Evaluation of *Echinacea purpurea* Extracts as Immunostimulants: Impact on Macrophage Activation

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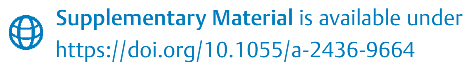

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ABSTRACT

Echinacea purpurea has been traditionally used to strengthen the immune system. Therefore, herein, we investigated the potential of *E. purpurea* aqueous extracts (AEs) obtained from flowers (F), leaves (L), or roots (R) as an immune booster in human primary monocyte-derived macrophages (hMDMs). Additionally, to identify the main class of compounds (phenolic/carboxylic acids vs. alkylamides) responsible for the bioactivity, the three AEs were fractioned by semi-preparative high-performance liquid chromatography (HPLC). The AEs and the isolated phenolic/carboxylic acidic fractions were not cytotoxic for hMDMs for all tested concentrations, as confirmed by the metabolic activity and DNA content assays. Moreover, AE drastically induced the production of the interleukin (IL)-6 and tumor necrosis factor (TNF)-α, with a minimal effect on IL-1β and prostaglandin E2 (PGE2), supporting their potential for macrophage activation. Interestingly, in the presence of the phenolic/carboxylic acidic fractions, this efficacy considerably decreased, suggesting a complementary effect between compounds. AE also triggered the phosphorylation of the extracellular signal-regulated kinase (ERK) 1/2 and p38 signaling pathways and upregulated the cyclooх-

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ygenase (COX)-2 expression in hMDMs. Overall, AE-F was demonstrated to be the most powerful immunostimulant extract that can be related to their higher number in identified bioactive compounds compared to AE-L and AE-R. These results highlight the efficiency of *E. purpurea* AE to enhance the function of a key cell type of the immune system and their potential as immunostimulant formulations for patients with a compromised immune system due to certain diseases (e.g., acquired immunodeficiencies) and treatments (e.g., chemotherapy).

ABBREVIATIONS

AE-F	aqueous extracts obtained from flowers
AE-L	aqueous extracts obtained from leaves
AE-R	aqueous extracts obtained from roots
AEs	aqueous extracts
COX	cyclooxygenase
cRPMI	complete RPMI (RPMI-1640 culture medium with 2 mM glutamine supplemented with 10% human serum, 1% penicillin/streptomycin and 1% HEPES)
DPBS	Dulbecco's phosphate-buffered saline
ERK 1/2	extracellular signal-regulated kinase
F	flowers
F1	phenol/carboxylic acid fraction
F2	alkylamide fraction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony-stimulating factor
hMDMs	human primary monocyte-derived macrophages
iNOS	inducible nitric oxide synthase
L	leaves
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
NF-κb	factor nuclear kappa B
PBMCs	peripheral blood mononuclear cells
PGE2	prostaglandin E2
PVDF	polyvinylidene fluoride
R	roots
RIPA	radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute 1640 media
RT	room temperature
SAPK/JNK	stress-activated protein kinases/jun amino-terminal kinases
STAT	signal transducers and activators of transcription

Introduction

Echinacea purpurea L. (Asteraceae family) preparations have been traditionally and highly used as an immune booster, being recognized as safe by the World Health Organization [1]. *E. purpurea* extracts can interact with different entities of the immune system, modulating their activity. The immunostimulatory activity of *E. purpurea* is related to the (*i*) activation of different immune cells (e.g., macrophages, dendritic cells, CD4 and CD8 T cells, natural killer cells, monocytes, myeloid progenitor cells, and splenocytes) [2–18], (*ii*) promotion of cytokine, chemokine, and immunoglobulin expression [19–24], (*iii*) stimulation of phagocytosis [21, 22], (*iv*) increase in respiratory burst [2, 9], (*v*) increase in cell proliferation (e.g., monocytes, B cells, CD4 and CD8 T cells, and natural killer cells) [5, 6, 14, 17, 25], and (*vi*) activation of immune cells (e.g., monocytes, dendritic cells, and natural killer cells) migration and mobility [25, 26]. This stimulatory effect may enhance, for instance, the surveillance to infections [27] and a faster resolution of the disease cause [28].

The immunostimulatory activity of *E. purpurea* has been mainly attributed to polysaccharides [5, 6, 9, 11, 27]. Alkylamides and caffeic acid derivatives can also present this bioactivity [2, 3, 20, 21]. However, there are incongruences in the literature regarding extracts bioactivity. Among different factors, the particular experimental cell procedures used in the several studies contribute to the reported differences between *E. purpurea* extracts bioactivity. For example, a butanol fraction of its stems and leaves significantly activated human-derived dendritic cells but not mouse-derived dendritic cells, suggesting that specific biochemical and cellular effects of plant extracts may differ between mammalian species [12, 26]. Therefore, the determination of the constituents' fingerprint and the use of reliable *in vitro* models that could mimic the human cell environment enable a greater accuracy in the identification of the compounds responsible for a specific activity of plant extracts.

Due to its immunostimulatory activity, *E. purpurea* formulations could present high added value in the therapy of diseases where the immune system is compromised (e.g., acquired immunodeficiencies). Particularly, the activation of a critical immune cell type, such as macrophages, in these individuals would benefit greatly the overall immune response [29]. In addition, there are several studies illustrating the immunostimulatory activity of *E. purpurea* extracts, the majority of them being performed with cell lines or murine primary macrophages [2, 9, 10, 18, 20, 22]. Indeed, to the best of our knowledge, only one study was carried out with human primary macrophages to demonstrate the ability of a commercial *E. purpurea* formulation to stimulate the production of cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and IL-10 [23]. However, its mechanism of action, as well as the active principles, were not investigated. Therefore, in this study, we aimed to explore the effects of the extract that most people consume as a tea, namely the aqueous extract (AE), in human primary monocyte-derived macrophage (hMDM) activity. Macrophages are often the first line of the body's defense against noxious stimuli [29], their activation being essential for the strengthening of the immune system. Different extracts were prepared by stirring flowers (F), leaves (L), and roots (R) of *E. purpurea* with water. Then, to determine which class of compounds were responsible for the immunostimulatory activity, each AE was fractionated by semi-preparative high-performance liquid chromatography (HPLC) into two fractions: phenolic/carboxylic

