MHz. A $^{1}H$ pulse program with 30-degree-pulses was used. For each sample 1024 scans were recorded with a spectral width of 20 ppm and a relaxation delay of 2.5 s. The spectra were referenced to a residual solvent peak (DMSO-d6) at 2.5 ppm Fig. 2.

**Table 2**
Components of the quality control samples. Components are as indicated in Fig. 2.

| Quality Control Sample Label | Components in the QC sample |
|---|---|
| QC1 | All samples |
| QC2 | Year two samples |
| QC3 | Year three samples |
| QC4 | Chopped samples |
| QC5 | Shredded samples |
| QC5 | Fresh samples |
| QC6 | Dry samples |

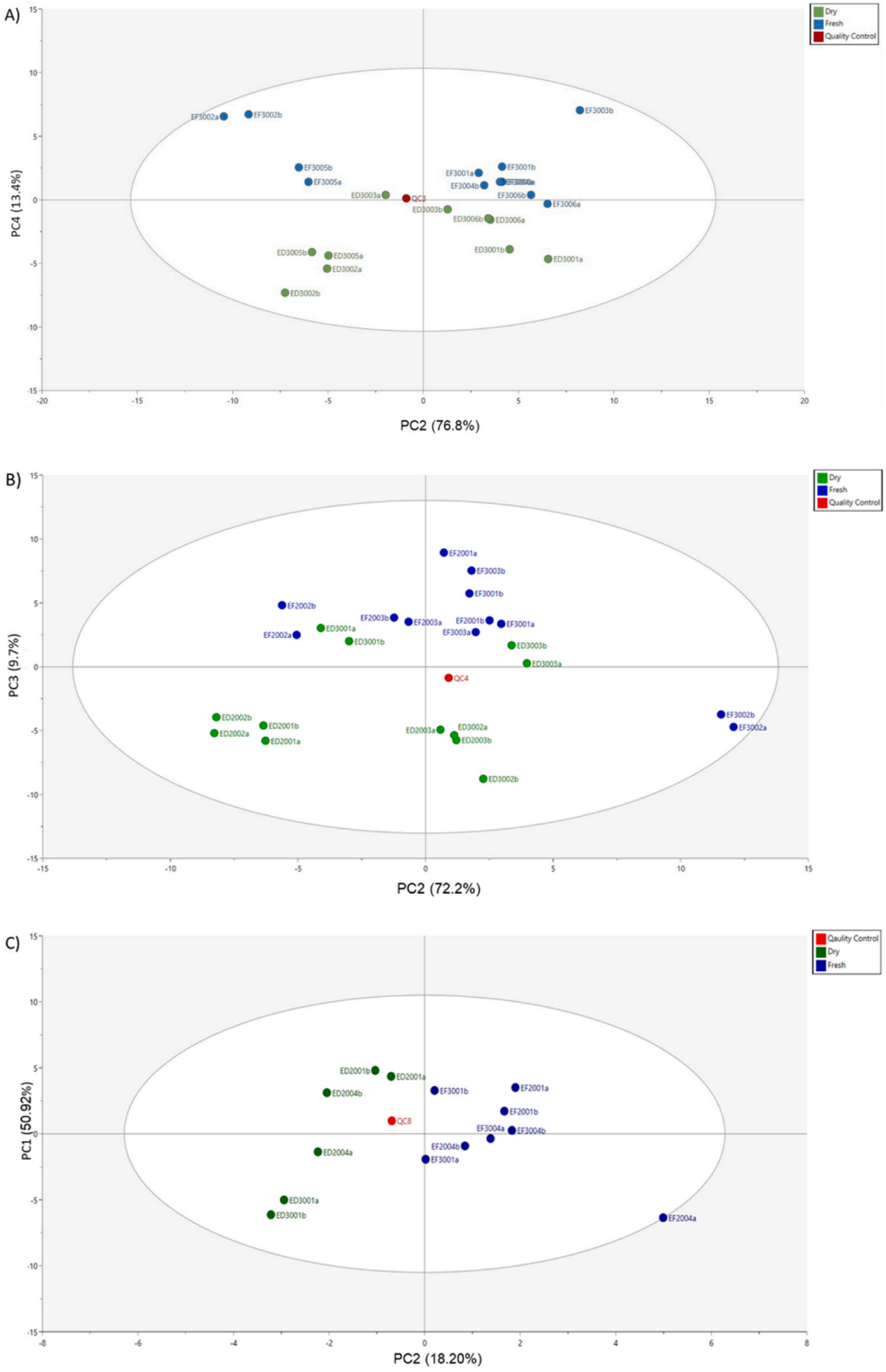


**Fig. 2.** Score plots generated from a PCA of $^{1}$H NMR data of E. purpurea tinctures. Data from tinctures prepared with dried plant material are shown in green and data from tinctures prepared with fresh plant material are shown in blue. The quality control sample is shown in red, and the ellipse represents the Hotelling T2 with 95 % confidence. A) PC2 vs PC4 of E. purpurea tinctures prepared with 3-year-old plant material. PC2 and PC4 account for 90.2 % of variance. The cumulative R2 value was 0.984 and the cumulative Q2 value was 0.928. B) PC2 vs PC3 of E. purpurea tinctures prepared with chopped plant material. PC2 and PC3 cumulatively account for 82.4 % of variance. The cumulative R2 value was 0.968 and the cumulative Q2 value was 0.901 C) PC1 and PC2 of E. purpurea tinctures prepared with herba. PC1 and PC2 cumulatively account for 69.12 % of variance. The cumulative R2 value was 0.943 and the cumulative Q2 value was 0.787.

### 2.6. Principal component analysis (PCA)

NMR spectra were processed using MestReNovax64 version 14.2.1 (Mestrelab Research, Santiago de Compostela, Spain). An auto-phase correction was used followed by a manual correction. The baseline was corrected to a polynomial fit in the second order. The spectra were then normalised to the largest peak area. NMR spectra were then binned using the binning function in MestReNovax64. The spectra were binned in widths of 0.04 ppm from 0.3 ppm to 12.0 ppm. Principal Component Analysis (PCA) was performed on the dataset using the SIMCA software version 16.02 (Umetrics, Umeå, Sweden).

### 2.7. Preparation of tincture extracts for cell studies

20 mL of the aqueous-ethanolic tinctures were subject to rotary evaporation (Rotavapor R-100, Büchi, Flawil, Switzerland) at 60 °C and 120 mbar for 20 min. Following this the samples were freeze dried (Lyovapor L-200, BUCHI, Switzerland) at −58°C and 2 mbar over 24 h. 100 μg of the dried samples were then reconstituted in 1 mL of biological grade DMSO (Sigma-Aldrich, St Louis, MO, USA) using a vortex mixer (Whirlimixer, Glasgow, United Kingdom).

### 2.8. iBMDM cell culture and treatment

Immortalized murine bone marrow derived macrophages (iBMDMs) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, CA, USA), a 1 % penicillin and streptomycin mixture (Gibco, Life Technologies, Carlsbad, CA, USA) and 0.2 % normocin (Invivogen, San Diego, CA, USA). Cells were split approximately every 2–3 days when they reached 70–80 % confluency. Cells were seeded in 96-well plates at a density of 5 × 105 cells/mL and incubated overnight at 37 °C. After 24 h, cells were treated at a concentration of 10, 30, 50, 75, or 100 μg/mL of the E. purpurea tincture extracts for 2 h, followed by lipopolysaccharide (LPS) stimulation to a final concentration of 200 ng/mL and incubated for 24 h. Cells treated with 0.2 % DMSO (Sigma-Aldrich, St. Louis, MO, USA) were deemed control.

### 2.9. THP-1 cell culture and treatment

Human monocytic leukaemia THP-1 cells obtained from American Type Culture Collection (ATCC) were maintained in tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany) in a humidified atmosphere at 37°C, 5 % $CO_2$ using RPMI 1640 medium (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10 % FBS (Gibco, Life Technologies, Carlsbad, CA, USA). For all experiments, cells were seeded at a density of 5 × 105 cells/mL in 96-well plates. Phorbol 12-myristate 13-acetate (PMA) (Invitrogen Molecular probes, Waltham, MA, USA) was added (at a final concentration of 60 ng/mL) to differentiate the cells to macrophages and the cells were then incubated for 48 h. Afterwards, cells were treated either with 10, 50 and 100 μg/mL of the *E. purpurea* extract for 24 h in the presence or absence of LPS which was added after 2 h at a final concentration of 200 ng/mL. The control cells (with or without LPS) were treated with 0.2 % DMSO.

### 2.10. Cells viability assay

Following 24 h incubation with the relevant extract, cell supernatants were transferred into new 96-well plates (to be used for cytokine levels analysis, see below). The cells, on the other hand, were incubated with resazurin solution. 100 μL of resazurin solution (prediluted with RPMI 1640 at 1:10 ration) were added to each well and the cells were incubated at 37 °C for 90 min. Afterwards, fluorescence was measured using a SpectraMax M2 plate reader (Molecular device, Sunnyvale, CA, USA) at excitation and emission wavelengths of 544 nm and 590 nm, respectively.

