Inhibition of pro-inflammatory cytokines and inducible nitric oxide by extract of Emilia sonchifolia L. aerial parts
Introduction

Medicinal plant extracts have been used successfully in traditional medicine to treat inflammatory conditions and other diseases with inflammatory components. Most often, the mechanisms of the anti-inflammatory activities of these extracts are hardly known or poorly understood. The inflammatory response itself is a complex cascade of steps that include an activation of white blood cells, the release of complements, and the production and release of pro-inflammatory mediators. Suppression of aberrant production of some of these pro-inflammatory mediators, such as nitric oxide (NO), prostaglandins, interleukin-1 (IL-1), IL-6, TNF-α, and/or interferon(1) is important to the actions of many conventional treatments of inflammation.

In the present study, we investigated the mechanisms of the anti-inflammatory activities of the leaf extract of *Emilia sonchifolia* (ES) L. DC. (Asteraceae). ES also known as lilac tasselflower is a small herbaceous plant found predominantly in the grassland regions of West Africa. The plant is used in traditional medicine to treat inflammation, sores, and convulsion.(2) In Cameroon, fresh juice squeezed from the leaves is believed to promote wound healing and is commonly applied to fresh wounds. The leaf-sap is also applied to cuts and wounds. Also the traditional medical literature describes...
its potential role as a domestic remedy for the relief of sore eyes and ears, night blindness and inflammation of the eyes.(3) The cytotoxic and antitumor properties of ES were reported by Shylesh and Padikkala (2000)4. ES is commonly used by the indigenous tribal population of Kerala in the Western Ghats region of India against inflammation, insect bites, conjunctivitis, rheumatism, and for cuts and wounds.(5) The plant is edible and is commonly used as a salad plant in South East Asia. The aerial part of this plant has been reported to contain alkaloids(6) and flavonoids (7) which are believed to be responsible for the biological activities. Anti-inflammatory and antinociceptive activities of ES extracts have also been reported in a preliminary study,8-10 but the mechanism through which these extracts produced these activities is not yet known.

Lipopolysaccharide (LPS) is a potent inducer of inflammation in monocytes cell lines and systemically in whole animal models. It can promote the secretion of many pro-inflammatory cytokines and mediators. Macrophages are a major cell population in the innate immune system. They play an important role in mounting inflammatory responses, by secreting a number of cytokines and mediators.(11) Activated macrophages produce potent pro-inflammatory cytokines such as NO, tumour necrosis factor α (TNFα), and IL-1β which though are beneficial to the host defence, yet can also trigger pathological conditions when expressed in excess.(12) Unregulated levels of these cytokines have been implicated as a pathogenic factor in the development of conditions associated with several chronic inflammatory diseases. In order to understand how the extract of ES produce its effects against inflammation, we investigated the effect of a methanol/methylene chloride extract of ES on the systemic levels of IL-1β and TNF-α in mice after an intraperitoneal LPS challenge. The effects of pre-treatment with ES on LPS-provoked release of interleukin (IL-1β), tumour necrosis factor (TNF)-α, and NO by culture of bone marrow-derived macrophages (BMDM) were also investigated. This study will be relevant in understanding the anti-inflammatory activities of ES and will contribute towards harnessing its potential as alternative anti-inflammatory therapy.

Materials and methods

Collection, extraction, and preparation of plant material

Fresh aerial parts of ES were collected and authenticated with the assistance of a plant taxonomist, Mr Alfred O. Ozioko, of the Bioresources Development and Conservation Programme Centre, Nsukka, Nigeria. The collected aerial parts were washed, air-dried, and pulverized. A portion of the powdered aerial parts (200g) was exhaustively extracted by maceration in methanol/methylene chloride mixture (1:1) for 48h. The extract solution was then filtered through Whatman No. 1 filter paper and concentrated by evaporation in vacuo to obtain 31.2 g (15.6% w/w recovery) of the extract (ES). The level of endotoxin was determined by a Limulus Amoebocytes Lysate kit (Endosafe®; Charles River, Sulzfeld, Germany) on a 1 mg ES/mL solution; the result showed that there was no gel clot formed and thus the level of any endotoxin present in the extract was below the detection level of the kit (0.03 EU). The extract was suspended in 3% Tween 80 for the in vivo studies and was solubilized in 5% solution of cell culture grade of dimethyl sulphoxide (DMSO) for the in vitro experiments. These vehicles were also used as control treatments in respective experiments.