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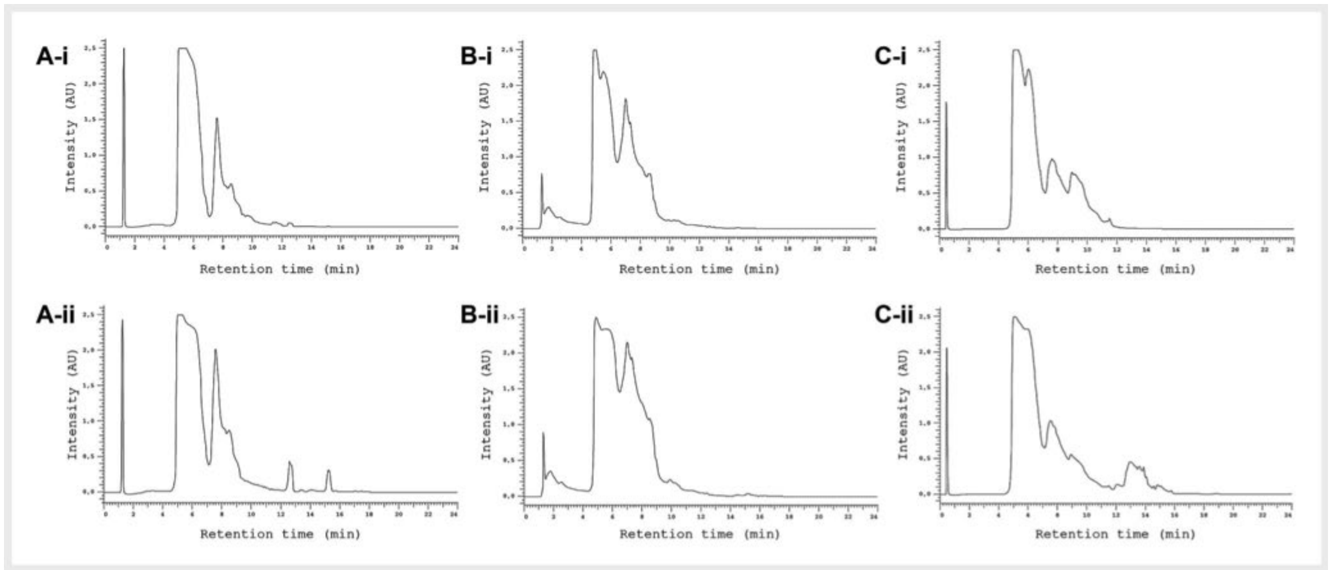

▶ **Fig. 1** Semi-preparative HPLC chromatograms of aqueous extracts obtained from flowers (**a**), leaves (**b**) and roots (**c**), at 30 mg/mL (400 µL of injection volume), detected at 330 nm (phenolic/carboxylic acids; a–i, b–i, and c–i) and 254 nm (alkylamides; a-ii, b-ii, and c-ii).

acids (F1) and alkylamides (F2) fractions. The production of the main pro-inflammatory cytokines (IL-6, TNF-α, IL-1β) and eicosanoids (prostaglandin – PG – E2) was first investigated. These inflammatory mediators trigger different biochemical cascades in target immune cells, determining the course of the immune response in a complex inflammatory process. Briefly, they can, e.g., stimulate chemotaxis and phagocytosis, promote the vascular permeability, and induce the cytotoxic and bactericidal activities of macrophages and neutrophils [30]. Hence, the increased levels of pro-inflammatory cytokines and eicosanoids can have a therapeutic action for individuals when the activation of the immune system is a challenge. Then, the mechanism of action underlying the pro-inflammatory activity was analyzed. The activation of macrophages is deeply related to the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signal transduction pathways [31], which are involved in the regulation of pro-inflammatory cytokine expression through phosphorylation of the transcription factors [32]. Based on that, different inflammatory signaling pathways were investigated, namely NF-κB p65, extracellular signal-regulated kinase – ERK – 1/2, p38, and cyclooxygenase – COX – 2 expression. The whole extract exhibited significantly higher immunostimulatory activity than the phenolic/carboxylic acids fractions (F1), suggesting a complementary effect between the two classes of compounds. To the best of our knowledge, this is the first study demonstrating the stimulation of hMDMs by *E. purpurea* AE, through the activation of ERK 1/2 and p38 of the MAPK signaling pathway, as well as by increasing COX-2 expression.

Results

▶ **Table 1** presents the identified phenolic/carboxylic acid compounds and alkylamides in each AE. Each extract exhibited different patterns of phenolic/carboxylic acids and alkylamides. Seven phenolic/carboxylic acids were identified in AE-F and AE-L. AE-R only present three phenolic/carboxylic acids. Sixteen and fourteen alkylamides were identified in AE-R and AE-F, respectively. AE-L did not contain any identified alkylamide.

The chromatograms obtained for AE by semi-preparative HPLC are present in ▶ **Fig. 1**. The three AEs show a higher number of phenolic/carboxylic acids compounds than alkylamides. AE-F displayed two individual peaks of alkylamides (▶ **Fig. 1 a–ii**), while AE-L did not exhibit any alkylamide peak (▶ **Fig. 1 b–ii**). AE-R revealed the highest amount of alkylamides, represented by an irregular peak (▶ **Fig. 1 c–ii**).

The metabolic activity and the relative DNA concentration of non-stimulated hMDMs in the absence or presence of the *E. purpurea* AE and their phenolic/carboxylic acid fractions at different concentrations are presented in ▶ **Fig. 2**. The cell metabolic activity and the DNA concentration were not affected in the presence of the AE and phenolic/carboxylic acid fractions at any tested concentration, showing results similar to those of the negative control (non-stimulated hMDMs without treatment, ▶ **Fig. 2**). As expected, the positive control (10% of DMSO) significantly decreased the viability of non-stimulated hMDMs.

The immunostimulatory activity of AEs and their phenolic/carboxylic acid fractions was evaluated by assessing the levels of pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) and the eicosanoid PGE2, produced by non-stimulated hMDMs in the cell culture medium. Non-stimulated hMDMs (negative control) produced basal amounts of IL-6 (5.9 ± 7.6 pg/mL), TNF-α (40.5 ± 28.9 pg/mL), IL-1β (1.1 ± 0.6 PG/mL), and PGE2 (311.7 ± 47.9 pg/mL). As expected, lipopolysaccharide (LPS)-stimulated hMDMs (positive control) significantly increased IL-6, TNF-α, and PGE2 production (▶ **Fig. 3**). The production of IL-1β was also enhanced ≈ 6 times compared with the negative control, but it was not statistically significant. When macrophages were incubated with the AE and their phenolic/carboxylic acids fractions, a significant increase in

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▶ **Table 1** Overview of the identified compounds (phenolic/carboxylic acids and alkylamides) in aqueous *E. purpurea* extracts (AEs) obtained from flowers (F), leaves (L), and roots (R) by LC-HRMS.

Compounds	**AE**		
	F	**L**	**R**
Phenolic/carboxylic acids			
Malic acid	+	+	+
Vanillic acid	+	+	–
Protocatechuic acid	+	+	–
Caftaric acid	+	+	–
Chlorogenic acid	–	–	–
Quinic acid	–	–	–
Vanillin	–	–	–
Caffeic acid	–	–	–
Benzoic acid	+	+	+
Cynarin	–	–	–
Echinacoside	–	–	–
p-coumaric acid	+	+	–
Chicoric acid	+	+	+
Rutin	–	–	–
Quercetin	–	–	–
Alkylamides			
Dodeca-2E,4Z,10E-triene-8-ynoic acid isobutylamide	+	–	+
Dodeca-2E,4Z,10Z-triene-8-ynoic acid isobutylamide	+	–	+
Dodeca-2,4,10-triene-8-ynoic acid isobutylamide (isomer 1)	–	–	–
Dodeca-2E,4E,10Z-triene-8-ynoic acid isobutylamide	–	–	+
Dodeca-2Z,4E,10Z-triene-8-ynoic acid isobutylamide	–	–	+
Dodeca-2E,4E,10E-triene-8-ynoic acid isobutylamide	+	–	–
Undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	+	–	+
Undeca-2E/Z-ene-8,10-diynoic acid isobutylamide	+	–	–
Undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	–	–	+
Undeca-2E/Z,4Z/E-diene-8,10-diynoic acid 2-methylbutylamide	–	–	–
Pentadeca-2E,9Z-diene-12,14-diynoic acid 2-hydroxyisobutylamide	–	–	–
Dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide	+	–	+
Undeca-2E,4E-diene-8,10-diynoic acid isobutylamide	–	–	–
Dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide	–	–	+
Dodeca-2E-ene-8,10-diynoic acid isobutylamide	+	–	–
Trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide	+	–	+
Dodeca-2,4-diene-8,10-diynoic acid 2-methylbutylamide	–	–	+
Dodeca-2Z,4Z,10Z-triene-8-ynoic acid isobutylamide	–	–	+
Trideca-2E,7Z-diene-10,12-diynoic acid 2-methylbutylamide	+	–	–
Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide	+	–	+