### 2.11. Analysis of cytokines levels

The levels of four proinflammatory cytokines tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β and RANTES, were measured in the media supernatants collected from cells treated with various *E. purpurea* extracts using highly binding 96-well plates (SPL Life Sciences, Geumgang, Naechon-Myeon, Korea) and ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. High-affinity 96 well ELISA plates were coated with 50 μL of rat monoclonal anti-IL-6, RANTES or TNF-α (R&D, Minneapolis, Canada) antibody at a dilution of 1:120 in 1 x PBS or rat anti-IFN-β (R&D, Minneapolis, Canada) antibody at a dilution of 1:1000 in carbonate buffer and incubated overnight at 4°C. 100 mL of carbonate buffer was prepared by adding 840 mg of sodium hydrogencarbonate (Sigma Aldrich, Burlington, MA, USA) and 356 mg of sodium carbonate (Sigma Aldrich, Burlington, MA, USA) to 100 mL of deionised water. Plates were washed 3–5 times in 1X PBS with 0.05 % Tween 20 (Sigma Aldrich, St Louis, MO, USA). The IL-6, RANTES and TNF-α plates were blocked with 1 % BSA solution in PBS and IFN-β plates were blocked with 10 % FBS in PBS and incubated at room temperature on a MX-M plate rocker (Orioner, Kuala Lumour, Malaysia) for 2 h. Cell supernatant was added to wells containing IFN-β at a 1:1 ratio in 10 % FBS and to wells containing RANTES, IL-6, and TNF-α at a ratio of 1:20 in 1 % BSA. Plates were then incubated at 4°C overnight. Polyclonal rabbit anti-mouse IL-6, RANTES and TNF-α antibody were prepared at a 1:60 dilution in 1 % BSA. Polyclonal rabbit anti-mouse IFN-β antibody was prepared at a 1:2000 dilution. 50 μL of the diluted antibody was added to each well and the plates were covered in foil and incubated at room temperature for 1 h. Plates were washed with 1 x PBS with 0.05 % Tween 20, and 50 μl of a 1:40 dilution of streptavidin in 1 % BSA were added per well (Invitrogen Molecular probes, Waltham, MA, USA). For detection 3,3′,5,5′-tetramethylbenzidine (TMB) solution A and B (1:1 ratio) (BD Biosciences, Berkshire, England) were mixed and 50 μL was added per well. When a strong colour developed, 25 μL of 5.4 % sulfuric acid (Sigma Aldrich, St Louis, MO, USA) was added to each well. Plates were then read on a Fluostar Optima plate reader (Logus Biosystems, Annandale, VA, USA) at 450 nm with a 620 nm wavelength correction.

### 2.12. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 for Windows, (GraphPad Software, San Diego, CA, USA). Where the means of more than two samples were compared one-way ANOVA with Tukey's post-test was performed. Where the means of two samples were compared a paired *t*-test was used.

## 3. Results and discussion

### 3.1. HPLC analysis of caffeic acid derivatives

A HPLC study was carried out in order to assess the effect of upstream processing on the concentrations of caftaric acid, chlorogenic acid, cynarin and chichoric acid in the final *E. purpurea* tinctures. These caffeic acid derivatives are outlined in the both the British and the US pharmacopeia as being of quantitative analytical interest because they have been correlated with biological activity and may be subject to degradation during harvesting, processing, and storage if proper procedures are not followed (Pharmacopeia, 2007). Caffeic acid derivatives, particularly chichoric acid, are sensitive to heat (Kim et al., 2000b; Stuart and Wills, 2003), to enzymatic degradation in the presence of moisture (Nüsslein et al., 2000) and to oxidation (Pasquet et al., 2024).

#### 3.1.1. Dried versus fresh plant material

The concentration of caffeic acid derivatives in *E. purpurea* tinctures prepared with dried and fresh plant material is shown in Table 3 and Table 4. These data illustrate that tinctures prepared with dried plant material have higher concentrations of cynarin and of chichoric acid ($n = 11$) than tinctures prepared with fresh plant material. This difference is statistically significant ($p \leq 0.0001$) when comparing pairs of equivalent samples with the exception of ED2002 versus EF2002 which were not significantly different. Tinctures prepared with dried plant material also had significantly ($p \leq 0.001$) higher concentrations of caftaric acid ($n = 10$) in comparison to their fresh counterparts, with the exception of EF3002 and EF3003 (when compared to ED3002 and ED3003, respectively). In addition, the tinctures prepared with dried plant material had higher concentrations of chlorogenic acid ($n = 10$), with the exception of EF2001 and EF2003 (when compared to ED2001 and ED2003, respectively). The difference in chichoric and caftaric acid concentrations in tinctures prepared with dried versus fresh plant material may be as a result of enzymatic degradation during processing of the fresh plant material. Polyphenol oxidases (PPOs) are reported as the enzymes which causes degradation of chichoric acid and caftaric acid (Nüsslein et al., 2000). This enzymatic activity is reduced by a low moisture content resulting in a lower water activity for enzymatic reactions (Barbagallo et al., 2013; Lavelli and Caronni, 2010; Wills and Stuart, 2000). A reduced activity of polyphenol oxidase may provide an explanation for the higher concentrations of chichoric and caftaric acid in tinctures prepared with dried plant material. PPO degrades chichoric acid derivatives by hydrolysing an ester bond. While chlorogenic acid and cynarin have an ester bond which is structurally similar to that found in chichoric and caftaric acid, degradation of chlorogenic and cynarin by PPO in *E. purpurea* tinctures has not been reported. However, degradation of chlorogenic acid by PPO in apple samples has previously been documented (Rocha et al., 1998). Degradation of cynarin by PPO has previously been reported in *E. angustifolia* samples (Wölkart et al., 2004)

#### 3.1.2. Three-year-old versus two-year-old plant material

Ontogenetic variation of active compounds is a well reported phenomenon (Kindlovits et al., 2016; Vaverková et al., 2007; Xu et al., 2013). Ontogenesis impacted the caffeic acid profile of the prepared *E. purpurea* tinctures. Tinctures prepared with dried plant material that was three years old at the time of harvest had significantly higher ($p \leq 0.001$) concentrations of caftaric acid ($n = 5$) than those prepared with plant material that was two years old at the time of harvest, with the exception of ED2004 (compared to EF3004). Similarly, tinctures prepared with dried plant material that was three years old at the time of harvest had significantly higher ($p \leq 0.05$) concentrations of chichoric acid ($n = 5$) than those prepared with plant material that was two years old at the time of harvest with the exception of ED2002 (compared to ED3002). This is consistent with a study by Stuart et al. which identified higher chichoric acid concentrations in dried *E. purpurea* that was two years old at the time of harvest than plant material that was one year old at the time of harvest (Stuart and Wills, 2000a).

HPLC analysis of tinctures prepared with fresh plant material that was two years old at the time of harvest showed them to have significantly higher ($p \leq 0.05$) concentrations of chlorogenic and chichoric acid ($n = 6$) than tinctures prepared with fresh plant material that was three years old at the time of harvest. Tinctures that were prepared with fresh plant material that was three years old at the time of harvest had significantly higher ($p \leq 0.05$) concentrations of caftaric acid and cynarin ($n = 6$).

#### 3.1.3. Chopped versus shredded plant material

Reducing the size of plant material used in a solid-liquid extraction systems has been reported to impact the concentration of bioactive secondary metabolites extracted from natural products including *Sargassum cristaefolium* (Prasedya et al., 2021), *Satureja montana* (Hudz et al., 2020) and *E. purpurea* (Stuart and Wills, 2000b). It is expected that the extract yield increases as the plant particle size decreases due to an increase in the contact area between the plant and the extraction solvent with a smaller particle size (Prasedya et al., 2021). A study by Stuart et al. showed that an extraction of dried *E. purpurea* radix had a 65 % higher concentration of chichoric acid when the particle size was in the range of 300–1200 μm compared to 1200–2800 μm (Stuart and Wills, 2000b). Our work corroborates these findings. Tinctures prepared with dried shredded plant material had significantly higher ($p \leq 0.01$) concentrations of chichoric acid than those prepared with dried chopped plant material ($n = 4$), with the exception of ED3005 and ED3006 (compared to ED3002 and ED3003). Tinctures prepared with dried, two-year-old plant material that was shredded before tincturing had significantly higher ($p \leq 0.01$) concentrations of caftaric acid ($n = 3$) than those tinctures prepared with plant material that was chopped before tincturing. In contrast, tinctures prepared with dried plant material that was chopped before tincturing had significantly higher ($p \leq 0.05$) concentration of chlorogenic acid ($n = 5$) than tinctures prepared with dried shredded plant material, with the exception of ED2006 (compared to ED2003).