Phytochemistry and HPLC identification and analysis of major constituents

Preliminary phytochemical tests were carried out on ES using standard procedures previously described.(13,14) A solution of 1 mg ES/mL was prepared in HPLC grade methanol. The solution was centrifuged and HPLC analysis was carried out on the supernatant with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235 nm. The separation column (125 x 4 mm; length x internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The various constituents present in ES and their relative abundance were determined by infer-ences based on High Performance Liquid Chromatography (HPLC) retention time, UV absorption spectra and a comparison of analytical data with a library of standards.

Animals

BALB/c mice (weighing 20–25 g) obtained from Centre d’Elevage (Janvier, France) were used for the study. The animals were housed in institutional facilities under standard conditions (25 ± 2°C and a 12-h light/ dark cycle) and maintained on standard pellets and drinking water ad libitum. The use and care of laboratory animals in the study were in accordance with ethical guidelines as contained in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (EEC Directive 86/609/EEC) of 1986.

Effects of ES on serum levels of IL-1β and TNF-α induced by LPS in mice

BALB/c mice were randomized into four groups (n = 5) and treated for three consecutive days with ES (100 and 250 mg/kg, i.p.) or vehicle. On the third day, 2 h after the ES treatment, the mice were treated with LPS (1 mg/kg; i.p.) (Escherichia coli, serotype 0128:B12); Sigma-Aldrich, Munich, Germany) or phosphate-buffered saline (PBS) in a 100-μL volume. Group treatments were as follows: Group 1 were naïve mice that were maintained only on feed and water throughout the experiment; Group 2 mice received no ES and were treated with LPS on Day 3; Groups 3 and 4 received daily i.p. administration of 100 and 250 mg ES/kg treatments, respectively, followed by LPS on Day 3 two hours after the administration of the
extracts. The animals were bled from the retro-orbital plexus 2h after the LPS (or PBS) treatment and the subsequently isolated sera were stored at −20°C until analyzed. Concentrations of IL-1β and TNF-α in the sera were determined with a cytokine ELISA kit (PeproTech, Hamburg, Germany).

Preparation and culture of BMDM
Murine BMDM were generated from the bone marrow cells of the tibia, humerus, and femur of BALB/c donor mice by a modification of the methods previously described.(15,16) BM cells were harvested and cultured in DC-medium containing RPMI 1640 medium (Gibco, Germany) supplemented with 5% heat-inactivated foetal calf serum (FCS), 50 μM 2-mercaptoethanol (Gibco, Germany), 1% L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 ng/mL of recombinant murine colony-stimulating factor (rmCSF-1; Immunotools, Friesoythe, Germany) in T-75 culture flasks. The cells were incubated at 37°C and 5% CO2 for 24 h to adhere and remove stromal cells and mature BM resident macrophages. Non-adherent cells, which are mainly progenitors, were recovered after 24 h of incubation and further incubated in cell culture flasks to expand and differentiate the cells under the influence of the rmCSF-1. After 7 days of culture, non-adherent cells were removed and the adherent cells were washed and harvested using a cell scraper. The viability of the generated macrophages was assessed by trypan blue exclusion. The BMDMs generated were plated and used for the in vitro studies.

Estimation of the viability of ES-treated BMDM by MTT assay
The viability of the BMDM after treatment with ES extract was determined using cellular respiration as an indicator. Cell viability was determined on the basis of mitochondrial dependent reduction of MTT to formazan.(17) BMDM were cultivated in 96-well plates (1 x 10^5 cells/well) for 24h. The cells were then treated with various concentrations (25, 100, 250, and 500 μg/mL) of ES in a fixed volume of 100 μL. After 24h of incubation at 37°C, the medium in each well was discarded; the cells were then incubated with fresh medium containing 5mg MTT/mL for 4h. The formazan blue that formed in the cells was then dissolved by addition of 150-μL DMSO to each well and the optical density of the solution in the well was measured at 550 nm in a multiwell microtiter plate reader (Tecan, Grödig, Austria).