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▶ Table 1 *Continued*

Compounds	AE		
	F	L	R
Dodeca-2E,4Z,10E-triene-8-ynoic acid 2-methylbutylamide *or* Dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide	+	–	+
Dodeca-2E,4E,8Z-trienoic acid isobutylamide (isomer 1)	–	–	–
Dodeca-2E,4E-dienoic acid isobutylamide (isomer 1)	–	–	–
Pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide	–	–	+
Dodeca-2E,4E,8Z-trienoic acid isobutylamide	+	–	+
Trideca-2Z,7Z-diene-10,12-diynoic acid 2-methylbutylamide	–	–	–
Dodeca-2E,4E,8Z,10E/Z-tetraenoic acid 2-methylbutylamide	+	–	–
Hexadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide	–	–	–
Dodeca-2E,4E,8Z-trienoic acid isobutylamide (isomer 2)	–	–	–
Dodeca-2E,4E-dienoic acid isobutylamide	+	–	+

Bold text corresponds to studied standards. Symbol "+" represents the presence of compound; symbol "–" represents the absence of compound.
E/Z stereochemistry is indicated here in accordance with the literature [33], but it should be highlighted that without conformational NMR spectra, it is not possible to conclusively distinguish between E and Z isomers.

those cytokines and the eicosanoid in the culture medium was observed (▶ **Fig. 3**), demonstrating its potential to stimulate naïve macrophages. In general, extracts from flowers and leaves were the most powerful in the stimulation of the production of pro-inflammatory mediators. Roots presented ≈ 1.3 times lower activity than the other parts of the plant.

AEs demonstrated an immunostimulatory profile for two important cytokines in inflammation, IL-6 (▶ **Fig. 3 a**) and TNF-α (▶ **Fig. 3 b**). IL-6 and TNF-α production was dramatically enhanced in the presence of 250 µg/mL of AE-F in, respectively, 20 164.9 ± 10 943.3 and 892.7 ± 209.9 times higher than non-stimulated hMDMs. AE-L demonstrated a comparable bioactivity to AE-F in stimulating IL-6 and TNF-α production (20 011.9 ± 8402.8 and 804.2 ± 154.4 times higher than non-stimulated hMDMs, respectively). AE-R was able to induce 18 616.9 ± 9241.5 and 742.9 ± 195.3 times the IL-6 and TNF-α production, respectively. Regarding the phenolic/carboxylic acids fractions, only AE-F-F1 was able to significantly stimulate hMDMs to produce IL-6 at 125 µg/mL (more than 7136.1 ± 2903.0 times the negative control, ▶ **Fig. 3 a**). Conversely, all the fractions significantly enhanced TNF-α production (▶ **Fig. 3 b**). However, the efficacy of all phenolic/carboxylic acid fractions, to stimulate IL-6 and TNF-α production, was dramatically lower in comparison with the whole extract. Comparing all the *E. purpurea* extracts, AE-F and AE-L exhibited the highest immunostimulatory activity for IL-6 and TNF-α production, followed by AE-R, AE-F-F1, AE-L-F1, and AE-R-F1. Moreover, all the AE demonstrated a similar or higher pro-inflammatory cytokine production than LPS-stimulated hMDMs.

IL-1β production was markedly improved 41.4 ± 21.7 and 30.8 ± 12.2 times in the presence of 250 µg/mL AE-F and AE-R, respectively. AE-L immunostimulatory activity was not significantly demonstrated for this pro-inflammatory cytokine (15.7 ± 1.0 times). Interestingly, AE-F-F1, at 250 µg/mL, demonstrated a superior immunostimulatory activity, enhancing 239.6 ± 94.6 times the IL-1β production. AE-R-F1 showed a comparable bioactivity to AE-R (44.0 ± 8.3 times, 250 µg/mL). IL-1β production by AE-L-F1 was not significantly enhanced (22.5 ± 10.1 times, 250 µg/mL). Comparing all the *E. purpurea* extracts, the AE-F-F1 was the fraction that was stronger in stimulating IL-1β production, followed by AE-R-F1, AE-F, AE-R, AE-L-F1, and AE-L. Furthermore, all the *E. purpurea* extracts showed higher IL-1β production than LPS-stimulated hMDMs.

PGE2 production was significantly enhanced 1.7 ± 0.1 and 1.4 ± 0.2 times in the presence of AE-F (250 µg/mL) and AE-L (50 µg/mL), respectively. AE-R did not demonstrate statistically significant differences in the induction of PGE2 production (1.3 ± 0.1, 125 µg/mL). In this case, the phenolic/carboxylic acids fractions obtained from AE were more effective for PGE2 production induction, showing an efficacy approximately 1.7 times higher compared to non-stimulated hMDMs for all tested fractions, at 250 µg/mL. Comparing all the *E. purpurea* extracts, AE-F-F1, AE-L-F1, AE-R-F1, and AE-F led to similar PGE2 production, followed by AE-L and AE-R. All the phenolic/carboxylic acid fractions and AE-F demonstrated a similar or higher induction of PGE2 production than LPS-stimulated macrophages.

To understand which mechanism of action was associated with the immunostimulatory activity of the *E. purpurea* AE, the signaling pathways of several pro-inflammatory molecules in hMDMs were investigated by Western blot. As AE at the highest tested concentration induced higher levels of IL-6 and TNF-α production than their phenolic/carboxylic acids fractions (F1), only these ones were investigated.