Tinctures prepared with fresh plant material that was chopped before tincturing had higher concentrations of caffeic acid derivatives than those tinctures prepared with fresh shredded plant material. Tinctures prepared with fresh shredded plant material do not have detectable concentrations of any of the studied caffeic acid derivatives. It is plausible that shredding the plant material breaks the cells open to a greater extent that chopping the plant material and thus releases more enzymes and induces an increased enzymatic activity. Furthermore, caffeic acids have a tendency to degrade under conditions of high moisture (Kim et al., 2000a). As discussed above, PPO is more active when the moisture content is high. It is possible that the shredding process releases more moisture from the plant material than the

**Table 3**
Concentration (μg/mL) of caftaric acid, chlorogenic acid, cynarin and chichoric acid in final E. purpurea tinctures prepared with dried plant material. Labels refer to samples as described in Fig. 1. *BLQ: Below the Limit of Quantification. *NPD: No Peak Detected (i.e. below the limit of detection).

| Sample Label | Plant age at time of harvest | Processing Method | Plant Part | Conc. of Caftaric Acid (μg/mL) | Conc. of Chlorogenic Acid (μg/mL) | Conc. of Cynarin (μg/mL) | Conc. of Chichoric Acid (μg/mL) |
|---|---|---|---|---|---|---|---|
| ED2001 | 2-years-old | Chopped | Herba | 37.95 ± 0.16 | NPD | 15.16 ± 0.38 | 357.8 ± 0.94 |
| ED2002 | 2-years-old | Chopped | Radix | 27.67 ± 0.97 | NPD | 40.39 ± 0.18 | 269.5 ± 2.60 |
| ED2003 | 2-years-old | Chopped | Combination | 71.32 ± 0.52 | NPD | 26.98 ± 0.36 | 368.8 ± 0.52 |
| ED2004 | 2-years-old | Shredded | Herba | 94.90 ± 0.50 | NPD | 41.99 ± 0.96 | 452.9 ± 2.73 |
| ED2005 | 2-years-old | Shredded | Radix | 43.31 ± 0.54 | NPD | BLQ | 335.4 ± 7.79 |
| ED2006 | 2-years-old | Shredded | Combination | 104.31 ± 0.49 | 13.66 ± 3.30 | 37.90 ± 0.74 | 488.4 ± 1.04 |
| ED3001 | 3-years-old | Chopped | Herba | 29.04 ± 0.69 | NPD | 54.65 ± 1.36 | 104.69 ± 0.78 |
| ED3002 | 3-years-old | Chopped | Radix | NPD | 54.01 ± 3.62 | BLQ | 395.03 ± 1.04 |
| ED3003 | 3-years-old | Chopped | Combination | NPD | 59.50 ± 0.32 | 29.13 ± 1.62 | 202.39 ± 0.52 |
| ED3004 | 3-years-old | Shredded | Herba | 55.97 ± 1.43 | NPD | 13.79 ± 0.27 | 318.26 ± 9.14 |
| ED3005 | 3-years-old | Shredded | Radix | NPD | 21.44 ± 0.08 | BLQ | 192.92 ± 5.19 |
| ED3006 | 3-years-old | Shredded | Combination | NPD | 12.96 ± 0.10 | 20.39 ± 1.62 | 65.77 ± 4.13 |

**Table 4**
Concentration (μg/mL) of caftaric acid, chlorogenic acid, cynarin and chichoric acid in final E. purpurea tinctures prepared with fresh plant material. Labels refer to samples as described in Fig. 1. *BLQ: Below the Limit of Quantification *NPD: No Peak Detected (i.e. below the limit of detection).

| Sample Label | Plant age at time of harvest | Processing Method | Plant Part | Conc. of Caftaric Acid (μg/mL) | Conc. of Chlorogenic Acid (μg/mL) | Conc. of Cynarin (μg/mL) | Conc. of Chichoric Acid (μg/mL) |
|---|---|---|---|---|---|---|---|
| EF2001 | 2-years-old | Chopped | Herba | BLQ | 20.21 ± 0.10 | BLQ | 101.87 ± 2.87 |
| EF2002 | 2-years-old | Chopped | Radix | NPD | BLQ | BLQ | 135.29 ± 0.73 |
| EF2003 | 2-years-old | Chopped | Combination | NPD | 19.30 ± 0.08 | BLQ | 120.09 ± 0.79 |
| EF2004 | 2-years-old | Shredded | Herba | NPD | NPD | BLQ | NPD |
| EF2005 | 2-years-old | Shredded | Radix | NPD | NPD | BLQ | NPD |
| EF2006 | 2-years-old | Shredded | Combination | NPD | NPD | BLQ | NPD |
| EF3001 | 3-years-old | Chopped | Herba | 8.15 ± 0.02 | BLQ | 16.32 ± 0.46 | 40.33 ± 1.52 |
| EF3002 | 3-years-old | Chopped | Radix | 7.99 ± 0.02 | BLQ | BLQ | 125.96 ± 2.73 |
| EF3003 | 3-years-old | Chopped | Combination | 7.55 ± 0.01 | BLQ | 14.05 ± 0.63 | 43.41 ± 1.88 |
| EF3004 | 3-years-old | Shredded | Herba | NPD | NPD | BLQ | NPD |
| EF3005 | 3-years-old | Shredded | Radix | NPD | NPD | BLQ | NPD |
| EF3006 | 3-years-old | Shredded | Combination | NPD | NPD | BLQ | NPD |

chopping process and that this also contributes to the lack of caffeic acid derivatives in the tinctures prepared with shredded and fresh plant material.

### 3.2. NMR-based principal component analysis

$^{1}$H NMR was the analytical tool of choice in this study owing to its reproducibility, ease of study expansion to include new samples, and the ability to provide a macro view of all the NMR-detectable metabolites in the samples (Tugizimana et al., 2013). Principal component analysis (PCA) is an unsupervised clustering method which acts to reduce the dimensionality of multivariate data while preserving most of the variance within it (Choi et al., 2004). In this study PCA was used to provide a comparative interpretation and visualisation of the metabolomic differences of *E. purpurea* tinctures. PCA was performed with a view to enhancing the understanding gained from the HPLC analysis which focused on caffeic acid derivatives.

Clustering of data points in the score plot (generated from the PCA) revealed that the use of fresh versus dried plant material in tincture preparation caused notable chemical variance in the final tincture (Fig. 2). Samples with a similar chemical profile cluster together on the score plot and are differentiated from other samples. The chemical variance in tinctures due to the use of fresh versus dried material is evident for tinctures that were prepared with 3-year-old plant material, as shown in Fig. 2A (PC2 vs. PC4). When tinctures are prepared with chopped plant material, a separation arises between tinctures prepared with fresh versus dried material (Fig. 2B, PC2 vs. PC3). A PCA was also performed on tinctures prepared with herba alone (Fig. 2C, PC1 vs. PC2) and this analysis also showed a distinction in tinctures prepared with fresh versus dried plant material. These PCA analyses illustrate that the use of fresh versus dry material was a cause of chemical variance in tinctures prepared with plant material of different sizes, of different ages and with different plant parts. This is of note as different preparation of *E. purpurea* are used in different studies. Most preparations used in clinical trials are prepared from fresh plant material while dried material is widely marketed and used in dietary supplements(Pharmacopeia, 2007).

The score plot in Fig. 3 (PC1 vs. PC2) shows a distinction in the chemical profile of tinctures prepared with herba versus radix. This may, in part, be due to the concentration of caffeic acid derivatives in the tinctures. Tinctures prepared with dried herba have significantly higher concentrations of chichoric and caftaric acid ($p \leq 0.05$) than those prepared with radix alone (Table 3). This finding is supported by Ávila et al., who found that chichoric and caftaric acid concentrations were higher in ethanolic extracts of *E. purpurea* leaf compared to extracts of *E. purpurea* radix (Ávila-Gálvez et al., 2024). The alkylamides concentration in the tinctures may also contribute to the distinction in chemical profile of tinctures prepared with herba versus radix (Qu et al., 2005). Although not directly measured in this study, alkylamide concentration is taken into account in the non-targeted NMR metabolomic analysis.

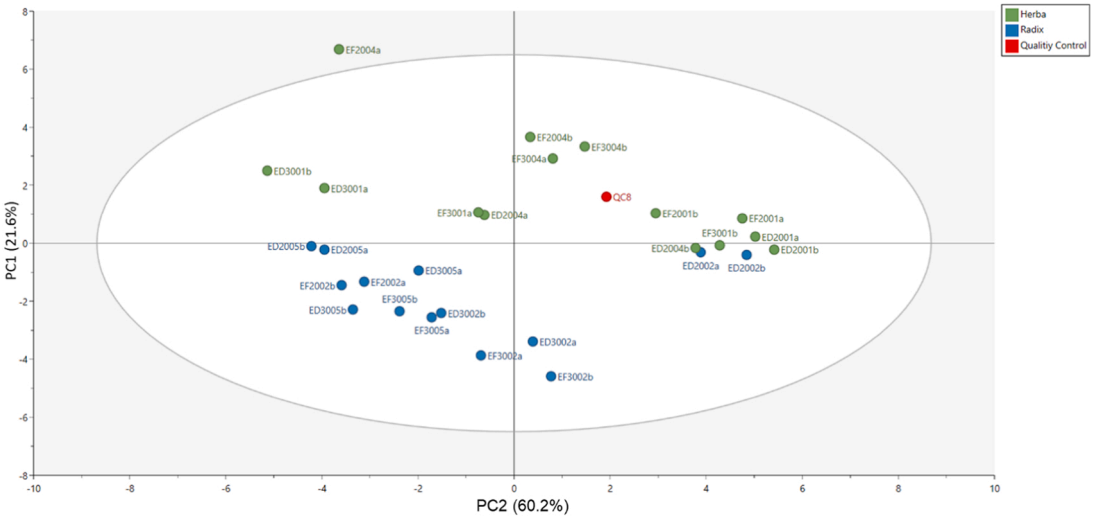


**Fig. 3.** PC1 vs PC2 of E. Purpurea tinctures prepared with herba or radix plant material where samples prepared with herba material are shown in green and samples prepared with radix plant material are show in blue. PC1 and PC2 cumulatively account for 60.3 % of variance. The cumulative R2 value was 0.922 and the cumulative Q2 value was 0.745. The quality control sample is shown in red, and the ellipse represents the Hotelling T2 with 95 % confidence.