Effect of ES treatment on pro-inflammatory cytokines expression by BMDM
BMDMs (5 x 10^5 cells/well) was cultured in 48-well plates for 24h. The cells were then pre-treated with ES (20, and 100 μg/mL) or with 100 μM Pyrrolidine dithiocarbamate, PDTC (Sigma-Aldrich, Munich, Germany) for 2h. After this period of pre-incubation, the cells were treated with 5 μg LPS/mL or vehicle. After 24-h incubation at 37°C, the cell-free medium in each well was collected and the concentrations of IL-1β and TNF-α in the culture supernatant were determined using commercially available ELISA kits, according to the manufacturer’s instructions (PeproTech, Germany).

Effect of ES treatment on inducible nitric oxide expression by BMDM
BMDM were cultivated in 48-well plates (5 x 10^5 cells/well) at 37°C in a 5% CO2 incubator for 24h. Thereafter, the cells were pre-treated with ES (20 and 100 μg/mL) or with PDTC (100 μM) for 2h. After pre-incubation, the cells were then treated with 5 μg/mL of LPS or vehicle (PBS) only. Supernatants were collected after 24 h of incubation and stored at −80°C. Nitrite levels in aliquots of each supernatant were measured in 96-well microtiters plates by mixing 100 μL of cell-free culture supernatant with an equal volume of Griess reagent (Applichem, Darmstadt, Germany) and then incubated at room temperature for 10 min. The Griess reagent contains equal volumes of 0.1% naphthylethlenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid. The NO concentration was determined at 550 nm in a multiwell microtiter plate reader (Tecan, Grödig, Austria) by extrapolation from a standard curve generated using NaNO2 standards that had been included in each measurement plate.

Statistical analysis
Results are presented as mean and standard error of the mean (SEM) of at least triplicate determinations for in vitro experiments and group size of n = 5 for in vivo experiments. To demonstrate statistical significance of data, a One-way Analysis of Variance (ANOVA) using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was performed. Generally, differences between test and control treatments or between any paired treatments of groups were assigned significance at p < 0.05.

Results
Phytochemical studies
Preliminary phytochemical analysis showed the presence of flavonoids, saponins, alkaloids, and terpenes in ES. HPLC ‘fingerprinting’ and UV analysis identified eleven major peaks representing eleven compounds in the extract (ES) – alkaloids (11.09%); Quercetin O- and C-glycosides derivatives (10.83%); Quecetrin-(15.94%); Kaemferol C-glycoside derivatives-(4.25%); Chlorophyll-(11.73%); Carrotenoid derivatives (10.64%); triterpenoids-(1.54%); and phenolic acids-caffeic acid derivatives (33.88%) (Table 1).

Inhibition of serum levels of IL-1β and TNF-α induced by LPS by ES extract in mice
Compared to untreated mice, the sera of mice treated with LPS after 2h showed a very significant induction of IL-1β and TNF-α (p < 0.001). The mean serum levels
of IL-1β induced by LPS were significantly (p < 0.05) reduced in groups of mice pre-treated with ES. Mean sera concentrations of IL-1β in ES (100 and 250 mg/kg) treated groups were reduced from 143 ± 0.05 pg/mL to 113 ± 4.3 pg/mL and to 90 ± 4.4 pg/mL which represent 20.70% and 37.07% inhibition, respectively (Figure 1a). Similarly, the mean sera concentrations of TNF-α in ES (100 and 250 mg/kg) treated groups were reduced from 143 ± 0.05 pg/mL to 113 ± 4.3 pg/ml and 90 ± 4.4 pg/mL which represent 46.88% and 64.58% inhibition, respectively (Figure 1b).

**Viability of BMDM treated with ES**

The toxicity of ES on BMDM was determined using a modification of the MTT cytotoxicity assay. The result of MTT assay showed that treatment of BMDM with 25–250 μg ES/mL did not significantly (p > 0.05) affect the viability of BMDM. The viability of BMDM was only significantly reduced (p < 0.05) by 31.67% in micro-wells treated with 500 μg ES/mL compared to the untreated cells.