Non-stimulated hMDMs showed basal levels of ERK 1/2 (▶ **Fig. 4 a1** and **b1**) and p38 phosphorylation (▶ **Fig. 4 a2** and **b2**). The phosphorylation of ERK 1/2 and p38 was significantly enhanced in LPS-stimulated hMDMs. All AEs, but mainly AE-L and

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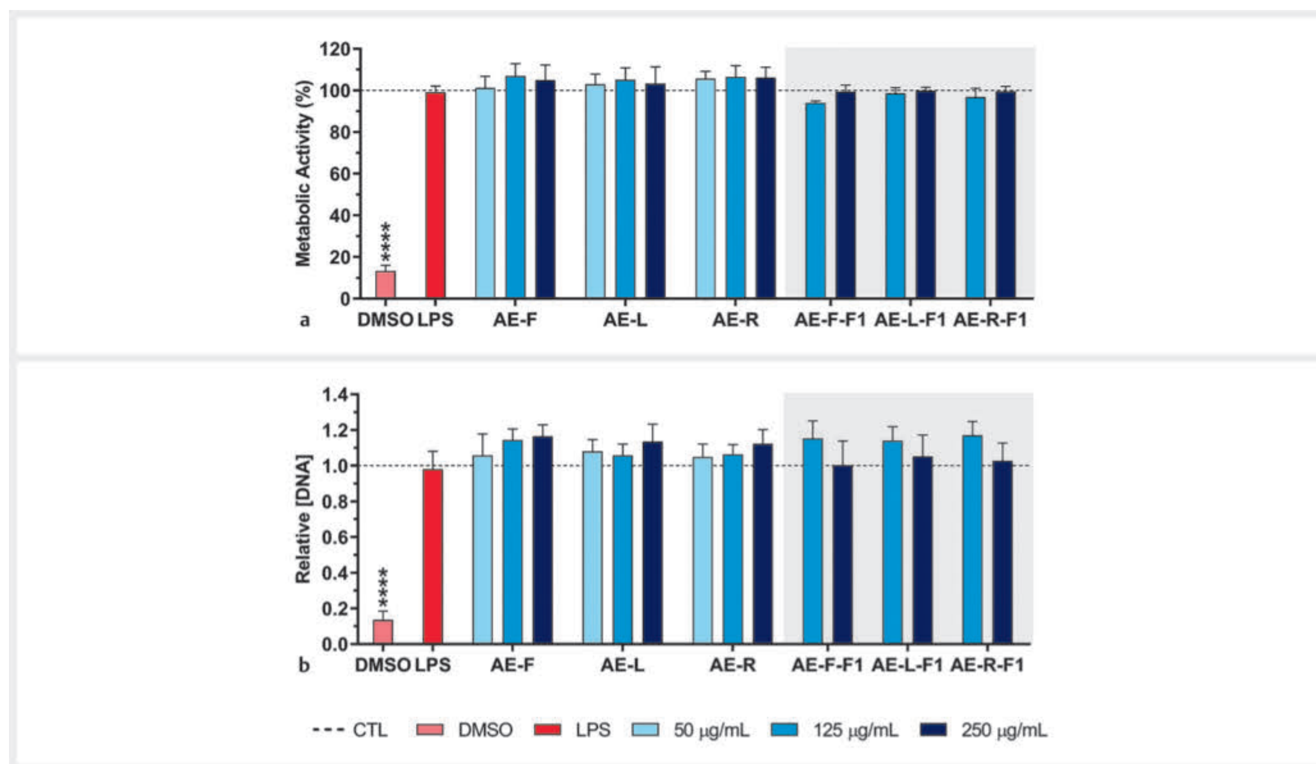

▶ **Fig. 2** Metabolic activity (**a**) and relative DNA concentration (**b**) of hMDM cultured in the presence of 10% DMSO, LPS, and different concentrations of the *E. purpurea* AEs obtained from flowers (F), leaves (L), roots (R) and their phenolic/carboxylic acid fractions (F1) for 24 h. The dotted line represents the metabolic activity and DNA concentration of the negative control (non-stimulated hMDMs without treatment). Statistically significant differences are **** ($p < 0.0001$) in comparison to the negative control (non-stimulated hMDMs without treatment). Data are represented as means ± SD ($n = 3$).

AE-R, also demonstrated the ability to drastically upregulate the expression of these inflammatory molecules, confirming their immunostimulatory activity. AE-R demonstrated a strong potential to upregulate the ERK 1/2 phosphorylation, being higher than that of LPS-stimulated hMDMs, followed by AE-L ≈ AE-F. The phosphorylation of p38 was similar between the different *E. purpurea* AEs, and the bioactivity was at similar level as that of LPS-stimulated hMDMs.

Basal levels of NF-*κ*B p65 were presented in non-stimulated hMDMs (▶ **Fig. 4 a3** and **b3**). Interestingly, the phosphorylation of NF-*κ*B p65 was not altered in the presence of LPS or AE for 24 h of culture (▶ **Fig. 4 a3** and **b3**).

Non-stimulated hMDMs did not present COX-2 expression, which was upregulated after their LPS-stimulation (▶ **Fig. 4 a4** and **b4**). AEs also showed a strong capacity to enhance the COX-2 expression, both AE-R and AE-L being the most powerful extracts compared to AE-F. The AEs had a similar or higher capacity to stimulate COX-2 expression in comparison with LPS.

Discussion

The immune system is a powerful tool resulting from the human body's evolution and is designed to keep it healthy and functional [34]. A healthy immune system is fundamental for recognizing several antigens, ranging from cancer to a common cold, and for developing and coordinating a complex defense response to maintain and protect the body. However, in individuals with a depressed or completely disabled immune system, an increased susceptibility to infections or illness is observed [35]. Therefore, the prescription of drugs to enhance the immune system is needed, *E. purpurea* being a strong candidate due to its traditional use in the prevention of infections. Indeed, the plants have been an excellent source of new and safer drugs with high therapeutic value in the clinic [36].

We investigated the *E. purpurea* AE – the most common beverage drunk by the world's population – composed by phenols/carboxylic acids and alkylamides as a promoter of hMDM activity. To identify the class of compounds present in AEs responsible for immunostimulatory activity, a fractionation by semi-preparative HPLC was further employed for all the three AEs. The optimized method perfectly enabled the fractionation of AE into phenolic/carboxylic acids (F1) and alkylamides (F2) due to the different polarities of the compounds (▶ **Fig. 1**). The bioactivity of the phenolic/carboxylic acid fractions obtained from AEs was also biologically evaluated to make conclusions about the class of compounds that mainly contribute to the immunostimulatory activity. Due to yield constrains, the bioactivity of the alkylamide fraction (F2) was not evaluated.

The identification of the bioactive compounds present in AE performed by LC-HRMS are in agreement with our previous work