### 3.3. Immunomodulating activity

Traditionally, preparations of E. purpurea were used to prevent and relieve a variety of inflammatory conditions (Kindscher, 1989). Modern interest in *E. purpurea* focuses on its immunomodulatory activity and in particular it is used for the prevention and treatment of upper respiratory tract infections (Barnes et al., 2005; Percival, 2000). *E. purpurea* tinctures have been assessed in several cell lines with a view to understanding it's immunomodulating and anti-inflammatory activity. These cell lines include the murine iBMDM (Fu et al., 2017), THP-1 human monocytic cell line (Vieira et al., 2022), the human bronchial epithelial cells BEAS-2B, the human peripheral blood mononuclear cells (McCann et al., 2007) and RAW 264.7 macrophage cells (Todd et al., 2015). *E. purpurea* extracts have shown an ability to both stimulate and inhibit the immune response. For example, *E. purpurea* extracts have been reported to promote both phenotypic and functional maturation of dendritic cells (Li et al., 2017) and activate and polarize M1 macrophages (Fu et al., 2017). *E. purpurea* extracts have also been shown to inhibit IL-2 production in T cells (Sasagawa et al., 2006) and TNF-α in macrophages (Thomsen et al., 2018b). The components of *E. purpurea* thought to be responsible for this activity include caffeic acid derivatives, alkylamides, polysaccharides and phenolics (Thomsen et al., 2018a). It is important to note that the overall immunostimulant activity appears to depend on the synergistic effect of a number of constituents (Böhmer et al., 2009).

The anti-inflammatory activity of tincture extracts was investigated in iBMDM and in THP-1 cells. The iBMDM cell line has the advantage of being very robust and of being fully differentiated *in vitro* from bone marrow stem cells, making it a favourable model in immunological studies (Marim et al., 2010). Furthermore, immortalised macrophages exhibit similar metabolism and polarisation to primary iBMDM cells (Xie et al., 2024). In spite of these advantages, there are limited number of studies that use iBMDM to study the immunomodulating activity of *E. purpurea.* One such study was carried out by Fu et al. who investigated the impact that polysaccharide-enriched extracts of *E. purpurea* had on iBMDMs. The study found that the extract activated the macrophage towards the M1 phenotype which would suggest that the E. purpurea extract enhances the phagocytic and bactericidal activity of the macrophage (Fu et al., 2017).

In the current work the immunomodulatory activity of the tincture extracts was also tested in human macrophages using the THP-1 cell line. This cell line is originally isolated from the peripheral blood of a childhood case of acute monocytic leukaemia (Bosshart and Heinzelmann, 2016) and has been widely used to study the immune response (Chanput et al., 2014). THP-1 cells resemble primary monocytes and macrophages in morphological and functional properties, including differentiation markers, which makes them an excellent model for investigating the immune response (Bosshart and Heinzelmann, 2016). Previous studies which investigated the impact of *E. purpurea* tinctures on THP-1 cells found that aqueous extracts of *E. purpurea* are capable of both a pro- and anti-inflammatory effect. The aqueous extracts stimulated the macrophages to produce pro-inflammatory cytokines TNF-α, IL-1β and IL-6. Conversely, under an inflammatory scenario, the aqueous tinctures reduced the pro-inflammatory mediators (Vieira et al., 2022).

#### 3.3.1. Murine Immobilised bone marrow derived macrophages (iBMDM)

Macrophages, a key immune cell in the first line of host defence, are activated in the presence of several signals, for example, LPS, a major constituent of the outer wall of Gram-negative bacteria (Medzhitov, 2010). When activated, intracellular signalling pathways are triggered, which in turn stimulates the production and release of several inflammatory mediators. In this work iBMDM were treated with LPS in order to induce a pro-inflammatory state. This mimics the cytokine storm which may be experienced following infection. The cells were then treated with *E. purpurea* tincture extracts.

A resazurin assay was used to assay the metabolic activity of the murine iBMDM in the presence of different concentrations of the prepared *E. purpurea* tincture extracts. The tincture extracts were assayed at concentration of 10, 30, 50, 75 and 100 μg/mL. The tincture extracts did not cause a significant decrease in metabolic function as compared to a DMSO control at a concentration of 100 μg/mg and so this concentration was used in subsequent studies. (as shown in the supplementary information, Figure S1)

IL-6, TNF-α, IFN-β and RANTES were investigated as markers of the immune response in an effort to understand the immunomodulating activities of the *E. purpurea* tinctures. The impact that *E. purpurea* tinctures have on these cytokines in murine iBMDMs has not previously been reported. In this study, none of the tincture extracts were seen to have a statistically significant effect on TNF-α or on RANTES when compared with LPS alone (as shown in the supplementary information, Table S1 and Table S2). The impact that the tincture extracts had on IL-6 and IFN-β will be further discussed.

#### 3.3.2. The impact of E. purpurea tincture extracts on IL-6 induction in iBMDMs

IL-6 is a pleiotropic cytokine which promotes the expansion and activation of T cells, the differentiation of B cells, and the regulation of the acute-phase response (Hunter and Jones, 2015). *E. purpurea's* ability to reduce concentrations of IL-6 in the LPS-stimulated murine macrophages, RAW 264.7, has previously been reported and demonstrates the tincture's ability to limit cytokine production in the case of excess inflammation (Papotti et al., 2023).

For 2-year-old *E. purpurea* material, there was a statistically significant difference in inhibition of IL-6 production in iBMDM depending on whether fresh or dried material was used in the tincturing process (Fig. 4). In each of the cases shown, tincture extracts prepared with dried *E. purpurea* plant material inhibited IL-6 production to a greater extent than their fresh counterparts. As shown in Section 3.1, tincture extracts prepared with dried *E. purpurea* material tended to have higher concentration of caffeic acid derivatives than their fresh counterparts, while the PCA carried out on the NMR data from these tinctures showed a clear distinction in the chemical profile of tinctures prepared with fresh versus dried plant material. This PCA analysis would have captured differing concentrations of compounds such as glycoproteins and alkylamides, as well as caffeic acid derivatives.

EF2003, EF2004 and EF2006 did not significantly inhibit LPS induced IL-6 (Fig. 4). It is interesting to note that these samples did not contain detectable (by HPLC) concentrations of caffeic acid derivatives. However, the low concentration of caffeic acid derivatives in these tinctures cannot necessarily be linked to their inability to reduce IL-6 concentrations, given that standards of caftaric acid, chlorogenic acid, cynarin and chichoric acid were also tested in the LPS-stimulated iBMDM model and they did not significantly lower IL-6 concentrations (as shown in the supplementary information, Figure S3).

The activity of tincture extracts made with dried and chopped *E. purpurea* plant on IL-6 production in iBMDM is shown in Fig. 5. Tincture extracts that were prepared with plant material that was two years old at the time of harvest (ED2001, ED2002 and ED2003) inhibited IL-6 release to a greater extent than their counterpart tincture extracts prepared with plant material that was three years old at the time of harvest (ED3001, ED3002, ED3003). It is interesting to note that ED2001, ED2002 and ED2003 have higher concentrations of caftaric acid as assayed by HPLC than ED3001, ED3002 and ED3003. However, as stated above, the concentration of caftaric acid is not likely to be linked to the tincture extracts' ability to reduce IL-6, as it did not show an ability to reduce the cytokine when tested alone (as shown in the supplementary information, Figure S3)

The impact of tincture extracts made with different parts of 2-year-old *E. purpurea* plant material (herba, radix or a combination of herba and radix) on IL-6 production in the iBMDM cell line is shown in Fig. 6. In this grouping it is evident that tinctures prepared with radix alone had a lesser ability to inhibit release of IL-6 than the tinctures prepared with