**Inhibition of LPS-induced IL-1β and TNF-α production in culture of BMDM treated with ES**

High levels of IL-1β and TNF-α were measured in culture supernatant following treatment of the BMDM with 5 μg LPS/mL alone; however, pre-treatment of the cells with ES (20 and 100 μg/mL) and PDTC (100 μM) significantly (p < 0.05) inhibited IL-1β and TNF-α levels in a concentration-related manner (Figure 2a and 2b) when compared to the LPS alone treated cells. Pre-treatment with 20 μg ES/mL resulted in a 33.0 and 47.08% mitigation of the LPS-induced IL-1β and TNF-α release. Pre-treatments of BMDM culture with the 100 μg ES/mL led to inhibitions of LPS-induced IL-1β and TNF-α by 41.63 and 53.85%, respectively. PDTC (100 μM), used as a standard comparator agent, similarly reduced LPS-induced IL-1β and TNF-α by as much as 78.42 and 81.82%, respectively when compared to cells treated with LPS alone.

**Inhibition of LPS-induced NO) release in culture of BMDM treated with ES**

Release of inducible nitric oxide (iNO) by macrophages in the culture medium was determined indirectly by measuring the concentration of accumulated nitrite (a stable product of NO conversion) by the Griess reaction. As illustrated in Figure 3, the levels of NO in LPS stimulated control cell cultures were significantly higher than those in normal unstimulated control cells. Similar to what occurred with respect to IL-1β and TNF-α expressions, LPS-stimulated NO production was inhibited in a concentration-related manner when the BMDM were pre-treated with 20 and 100 μg ES/mL and these inhibitions were found to be significant (p < 0.05). Pre-treatment of BMDM with 20 μg ES/mL resulted in a 46.69% mitigation of the LPS-induced response and treatment with 100 μg ES/mL led to a reduction in formation of iNO by 56.45% compared to cells treated with LPS alone. Similarly, PDTC also suppressed the expression of LPS induced nitric oxide (iNO) by 76.51%.

**Table 1. Major phytochemical constituents of *Emilia sonchifolia* extract (ES).**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Relative peak area (%)</th>
<th>Class of secondary metabolite</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.53</td>
<td>11.09</td>
<td>Alkaloid</td>
</tr>
<tr>
<td>2</td>
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<td>6.77</td>
<td>Caffeic acid derivative</td>
</tr>
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<tr>
<td>5</td>
<td>19.96</td>
<td>9.36</td>
<td>Quercetin C-glycoside derivate</td>
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<tr>
<td>6</td>
<td>21.23</td>
<td>15.94</td>
<td>Quercetin</td>
</tr>
<tr>
<td>7</td>
<td>25.06</td>
<td>1.47</td>
<td>Quercetin O-glycoside derivate</td>
</tr>
<tr>
<td>8</td>
<td>29.37</td>
<td>1.54</td>
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<tr>
<td>11</td>
<td>39.38</td>
<td>2.56</td>
<td>Carrotinoid derivative</td>
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**Figure 1.** Effect of ES on LPS-induced IL-1β release *in vivo*. Mice were treated daily for three consecutive days with ES (100 or 250 mg/kg, i.p.). On day 3, 2h after administration of ES, the mice were administered 1 mg LPS/kg (100 μL; i.p.). Blood sample was obtained from each mouse 2h after the LPS treatment and the serum analyzed for IL-1β (Figure 1a) and TNF-α (Figure 1b) by ELISA. Values shown are the mean (±SEM) from n = 8/treatment group. *p < 0.05. LPS, lipopolysaccharide; ES, *Emilia sonchifolia* extract; IL-1β, interleukine-1β.
Discussion

ES is used in ethnomedicine for the treatment of a wide array of inflammatory disorders and this practice has also been supported by scientific reports which showed that extracts of ES possess anti-inflammatory and antinociceptive effects in rodents.6–8 However, the mechanism(s) through which the extracts of this plant produce these effects is not known. The effects of the extract on LPS-induced escalation of IL-1β, TNF-α, and NO were studied using both in vivo and in vitro models. Unregulated levels of pro-inflammatory cytokines has been implicated as a potential aetiological factor in the development of several chronic inflammatory diseases, including type-2 diabetes, cardiovascular diseases, inflammatory bowel diseases, rheumatoid arthritis, major depression, and even normal aging.8–10 Treatments for most inflammatory disorders often result in reducing or suppressing the release of these pro-inflammatory mediators.1