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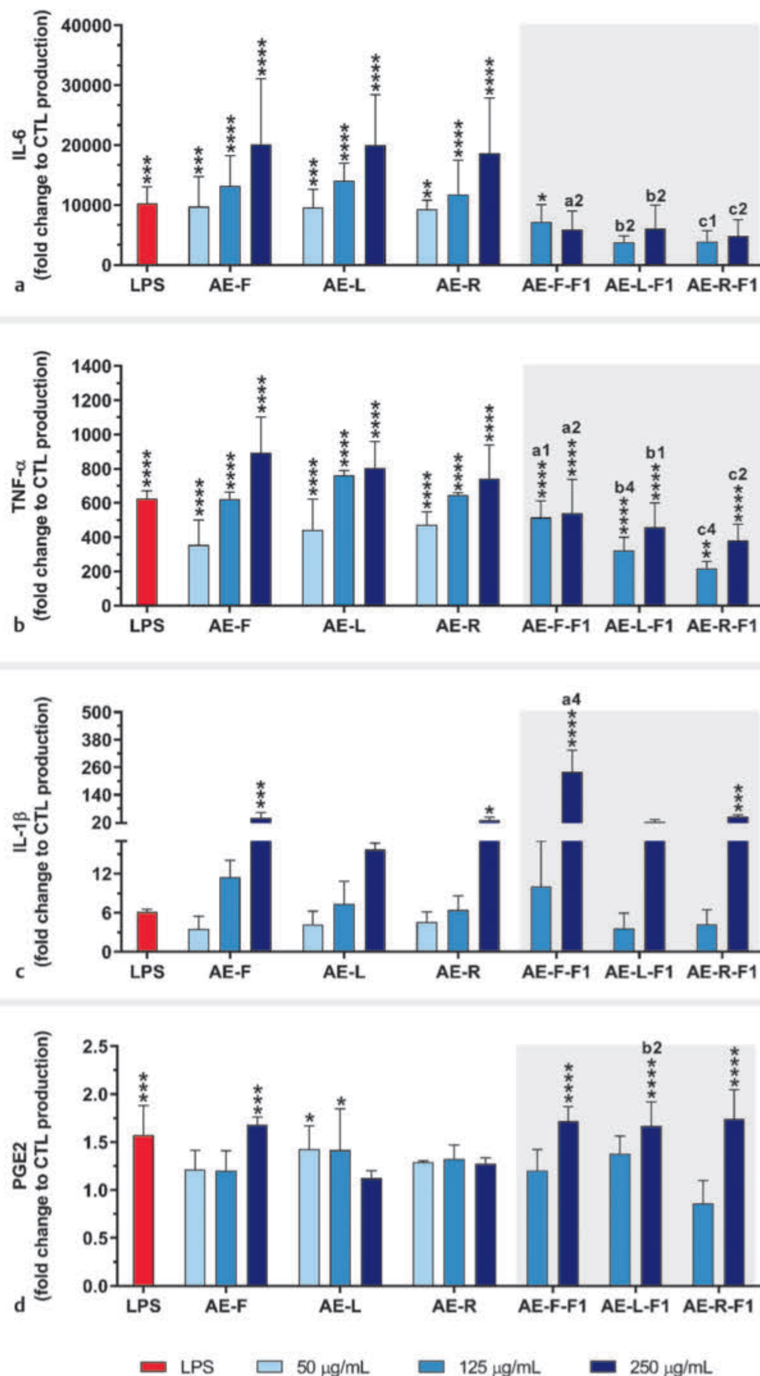

▶ **Fig. 3** IL-6 (**a**), TNF-*α* (**b**), IL-1*β* (**c**), and PGE2 (**d**) production of non-stimulated hMDMs cultured in the presence of LPS and different concentrations of the *E. purpurea* AEs obtained from flowers (F), leaves (L), roots (R) and their phenolic/carboxylic acid fractions (F1) for 24 h. Statistically significant differences are * ($p < 0.0395$), ** ($p < 0.0047$), *** ($p < 0.0009$), and **** ($p < 0.0001$) in comparison to the negative control (non-stimulated hMDMs without treatment) for each different tested *E. purpurea* AE and F1 concentrations, and 1 ($p < 0.0438$), 2 ($p < 0.0093$), and 4 ($p < 0.0001$) in comparison with a (AE-F vs. AE-F-F1), b (AE-L vs. AE-L-F1), and c (AE-R vs. AE-R-F1). Data are represented as means ± SD ($n = 3$).

[33]. Only AEs were chemically identified since no significant biological activity was observed in F1. AEs, exhibiting different fingerprint patterns considering the plant part, are enriched in phenolic/carboxylic acid compounds, with small amounts of alkylamides (▶ **Table 1**). Briefly, in flowers and leaves, there were seven phenolic/carboxylic acids identified, namely malic acid, vanillic acid, protocatechuic acid, caftaric acid, benzoic acid, p-coumaric acid, and chicoric acid. Only three of these (malic acid, benzoic acid,

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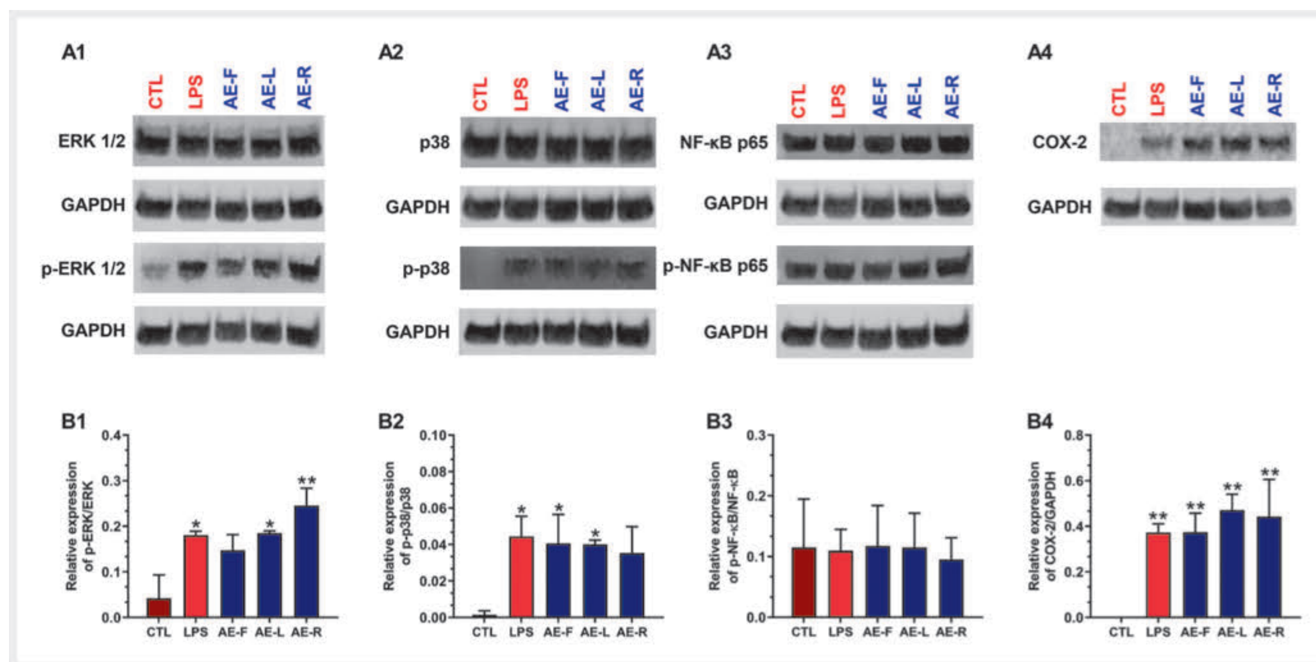

▶ **Fig. 4** ERK 1/2 (**a1** and **b1**), p38 (**a2** and **b2**), NF-*κ*B p65 (**a3** and **b3**), and COX-2 (**a4** and **b4**) signaling pathway activation of non-stimulated hMDMs cultured in the presence of LPS and *E. purpurea* aqueous extracts (AEs) obtained from flowers (F), leaves (L), and roots (R), at 250 µg/mL, for 24 h. Statistically significant differences are * ($p < 0.0457$) and ** ($p < 0.0078$) in comparison to the negative control (non-stimulated hMDMs without treatment) for each different tested *E. purpurea* AE. Data are represented as means ± SD ($n = 3$).