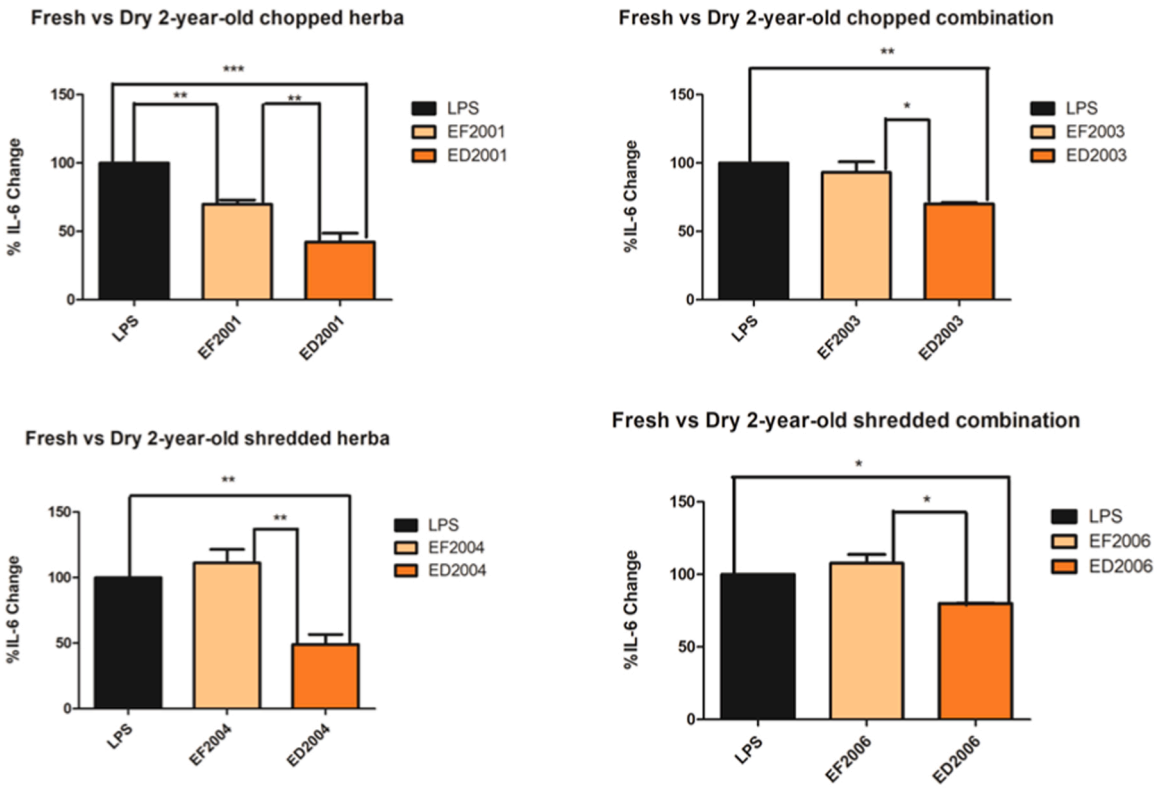


**Fig. 4.** Effects of E. purpurea tincture extracts made with 2-year-old plant material on the IL-6 production in iBMDM cells following treatment with LPS. Where the bar is coloured light orange the impact of tincture extracts made with fresh plant material and where the bar is coloured dark orange the impact of tincture extracts made with dried plant material is indicated. *, * *, * ** significant reduction of cytokines production at $p \leq 0.05$, 0.01, and 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test.

herba or with a combination of herba and radix. The PCA studies carried out on the NMR data of these tinctures shows a clear distinction in the chemistry of tinctures prepared with herba and those prepared with radix which might explain the difference in activity.

*3.3.2.1. The impact of E. purpurea tincture extracts on IFN-β production in iBMDM.* IFN-β plays an important role in both the innate and adaptive immune response. Echinacea treatment has been previously reported to induce Type I interferon expression in human peripheral leucocytes (Randolph et al., 2003). The impact of upstream processing on this cytokine was also investigated in the current study. IFN-β production were significantly inhibited by the tinctures (n = 21), with the exception of ED3001, ED3005 and EF3003 (as shown in the supplementary information, Table S1 and S2).

The use of fresh versus dry plant material in the tincturing process resulted in a significant difference in the ability of the tincture extracts to reduce IFN-β release. Similar to IL-6, tincture extracts prepared with dried plant material reduced the levels of IFN-β to a greater extent than those prepared with fresh plant material (Fig. 7).

The tinctures prepared with dried and shredded plant material (ED2001, ED2002) did have higher concentrations of caffeic acid derivatives than tinctures prepared with dried and shredded plant material (ED2004, ED2005) as assayed by HPLC (Table 3) with the exception of cynarin concentrations in ED2002. There was a significant difference in the activity of tincture extracts that were prepared with fresh and chopped versus fresh and shredded plant material, with tincture extracts prepared with fresh chopped material displaying more potent reductions in IFN-β production (Table 5). It is interesting to note that tinctures prepared with fresh chopped plant material had significantly higher concentrations of caffeic acid derivatives than tinctures prepared with fresh shredded plant material, with the latter containing no quantifiable concentrations of any of the caffeic acid derivatives assayed by HPLC (Table 4). The ability of the caffeic acids derivatives caftaric acid, chlorogenic acid, cynarin and chichoric acid to influence IFN-β levels was tested and all the compounds significantly reduced production of the cytokine in LPS-stimulated iBMDMs (as shown in the supplementary information, Figure S3).

Table 5 also compares the ability of tincture extracts prepared with dried and chopped versus dried and shredded plant material to modulate IFN-β production in iBMDMs. There is no significant difference in the activity of tincture extracts prepared with chopped versus shredded plant material when using dried plant material. However, the tinctures prepared with dried and shredded plant material (ED2001, ED2002) did have higher concentrations of caffeic acid derivatives than tinctures prepared with dried and shredded plant material (ED2004, ED2005) as assayed by HPLC (Table 3) with the exception of cynarin concentrations in ED2002.

### 3.3.3. THP-1 cell line

The anti-inflammatory potential of the *E. purpurea* tincture extracts were also examined in a human macrophage cell line, with THP-1 cells being used for this purpose. The suitability of THP-1 cells for such a study is highlighted by a gene expression analysis which was carried out by Declerk et al. This revealed that *E. purpurea* constituents tigger innate immunity pathways involving the upregulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which can promote downstream production of pro-inflammatory cytokines (Declerck et al., 2021).

A resazurin assay was used to measure the metabolic activity of the THP-1 cell in the presence of the *E. purpurea* tincture extracts at a concentration of 10, 50 and 100 μg/mL. The tinctures did not cause a significant decrease in metabolic function as compared to a DMSO control at a concentration of 100 μg/mL (as shown in the supplementary information, Figure S2) and so this concentration was used in subsequent

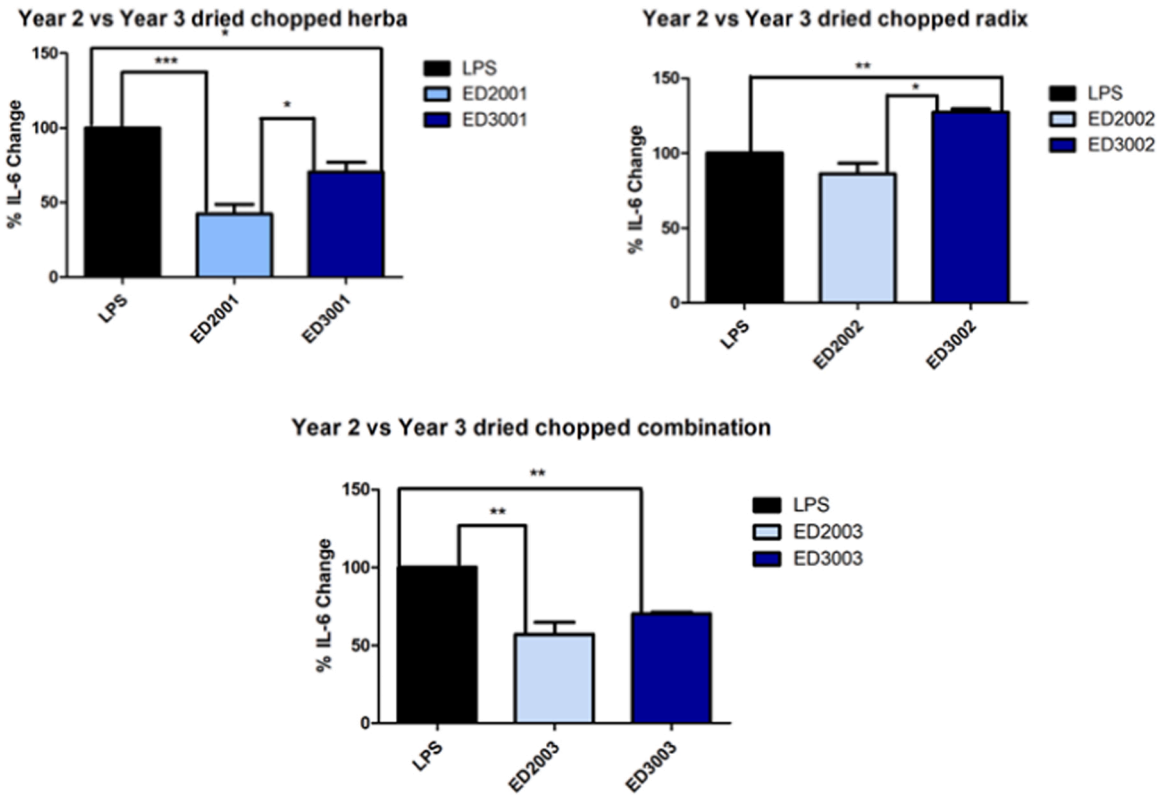


**Fig. 5.** Effect of E. purpurea tincture extracts prepared with plant material that was chopped and dried before tincturing on the IL-6 production in iBMDM cells following treatment with LPS. Where the bar is coloured light blue the impact of tincture extracts made with 2-year-old plant material is shown and where the bar is coloured dark blue the impact of tincture extracts made with 3-year-old plant material is indicated. *, * *, * ** significant reduction of cytokines production at $p \leq 0.05$, 0.01, and 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test.