and chicoric acid) were present in AE obtained from roots. The alkylamides were more concentrated in roots (16 compounds) than in flowers (14 compounds). Dodeca-2E,4Z,10E-triene-8-ynoic acid isobutylamide, dodeca-2E,4Z,10Z-triene-8-ynoic acid isobutylamide, undeca-2E,4Z-diene-8,10-diynoic acid isobutylamide, dodeca-2E,4Z-diene-8,10-diynoic acid isobutylamide, trideca-2E,7Z-diene-10,12-diynoic acid isobutylamide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide, dodeca-2E,4Z,10E-triene-8-ynoic acid 2-methylbutylamide or dodeca-2E-ene-8,10-diynoic acid 2-methylbutylamide, dodeca-2E,4E,8Z-trienoic acid isobutylamide, and dodeca-2E,4E-dienoic acid isobutylamide were present in both roots and flowers. Dodeca-2E,4E,10Z-triene-8-ynoic acid isobutylamide, dodeca-2Z,4E,10Z-triene-8-ynoic acid isobutylamide, undeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, dodeca-2Z,4E-diene-8,10-diynoic acid isobutylamide, dodeca-2,4-diene-8,10-diynoic acid 2-methylbutylamide, dodeca-2Z,4Z,10Z-triene-8-ynoic acid isobutylamide, and pentadeca-2E,9Z-diene-12,14-diynoic acid isobutylamide were only identified in roots. Conversely, dodeca-2E,4E,10E-triene-8-ynoic acid isobutylamide, undeca-2E/Z-ene-8,10-diynoic acid isobutylamide, dodeca-2E-ene-8,10-diynoic acid isobutylamide, trideca-2E,7Z-diene-10,12-diynoic acid 2-methylbutylamide, dodeca-2E,4E,8Z,10E/Z-tetraenoic acid 2-methylbutylamide were only present in flowers. The extracts obtained from leaves did not present any identified alkylamide (▶ **Fig. 1**). Analyzing the three AE, flowers exhibited the highest number of identified bioactive compounds (21 compounds), followed by roots (19 compounds) and leaves (7 compounds).

In a first approach, we investigated the capacity of cytocompatible AEs and their phenolic/carboxylic acid fractions to induce the overexpression of the main mediators of the acute phase response, namely pro-inflammatory cytokines (IL-6, TNF-*α*, IL-1*β*) and an eicosanoid (PGE2), in hMDMs [30]. The AE and their phenolic/carboxylic acid fractions were not cytotoxic for hMDMs at any tested concentration, demonstrating their cytocompatibility (▶ **Fig. 2**). In addition, AEs and their phenolic/carboxylic acid fractions induced the production of IL-6 and TNF-*α* by hMDMs, having minimal effect in IL-1*β* and PGE2 (▶ **Fig. 3**). IL-1*β* is synthesized as a precursor peptide (pro-IL-1*β*) and secreted in the mature form (IL-1*β*) by activated macrophages [30]. Accordingly, AE may not be able to activate certain signals required for the complete maturation of IL-1*β*. Conversely, AE-F-F1 was incredibly efficient to activate the mature form of IL-1*β* (▶ **Fig. 3**). Interestingly, when the AEs were separated into phenolic/carboxylic acid fractions, in general, the immunostimulatory activity considerably decreased. All these results highlight the possibility that a complementary effect between phenolic/carboxylic acid compounds and alkylamides can occur, as we previously hypothesized with a human cell line [33]. Therefore, the multiplicity of bioactive compounds present in the whole AE may provide a greater immunostimulatory activity [23]. However, AE-L did not present any alkylamide [33], and its immunostimulatory activity was similar to AE-F, which led us to make conclusions about the presence of other bioactive compounds, such as polysaccharides, that can also be responsible for the stimulation of hMDMs [5, 6, 9, 11, 27]. Flowers demonstrated an enhanced potential in hMDM activation, which may be correlated with the highest number of identified bioactive

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compounds (▶ **Fig. 3**). We also investigated the mechanism of action of the AE underlying the immunostimulatory activity in hMDMs. No significant effects were observed in NF-κB p65 phosphorylation when hMDMs were treated with AE (▶ **Fig. 4**). Matthias et al. also reported similar results with the Jurkat human T cell line treated with *E. purpurea* ethanolic extracts and their fractions [37]. The *E. purpurea* AE triggered the phosphorylation of the ERK 1/2 and p38 inflammatory proteins in the MAPK signaling pathway in hMDMs (▶ **Fig. 4**). Additionally, for the first time, we demonstrated the upregulation of COX-2 expression in the presence of AE. These events suggest an activation of macrophages. However, further research is needed to confirm direct evidence for the suggested causative link. Sullivan et al. described comparable results, reporting an *E. purpurea* polysaccharide fraction that activated murine-derived macrophages to produce pro-inflammatory cytokines (IL-6, TNF-α, and IL-12p70) through the activation of MAPK pathways (ERK, p38, and JNK) [9].

The efficacy of *E. purpurea* AEs and their phenolic/carboxylic acid fractions in the stimulation of hMDMs depends on its phytochemical composition. All AEs demonstrated higher pro-inflammatory capacity in comparison with their phenolic/carboxylic acid fractions, pointing to the existence of a complementary effect between phenols/carboxylic acids and alkylamides. This study also shows the importance of fractionating and chemically characterizing the plant extracts to identify the therapeutic profile of different classes of compounds. Different studies pointed out the bioavailability of echinacea constituents. Alkylamides are interesting in the pharmaceutical field since they have been shown to have good permeability using a Caco-2 cell monolayer [38, 39], demonstrating their potential oral bioavailability. Additionally, alkylamides were detected in plasma and blood following an oral dose of echinacea as a tablet formulation, in contrast to phenols [40, 41]. As the aqueous extracts are more enriched in phenols/carboxylic acids, advanced techniques for encapsulating those bioactive compounds must be addressed to overcome the poor bioavailability.

AEs strongly induce the production of IL-6 and TNF-α by stimulated hMDMs. However, a minimal effect in IL-1β and PGE2 concentrations was observed. Based on the presented results, these events can be correlated with the activation of the ERK 1/2 and p38 signaling pathways and the upregulation of COX-2 expression. Nonetheless, it is important to note that while these associations were observed, additional research is needed to establish whether these signaling events directly influence cytokine production and macrophage activity. Flowers, presenting the highest number of identified bioactive compounds, demonstrated the greatest immunostimulatory activity. Consequently, the activated macrophages can provide an efficient defensive response against noxious stimulus, protecting the body from infections and diseases. These promising results demonstrate the therapeutic value of *E. purpurea* AEs in the activation of crucial immune cells, highlighting the potential of this immunostimulant plant-based formulation in disorders in which the immune system is impaired. In summary, the data presented in this proof-of-concept study provide a basis for further investigation. Future work will include the use of pathway inhibitors, knockdowns, and additional molecular targets to thoroughly elucidate the mechanisms of action through which the bioactive compounds in AE extracts exert their effects. Further studies supporting the role of AE in complex models of inflammation should also be explored.