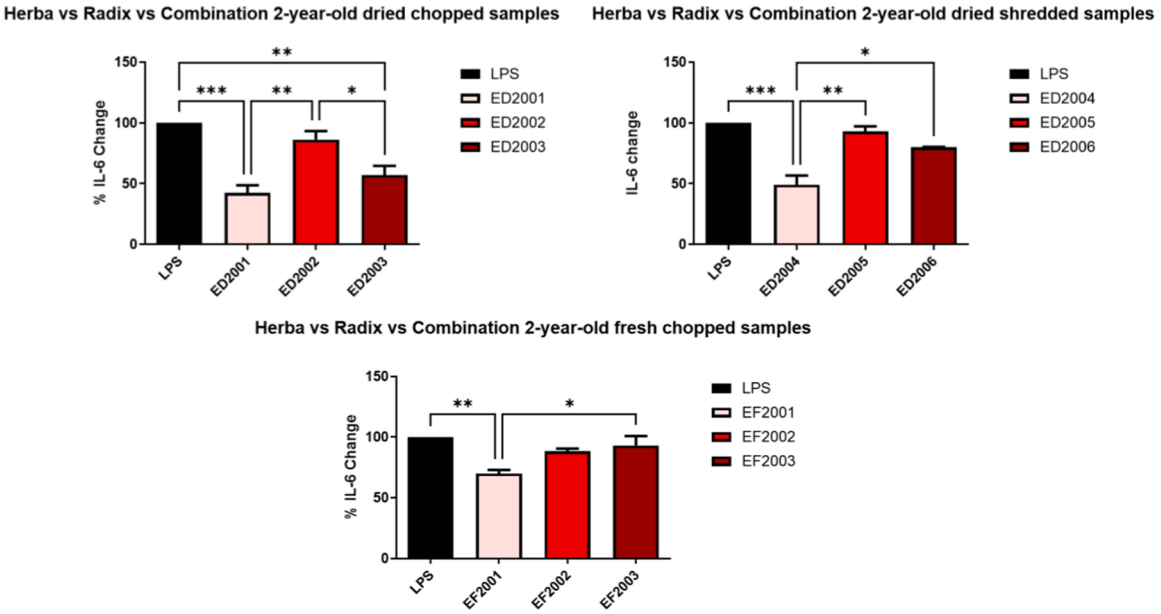


**Fig. 6.** Effects of E. purpurea tincture extracts made with plant material that was 2 years old at the time of harvest on IL-6 production in iBMDM cells following treatment with LPS. Where the bar is coloured pink the impact of tincture extracts made with herba is shown, where the bar is coloured red the impact of radix is shown and where the bar is coloured dark red the impact of tincture extracts made with a combination of herba, and radix is indicated. *, * *, * ** significant reduction of cytokines production at $p \leq 0.05$, 0.01, and 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test.

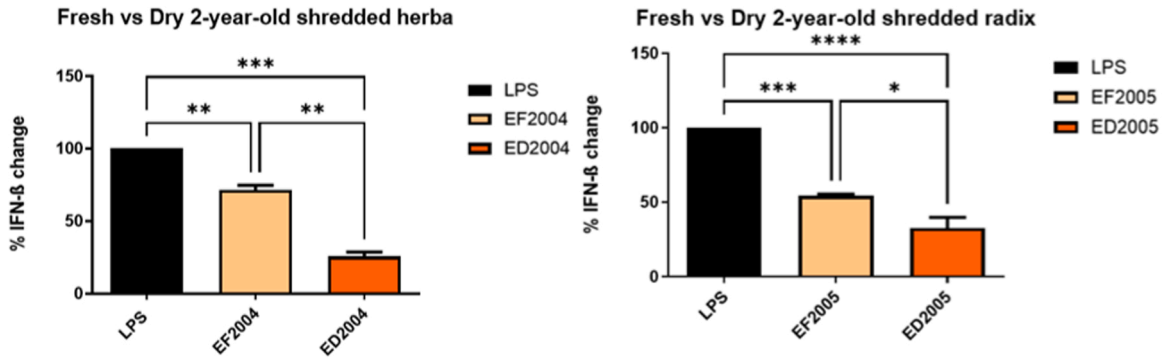


**Fig. 7.** Effects of E. purpurea tincture extracts made with plant material that was two years old at the time of harvest and was shredded before tincturing on IFN-• production by iBMDM cells following treatment with LPS. Where the bar is coloured light orange the impact of tincture extracts made with plant material that was used fresh in the tincturing is shown, where the bar is coloured dark orange the impact of tincture extracts that were made with shredded plant material is shown. * , * *, * ** and * ** * significant reduction of cytokines production at $p \leq 0.05$, 0.01, 0.001, 0.0001 when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test.

**Table 5**
Effects of E. purpurea tinctures made with plant material that was two years old at the time of harvest on IFN-α production in iBMDM cells following treatment with LPS. P values were measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test. SD indicates standard deviation.

| Sample Label | % IFN-β Change + /- SD | P value |
|---|---|---|
| Chopped vs Shredded 2-year-old fresh herba.<br>EF2001<br>EF2004 | 68.42 ± 7.678<br>37.50 ± 13.03 | ≤ 0.05 |
| Chopped vs Shredded 2-year-old fresh radix.<br>EF2002<br>EF2005 | 73.09 ± 1.579<br>45.75 ± 1.662 | ≤ 0.0001 |
| Chopped vs Shredded 2-year-old dried herba.<br>ED2001<br>ED2004 | 67.24 ± 17.16<br>58.94 ± 21.77 | NS |
| Chopped vs Shredded 2-year-old dried radix.<br>ED2002<br>ED2005 | 51.62 ± 2.015<br>67.36 ± 10.12 | NS |

studies.

IL-6, TNF-α, IL-1β and RANTES were investigated as markers of the immune response in the THP-1 cell line. None of the tinctures showed a statistically significant effect on the production of IL-1α when compared with LPS alone in a paired *t*-test (as shown in the supplementary information, Table S3 and S4).

*3.3.3.1. The impact of E. purpurea tincture extracts on IL-6 production in THP-1 cells.* Tincture extracts prepared with fresh *E. purpurea* herba reduced the production of IL-6 in LPS-stimulated THP-1 cells. These findings are consistent with a study by Vieira et al. which found that aqueous extracts of *E. purpurea* flowers reduced IL-6 levels in THP-1 cells by 61.8 ± 12.3 % (Vieira et al., 2022). Although Vieira's tinctures are not a direct comparison with ours as they were prepared with plant material that was harvested after one year of growth. Fig. 8 shows that when tincture extracts were prepared with fresh herba, there was a significant difference in the activity of tincture extracts prepared with shredded versus chopped plant material. Tincture extracts prepared with chopped plant material reduced IL-6 production to a greater extent than their shredded counterparts. It is interesting to note that tinctures prepared with fresh and shredded plant material had no quantifiable concentrations of any of the caffeic acid derivatives, as measured by HPLC. In contrast tinctures prepared with fresh and chopped plant


**Fig. 8.** Effects of E. purpurea tincture extracts made with fresh plant material on IL-6 production in THP-1 cells following treatment with LPS. Where the bar is coloured dark green the impact of tincture extracts made with plant material that was chopped before tincturing on IL-6 production is shown, where the bar is coloured light green the impact of tincture extracts that were made with shredded plant material is shown. * , * *, * ** indicate significant reduction of cytokines production at $p \leq 0.05$, 0.01, 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test. ns indicates no significant difference.

**Table 6**
Effects of E. purpurea tinctures made with herba on IL-6 production in THP-1 cells following treatment with LPS. P value is measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test. SD indicates standard deviation.

| Sample Label | % IL-6 Change + /- SD | P value |
|---|---|---|
| 2-year-old vs 3-year-old dried shredded herba. | 43.01 ± 8.37 | ≤ 0.01 |
| ED2004 | 82.60 ± 8.87 | |
| ED3004 | | |
| 2-year-old vs 3-year-old fresh chopped herba. | 52.32 ± 7.18 | ≤ 0.05 |
| EF2001 | 33.40 ± 4.12 | |
| EF3001 | | |
| 2-year-old vs 3-year-old fresh shredded herba. | 97.86 ± 8.85 | ≤ 0.01 |
| EF2004 | 82.60 ± 8.87 | |
| EF3004 | | |

material did have quantifiable concentrations of one or more of the evaluated caffeic acid derivatives (Table 4).

The ability of tincture extracts prepared with E. purpurea herba to reduce production of the IL-6 cytokine in THP-1 cells is shown in Table 6. There was a significant difference in the activity of the tincture extracts prepared with dried herba that was harvested at two years old (ED2004) versus the tincture that was prepared with dried herba that was harvested at three years old (ED3004), with the latter tincture extract reducing IL-6 production more potently than the former. There was a significant difference in the concentration of caftaric acid, chichoric acid and cynarin in these tinctures, with ED2004 having a higher concentration of each of these caffeic acid derivatives. Tincture extracts prepared with fresh herba that was two years old at the time of harvest reduced IL-6 production to a greater extent than tinctures extracts prepared with fresh herba that was three years old at the time of harvest. It is notable that EF3004 did not contain quantifiable concentrations of caffeic acid derivatives as detected by HPLC (Table 4) and yet significantly reduced IL-6 production ($p \leq 0.01$).

The ability of tincture extracts prepared with fresh versus dried plant material to reduce the production of IL-6 in THP-1 cells is shown in Fig. 9. Tinctures extracts prepared with two-year-old dried plant material had greater activity than tincture extracts prepared with two-year-old fresh plant material. The opposite is true of tinctures extracts prepared with plant material that was three years old at the time of harvest. In this grouping tinctures extracts that were prepared with fresh plant material reduce IL-6 production more potently than those prepared with dried plant material. The PCA analysis highlighted chemical differences between tinctures prepared with fresh versus dried plant material (Fig. 2).

Fig. 10 shows the ability of tincture extracts prepared with herba, radix or a combination of herba and radix to reduce IL-6 production in LPS stimulated THP-1 cells. These graphs illustrate that tincture extracts prepared with herba more potently reduced IL-6 production than those prepared with radix or with a combination of herba and radix. The impact of the use of different plant parts in tincture preparation was also

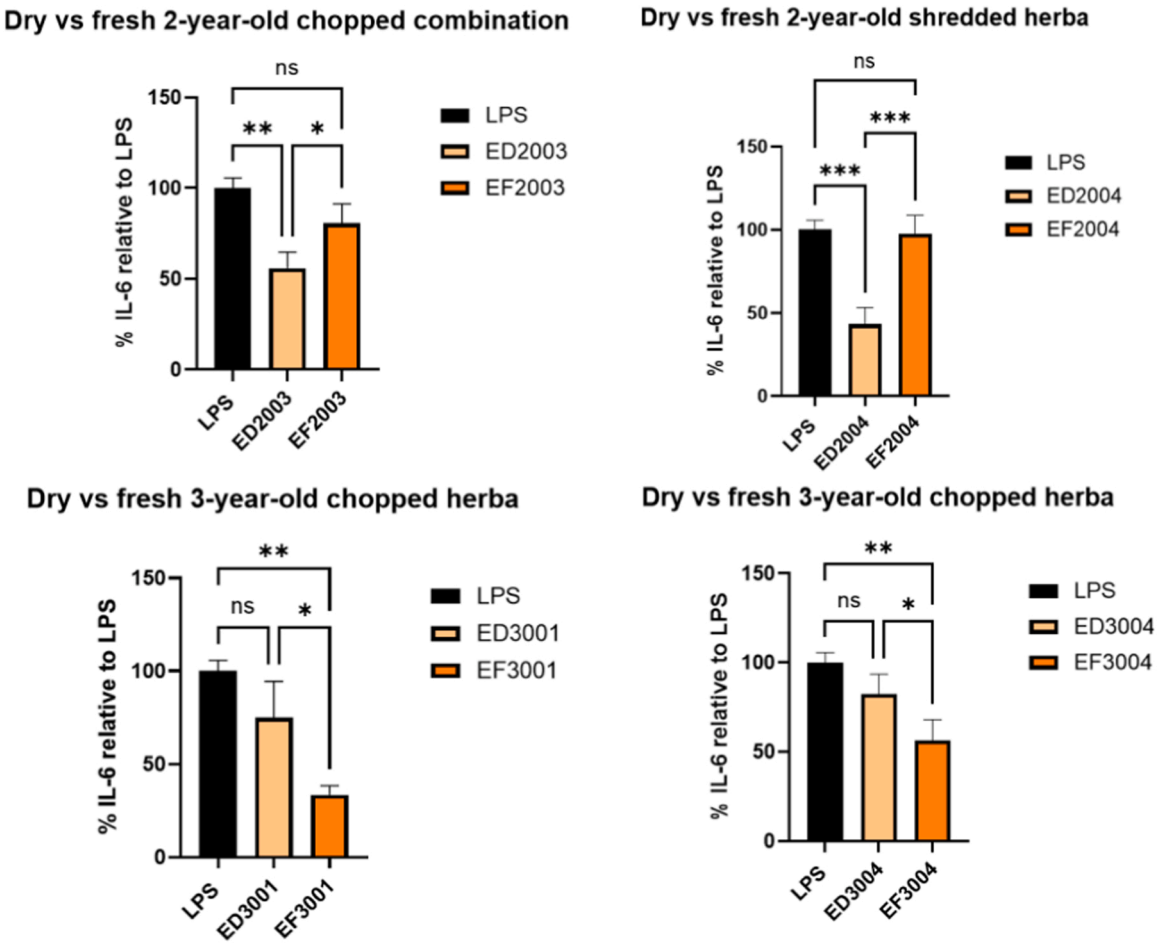


**Fig. 9.** Effects of E. purpurea tincture extracts made on IL-6 production in THP-1 cells following treatment with LPS. Where the bar is coloured light orange the impact of tincture extracts made with plant material that was dried before tincturing on IL-6 production is shown, where the bar is coloured dark orange the impact of tincture extracts that were prepared with plant material that was used fresh in the tincturing process is shown. *, * *, * ** indicate significant reduction of cytokines production at $p \leq 0.05$, 0.01, 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test. ns indicates no significant difference.

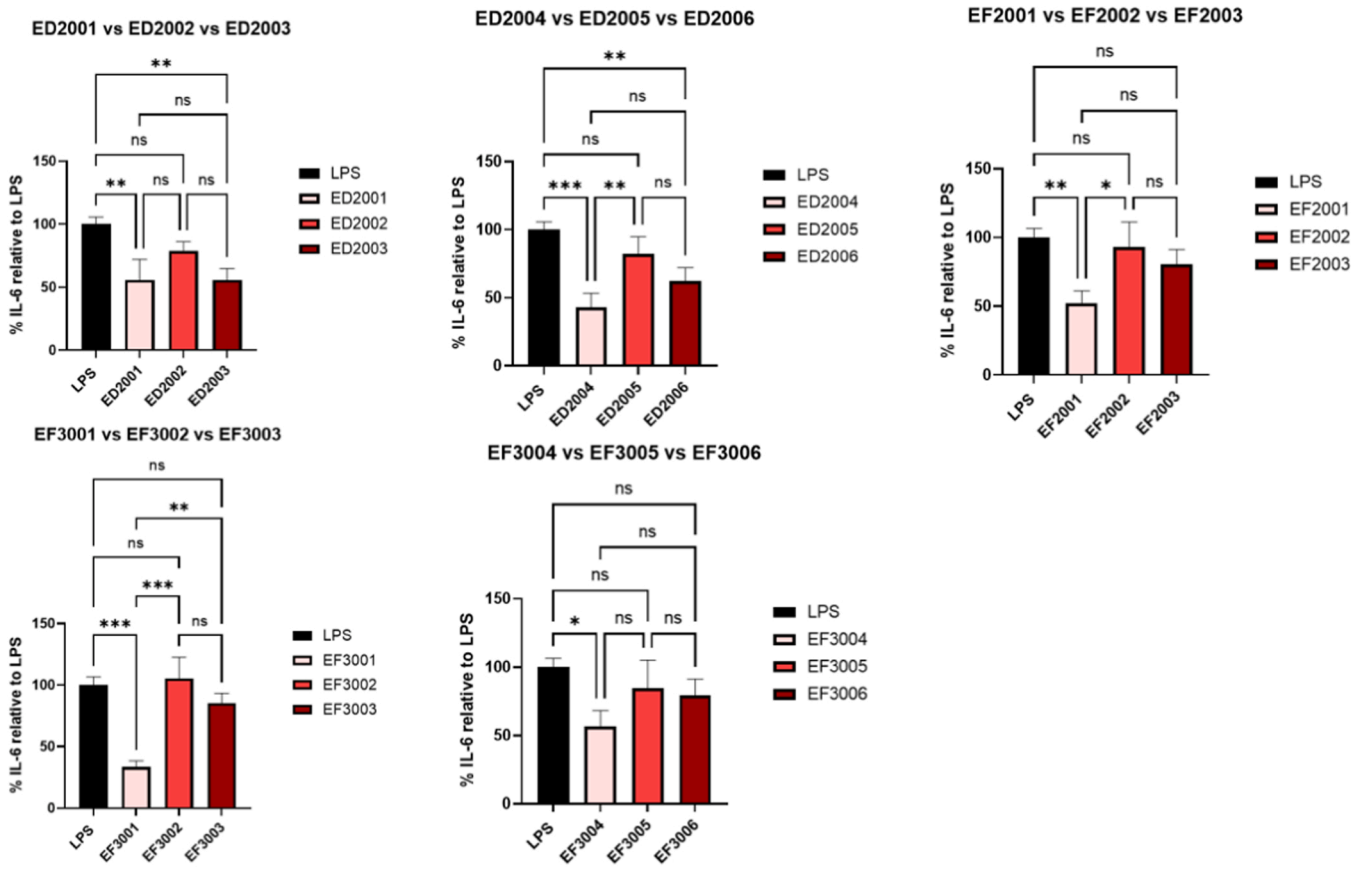


**Fig. 10.** Effects of E. purpurea tinctures on IL-6 production in THP-1 cells following treatment with LPS. Where the bar is coloured pink the impact of tinctures made with herba on IL-6 production is shown, where the bar is coloured red the impact of radix is shown and where the bar is coloured dark red the impact of tinctures made with a combination of herba, and radix is indicated. *, * *, * ** indicate significant reduction of cytokines production at $p \leq 0.05$, 0.01, 0.001, when compared with LPS as measured using a 1-way ANOVA analysis followed by a Tukey's multiple comparison test. ns indicates no significant difference.

highlighted in the PCA, where a clear distinction in the chemical profile of herba and radix was identified (Fig. 3).

*3.3.3.2. The impact of E. purpurea tincture on TNF-α production in THP-1 cells.* TNF-α is a pleiotropic cytokine that induces the proliferation of immune cell and stimulates the differentiation of naïve immune cells (Faustman and Davis, 2013). Non-LPS stimulated control THP-1 cells were treated with *E. purpurea* tincture extracts in order to evaluate their ability to enhance the production of cytokines and thus assess their prophylactic capabilities. Fig. 11A illustrates the ability of *E. purpurea* tincture extracts prepared with plant material that was two years old at the time of harvest to induce TNF-α production in THP-1 cells, relative to a DMSO control. The activity was significantly different in tincture extracts prepared with fresh versus dried plant material (with the exception of ED2001 vs ED2002). Tincture extracts prepared with dried plant material induced higher production of TNF-α than those prepared with fresh plant material. Similarly, tincture extracts prepared with plant material that was three years old and fresh at the time of harvest and dried induced significantly higher TNF-α production than those prepared with plant material that was three years old at the time of harvest and fresh (Fig. 11B). The PCA data showed that tinctures prepared with fresh versus dried plant material differ in their chemical profiles (Fig. 2).