Materials and Methods

Reagents and chemicals

E. purpurea, commonly known as purple coneflower, was purchased from Cantinho das Aromáticas in May 2017. The plants were immediately transferred to the soil and were allowed to grow following a sustainable agriculture procedure (41°37′04.5″ N, 7°16′14.4″ W). After one year of cultivation, the flowers and leaves were collected in a full bloom phase (June and July 2018), while the roots, including rhizomes, were harvested in autumn (October 2018). The plants were dried in the dark and stored at room temperature (RT) protected from the light. A voucher specimen of both aerial parts (DB-16-EPT) and roots (DB-15-EPR), identified by Prof. Alberto C. P. Dias, was deposited at the Department of Biology, University of Minho, Portugal. Ultra-pure water was obtained from a Milli-Q Direct Water Purification System (Milli-Q Direct 16, Millipore). Roswell Park Memorial Institute (RPMI)-1640 media, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution 1 M, penicillin-streptomycin (10 000 U/mL), Dulbecco's phosphate-buffered saline (DPBS), Pierce Chromogenic Endotoxin Quant Kit, Quant-iT PicoGreen dsDNA Kit, Pierce Phosphatase Inhibitor Mini Tablets, PageRuler Plus Prestained Protein Ladder (10 to 250 kDa), Bolt Sample Reducing Agent, Bolt LDS Sample Buffer, Bis-Tris Bolt 8 %, Bolt MES SDS Running Buffer, and iBlot 2 Transfer Stacks (polyvinylidene fluoride, PVDF) were purchased from Thermo Fisher Scientific. An OctoMACS separator, human CD14 microbeads, MS columns, and human recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) were obtained from Miltenyi Biotec. AlamarBlue, Bio-Rad Protein Assay Dye Reagent Concentrate, and Tween20 were purchased from Bio-Rad. Human IL-1β, IL-6, and TNF-α, DuoSet Enzyme-linked immunosorbent assay (ELISA) and DuoSet ELISA Ancillary Reagent Kit 2 were purchased from R&D Systems. Ethanol, acetonitrile (ACN) HPLC grade, methanol HPLC grade, formic acid analytical grade, Histopaque-1077, human serum, lipopolysaccharide (LPS; *Escherichia coli* O26:B6), radioimmunoprecipitation assay (RIPA) buffer, complete mini protease inhibitor cocktail tablets, bovine serum albumin (BSA), tris-base, and high-purity standards of echinacoside (purity 100 %), chicoric acid (purity 100 %), caftaric acid (purity > 97 %), caffeic acid (purity 100 %), chlorogenic acid (purity 100 %), and cynarin (purity > 98 %) were obtained from Sigma-Aldrich. An echinacea isobutylamide standards kit, composed of undeca-2E/Z-ene-8,10-diynoic acid isobutylamide (purity > 99 %), dodeca-2E-ene-8,10-diynoic acid isobutylamide (purity 100 %), and dodeca-2E,4E-dienoic acid isobutylamide (purity > 93 %), was acquired from ChromaDex. High-purity standard dodeca-2E,4E,8Z,10E/Z-tetraenoic acid isobutylamide (purity > 99 %) was obtained from Biosynth Carbosynth. A PGE2 ELISA Kit and rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were acquired from Abcam. IRDye 800CW Goat anti-Rabbit IgG and IRDye 680RD Goat anti-Rabbit IgG secondary antibodies were obtained from LI-COR Biosciences. Monoclonal rabbit

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NF-κB p65, monoclonal rabbit p44/42 MAPK (ERK 1/2), monoclonal rabbit p38 MAPK, monoclonal rabbit COX-2, monoclonal rabbit phospho-NF-κB p65, monoclonal rabbit phospho-p38 MAPK, and monoclonal rabbit phospho-p44/42 MAPK (ERK 1/2) were purchased from Cell Signaling. Sodium chloride (NaCl) was purchased from PanReac AppliChem. Coffee filter paper N4 was acquired in a local supermarket.

Bioactive compounds extraction

The plant material was ground immediately before extraction. Dried flowers (F) and leaves (L) were ground using a blender (Picadora Clássica 123 A320R1, Moulinex), while dried roots (R) were ground using an Analytical Sieve Shaker (AS200 Digit, Retsch, Germany). The AEs were prepared by stirring 20 g of sample in 150 mL of ultra-pure water at RT for 24 h, as previously described by Vieira et al. [33]. The water was changed after 12 h of the extraction process. After extraction, the AE was filtrated using a coffee filter paper N4. Both solutions were mixed, frozen at − 80 °C and then freeze-dried (Lyoquest − 85 °C Plus Eco, Telstar). The lyophilized extracts were stored at − 80 °C until further use. The AE tested negative for endotoxin contamination by the limulus amebocyte lysate (Pierce Chromogenic Endotoxin Quant kit).

Fractionation of the AE

The dry powder of AE was dissolved in ultra-pure water (30 mg/mL) and centrifuged at 10 000 × g for 5 min (ScanSpeed Mini, Labogene). The collected supernatants were injected (200–400 µL) in the LaChrom Merck Hitachi system equipped with a D-7000 Interface, an L-7100 Pump, an L-7200 autosampler, an L-7455 diode array detector (DAD), and an HPLC System Manager HSMD-7000, version 3.0. The fractionation method previously optimized by our research group was employed to fractionate AE [42]. Briefly, the chromatographic separation was performed on a Uptisphere WOD homemade semi-preparative column (250 mm × 10 mm, 5 µm, interchrom, Interchim, Montluçon, France), employing a gradient elution composed of 0.1 % formic acid and ACN. The flow rate was 2 mL/min. Two fractions, phenolic/carboxylic acid (F1, 2–11 min) and alkylamide (F2, 11–20 min) fractions, were obtained through the eluent collection. The organic solvent was evaporated in a rotavapor (R210 Buchi, Switzerland). Then, the fractions were freeze-dried (LyoQuest Plus Eco, Telstar) to remove the water content. The powder obtained was stored at − 80 °C until further use.

Chemical characterization of the AE

The chemical characterization of the AE was performed according to the liquid chromatography–high-resolution mass spectrometry (LC-HRMS) analysis method described by us [33]. AEs were dissolved in ultra-pure water at 5.0 mg/mL. The AEs were centrifuged (10 000 × g, 5 min), and the supernatant was collected and injected into the UltiMate 3000 Dionex ultra-high-performance liquid chromatography (UHPLC, Thermo Scientific, Lisbon, Portugal), coupled to an ultra-high-resolution quadrupole-quadrupole time-of-flight (UHR-QqTOF) mass spectrometer (Impact II, Bruker). Bruker Compass DataAnalysis 5.1 software (Bruker) was used to process the LC-HRMS-acquired data. The identification of the chemical compounds present in AE was confirmed by the matching of the retention time (t_R, min), mass-to-charge ratio (m/z) of the molecular ion, and MS/MS fragmentation patterns with the standards. When the t_R and MS data did not match with the available standards, the potential identity of the compound was assigned by comparing the MS/MS spectra with the theoretical data MS/MS fragments and data in the literature [33].