In this work, we demonstrated that upstream processing of *E. purpurea* plant material has a very significant impact on both the chemical profile and the immunomodulating activity of *E. purpurea* tinctures. The use of fresh versus dried plant material was one such process parameter. All tinctures (n = 8), except four tinctures that were prepared with dried plant material had higher concentrations of caffeic acid derivatives than tinctures prepared with fresh plant material. A distinction in the chemical profile of tinctures prepared with fresh and dried plant material was also clear from the PCA. Studies probing the immunomodulating activity of *E. purpurea* showed that tincture extracts prepared with dried plant material reduced production of IL-6 in LPS-stimulated iBMDM and THP-1 cells and increased production of TNF-α in THP-1 cells more potently than tincture extracts prepared with fresh plant material. Thus, *E. purpurea* tincture extracts have demonstrated an ability to stimulate cytokine production *in vitro*, which is indicative of a potential to initiate a protective immune response. The tincture extracts have also demonstrated an ability to limit cytokine production in the case of excess production.

The size of the plant material used in the tincture also had an impact on both the chemical profile and the biological activity of the tinctures. When samples were prepared with fresh and chopped *E. purpurea,* higher concentrations of caffeic acid derivatives were detected then compared with tinctures that were prepared with plant material that was fresh and shredded. In fact, tinctures prepared with fresh and shredded plant material did not have quantifiable concentrations (as assayed by HPLC) of any of the caffeic acid derivatives studied. Despite the absence of caffeic acid derivatives a number of tinctures prepared with fresh and shredded plant material still had significant immunomodulating activity, one such example being EF3004. Thus, while caffeic acid derivatives are indicated in the British and US Pharmacopoeias as markers of quality of *E. purpurea* plant material, they are not necessarily the best indicators of immunomodulating activity of *E. purpurea* tinctures.

The impact of the age of the plant material at the time of harvest on the chemical profile and biological activity of *E. purpurea* tinctures was also considered. This parameter was seen to impact the extent to which the tincture extracts reduced concentrations of IL-6 in iBMDM. Tincture

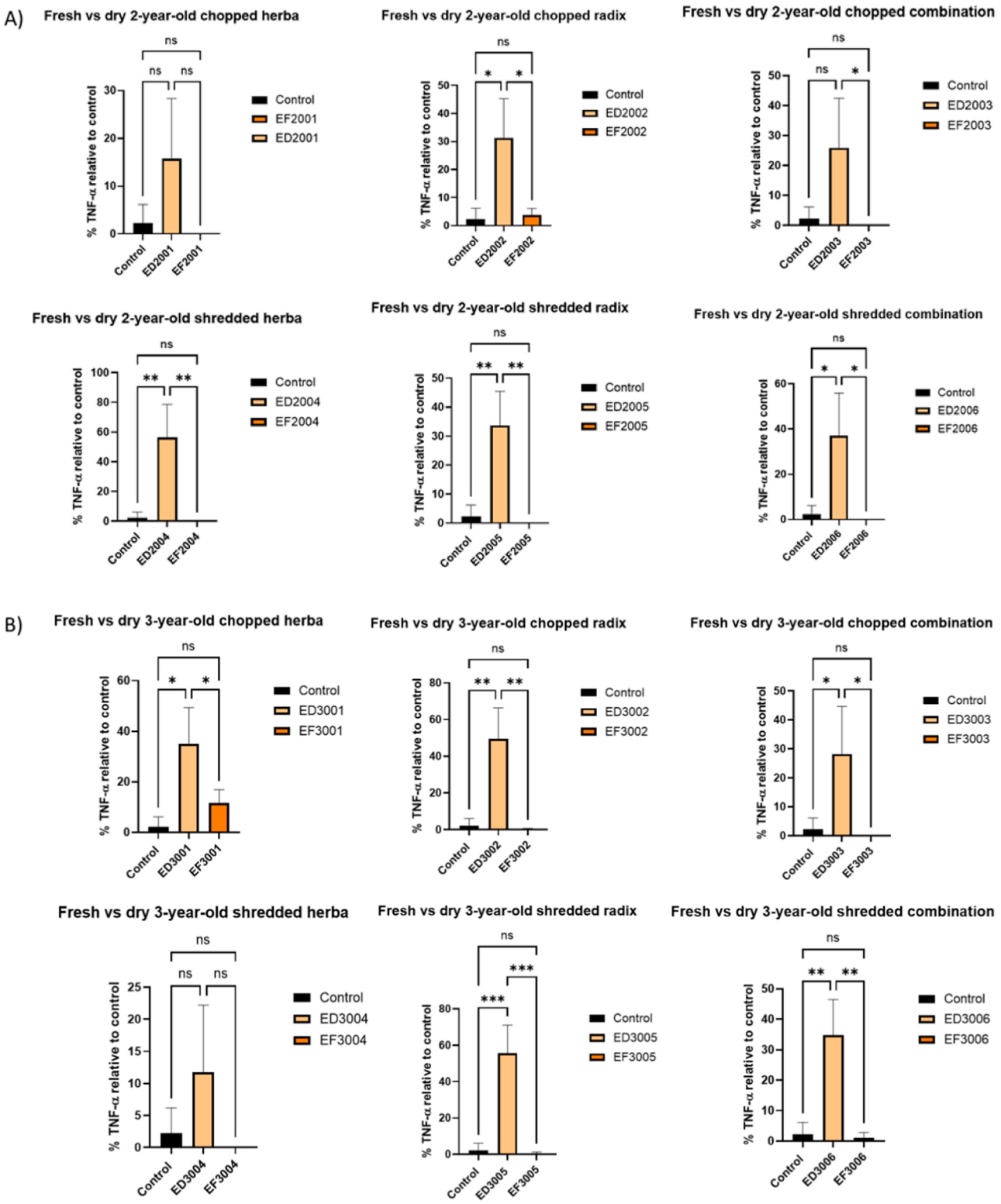


**Fig. 11.** Effects of E. purpurea tincture extracts on TNF-α production in THP-1 cells. Where the bar is coloured light orange the impact of tincture extracts made with dried plant material on is shown and where the bar is coloured dark orange the impact of tincture extracts made with fresh plant material is shown. A) shows the impact of tincture extracts prepared with plant material that was 2 years old at the time of harvest B) shows the impact of tincture extracts prepared with tinctures that were 3 years old at the time of harvest. * , * *, * ** and indicate significant reduction of cytokines production at $p \leq 0.05$, 0.01, 0.001, when compared with a DMSO control as measured using a 1-way ANOVA. ns indicates no significant difference.

extracts prepared with two-year-old plant material had a greater impact on IL-6 production than tincture extracts prepared with three-year-old plant material. However, a distinct difference in the chemical profile of tincture prepared with plant material that was two versus three years old at the time of harvest was not observed. A further analysis exploring compounds such as alkylamides may reveal why tinctures prepared with plant material that was 2 or 3 years old at the time of harvest have different chemical profiles.

Finally, the inclusion of herba in the tincture extracts was seen to significantly improve modulation of the IL-6 cytokine in both iBMDM and THP-1 cells. The PCA revealed a distinction in the chemical profile of tinctures prepared with herba versus with radix. Many commercially available *E. purpurea* formulations include a combination of radix and herba. These *in vitro* findings indicate the importance of including herba in tinctures of *E. purpurea* intended for IL-6 modulation, however *in vivo* studies are warranted to understand if the inclusion of herba in tinctures leads to improved immunomodulating effects *in vivo*.

This study focused on *in vitro* results and did not explore the bioavailability of the *E. purpurea* tinctures. As such, future work could explore the impact of upstream processing on *E. purpurea* tincture's immunomodulating activity *in vivo* as well as undertaking further chemical analysis aimed at identifying marker compounds which are indicative of bioactivity. Investigations into the role of gut microbiota in the transformation of *E. purpurea* phenolics (as highlighted by Ávila-Gálvez et al., 2024) and/or other bioactives *in vivo* would also be of interest.

## 4. Conclusion

This work demonstrated that using fresh or dried plant material, that was chopped (>2000μm) or shredded (500–2000μm), that was two or three years old at the time of harvest and the use of herba, radix or a combination has an impact on both the chemical profile and the immunomodulating activity of *E. purpurea* tinctures. The study indicates that including dried, rather than fresh *E. purpurea* plant material in a tincture intended for immunomodulating purposes would be advantageous. The importance of including herba in a tincture intended for immunomodulating activity is also highlighted. Although further studies are required in order to understand if these trends translate to *in vivo* models. The impact that upstream processing has on caffeic acid derivatives is highlighted but the concentration of these compounds is not always linked to an immunomodulating activity. This indicates that the use of caffeic acid derivatives alone as markers of the quality of *E. purpurea* preparations may be questionable, and also indicates the need to identify (a) marker compound(s) which is(are) indicative of the plant's bioactivity. The study underscores the necessity of selecting appropriate upstream processing methods to ensure the production of *E. purpurea* tinctures that have a consistent chemical profile and potent immunomodulating activity.

## CRediT authorship contribution statement

**Tolan Kate:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Selo Mohammed Ali:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Obaid Ismael:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Carty Michael:** Writing – review & editing, Methodology. **Sheridan Helen:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Healy Anne Marie:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Peter O'Connell:** Writing – review & editing, Methodology, Data curation. **Nagar Shipra:** Writing – review & editing, Formal analysis.

## Declaration of Competing Interest

The authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence or be perceived to influence this work.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phytol.2025.102971.

## Data availability

Data is available within the article and the supplementary information

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