Preparation of AE and fractions solutions

Stock solutions of AE (5.1 mg/mL, F, L and R) and phenolic/carboxylic acid fractions (60.0 mg/mL) were prepared in ultra-pure water and sterilized with a 0.22 µm filter. The alkylamine fraction prepared from the AE was not biologically studied, since no measurable amounts were obtained.

Aliquots of the stock solutions of each extract and phenolic/carboxylic acid fractions were prepared and stored at − 80 °C. Then, serial dilutions were made with complete RPMI-1640 culture medium with 2 mM glutamine supplemented with 10 % human serum, 1 % penicillin/streptomycin, and 1 % HEPES (cRPMI). Different final concentrations of AE (50, 125, and 250 µg/mL) and phenolic/carboxylic fractions (125 and 250 µg/mL) were tested in the biological studies.

Biological studies

Ethics statement

The collection of peripheral blood from healthy volunteers at Hospital of Braga, Portugal, was approved on the 14th of December, 2018, by the Ethics Subcommittee for Life and Health Sciences (SECVS) of the University of Minho, Portugal (No. 014/015). The principles expressed in the Declaration of Helsinki were followed, and participants provided signed informed consent.

Isolation and differentiation of monocytes

Monocytes were isolated from buffy coats, as previously described by Gonçalves et al. [43]. Briefly, peripheral blood mononuclear cells (PBMCs) were subjected to a density gradient centrifugation using a Histopaque-1077 solution. The PBMC ring was carefully collected and washed twice with PBS. Then, the monocytes were isolated from the PBMCs using positive magnetic beads separation with CD14 microbeads, according to the instructions of the manufacturer. Isolated monocytes were resuspended in cRPMI. The monocytes (5×10^5 cells/well in adherent 24-well plates) were seeded in the presence of 20 ng/mL of recombinant human GM-CSF and incubated at 37 °C in a humidified atmosphere at 5 % CO_2 for 7 days. The culture medium was replaced every 3 days, and the acquisition of macrophage morphology was confirmed by visualization under an inverted microscope (Axiovert 40, Zeiss).

Pro-inflammatory activity evaluation

The hMDMs were incubated with AEs and their phenolic/carboxylic acid fractions at different concentrations for 24 h. Afterward, the culture medium was harvested (the triplicates were mixed and homogenized) and stored aliquoted at − 80 °C until cytokines quantification. The cells were washed with warm sterile DPBS, and the metabolic activity and DNA quantification were determined as described in the following section. The hMDMs cultured without treatment (only with culture medium) and stimulated

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with 100 ng/mL of LPS for 24 h were used as negative and positive controls in the production of the pro-inflammatory mediators, respectively [42].

Metabolic activity and DNA quantification

The metabolic activity and DNA concentration of hMDMs incubated with AEs and their phenolic/carboxylic acid fractions were determined using an alamarBlue assay and a fluorimetric dsDNA quantification kit, respectively, as previously described by Vieira et al. [44]. The hMDMs cultured without treatment (only with culture medium) and treated with 10 % DMSO were used as a negative and positive control, respectively, to access cytotoxicity. The results of metabolic activity were expressed in percentages related to the negative control. DNA contents were expressed in relative concentrations of the negative control.

Cytokine and eicosanoid quantification

The amounts of IL-1β, IL-6, TNF-α, and PGE2 were assayed using ELISA kits, according to the instructions of the manufacturer. The obtained values were normalized by the respective DNA concentrations. The results were expressed as fold change relative to the non-stimulated hMDM condition (negative control).

Western blot analysis

To determine the expression of inflammatory proteins and phosphorylation of the signaling pathways involving the activation of macrophages, Western blot analysis was performed. The hMDMs (5×10^5 cells/well in 24-well plates) were cultured with AE for 24 h, at 37 °C and 5 % CO_2. After that period of time, the medium was removed, and the cells were washed with ice DPBS. Then, the cells were lysed in RIPA buffer containing a mixture of protease and phosphatase inhibitors, at 4 °C for 30 min, under shaking. Then, samples were collected into an Eppendorf tube and centrifuged (2000 rpm, 20 min). The supernatant was used to determine the protein content using the Bio-Rad Protein Assay. Bolt sample-reducing agent and bolt LDS sample buffer were added to 30–40 µg of protein. Then, the samples were heated and denatured at 70 °C (20 min) and 95 °C (5 min). The centrifuged samples (13 000 × g, 1 min) were loaded and separated on an 8 % precast polyacrylamide gel set on a Mini Gel Tank (Invitrogen, ThermoFisher Scientific). The proteins were transferred from the gel to a PVDF membrane using the iBlot 2 Gel Transfer Device (Invitrogen, ThermoFisher Scientific). After blocking for 30 min at RT with 5 % BSA in tris-buffered saline with Tween20 (TBST), the membranes were incubated overnight at 4 °C with the following primary antibodies diluted in blocking solution: NF-κB p65 (1 : 1000), p44/42 MAPK (ERK 1/2) (1 : 1000), p38 MAPK (1 : 1000), COX-2 (1 : 500), phospho-NF-κB p65 (1 : 1000), phospho-p38 MAPK (1 : 1000), phospho-p44/42 MAPK (ERK 1/2) (1 : 1000), and GAPDH (1 : 10 000). The membranes were washed three times for 5 min with TBST, and then, IRDye 800CW Goat anti-Rabbit IgG or IRDye 680RD Goat anti-Mouse IgG secondary antibodies, both diluted in TBST (1 : 15,000), were incubated for 1 h at RT in the dark. The Odyssey Fc Imaging System (LI-COR Inc., 2800, Nebraska, USA) was used for image acquisition of the Western blots using a near-infrared method set to 700 or 800 nm. The intensity of the bands was quantified with Image Studio Lite software (LI-COR, Inc., Version 5.2.5). The data were normalized to the housekeeping GAPDH. The phenolic/carboxylic acid fractions obtained from AE were not further studied, since no significant immunostimulatory activity was observed.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) of three independent experiments, with a minimum of three replicates for each condition. Statistical analyses were performed using GraphPad Prism 8.0.1 software. Two-way analysis of variance (ANOVA) and Dunnett's multiple comparison test or Sidak's multiple comparisons test were used for cell assays. Differences between experimental groups were considered significant with a confidence interval of 99 %, whenever $p < 0.01$.

Contributors' Statement

Sara F. Vieira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Resources, Validation, Visualization, Writing – original draft. Samuel M. Gonçalves: Methodology, Validation, Visualization, Writing – review & editing. Virgínia M. F. Gonçalves: Methodology, Validation, Visualization, Writing – review & editing. Maria E. Tiritan: Methodology, Validation, Visualization, Writing – review & editing. Cristina Cunha: Methodology, Validation, Visualization, Writing – review & editing. Agostinho Carvalho: Methodology, Validation, Visualization, Writing – review & editing. Rui L. Reis: Funding acquisition; Project administration; Resources; Validation, Visualization, Writing – review & editing. Helena Ferreira: Conceptualization, Methodology, Validation, Supervision, Visualization, Writing – review & editing. Nuno M. Neves: Conceptualization, Funding acquisition; Methodology, Project administration; Resources; Validation, Supervision, Visualization, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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