In this study, pre-treatment of mice with extract of ES (100 and 250 mg/kg) resulted in decreased mean serum levels of TNF-α and IL-1β. TNF-α is a member of the pro-inflammatory cytokines family and increased plasma TNFα levels during sepsis contribute to lethality in the host. It has been found that host treated with TNF-α-neutralizing antibodies yields significant protective effects during an episode of acute sepsis that is usually associated with the release of several pro-inflammatory mediators.11 LPS is a potent inducer of inflammation in monocytes cell lines such as macrophages and systemically in whole animal models; it can induce the secretion of many pro-inflammatory cytokines and mediators. As such, the inflammation model stimulated by LPS is frequently used to evaluate the effect of pharmacological treatment on the inflammatory response.12,13

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the macrophages. Treating the BMDM with concentration up to 250 µg ES/mL did not significantly (p > 0.05) affect the viability of the cells in the MTT assay. The inhibition of iNO release by activated macrophages by the extract of ES is also an important mechanism in its anti-inflammatory effect. NO is produced by NO synthase (NOS) (26); after exposure to LPS, inducible NOS (iNOS) is induced quantitatively. (27) The role of NO in host defence against microorganisms and tumour cells is well-recognized. (28–30) Nevertheless, excess production of NO is also associated with several diseases, e.g. arthritis, autoimmune diseases, septic shock, as well as in several chronic inflammatory diseases. In these disorders NO is known to contribute to the inflammation cascade by increasing vascular permeability and extravasations of fluid and proteins at inflammatory sites. (31–33) As such, the inhibition of high-output NO production has been a therapeutic strategy increasingly used for the treatment of various inflammatory diseases. In this study, we demonstrated that the plant extract, ES significantly inhibited NO production in LPS-stimulated macrophages. Pre-treatment of macrophage culture with Pyrrolidine dithiocarbamate (PDTC) caused an inhibition of the release of pro-inflammatory mediators release. PDTC is a thiol compound widely used to study the activation of redox-sensitive transcription factors such as NF-κB (34,35).

Macrophages are versatile cells that play many important roles in host innate and adaptive defence mechanisms. As scavengers, they rid the body of worn-out cells and other debris. Together with dendritic cells and B cells, they are involved in ‘antigen presentation,’ a crucial role in initiating adaptive immune response. As secretory cells, macrophages are vital to the regulation of immune responses and the development of inflammation. When macrophages are activated; they inhibit the growth of a wide variety of tumour cells and pathogenic organisms. (36,37) They are activated through some pathogen associated molecular patterns (PAMPs) receptors otherwise known as the Toll-like receptors (TLRs) on their surface. The activation of TLRs triggers a complex cellular response that activates multiple intracellular signalling pathways (38–40) and the control of the activation of these pathways in macrophages and other monocyte-derived cells is critical. Excessive activation can lead to chronic inflammatory disorders, whereas insufficient activation can render the host susceptible to infection. For example, TLR4 recognizes and binds to LPS, a gram-negative bacterial component, to trigger the myeloid differentiation primary-response protein 88 (MyD88)-dependent signalling pathway and/or the MyD88-independent signalling pathway leading to NF-κB activation. (41,42) TLR-induced activation of these intracellular signal transduction cascades result in the production of inflammatory molecules which are critical components of the host innate defence system. While TLR-mediated inflammatory responses are important for controlling infections, overwhelming activation of TLR signalling is deleterious and can cause severe inflammatory disease. Thus, the activation of TLRs should be tightly regulated in vivo. Various anti-inflammatory agents and mechanisms are employed to regulate TLR triggered inflammatory immune responses.

Phytochemical studies showed the presence of eleven major compounds in large amounts in the extract of ES alkaloids, caffeic acid derivatives, flavonoids (quercetin and kaemferol C- and O- glycosides), and triterpenoids. Although it is difficult to speculate on the specific contribution of these bioactive compounds to these effects of ES, some of these phytoconstituents have been reported in previous studies to suppress the production and release of pro-inflammatory mediators by phagocytic cells. (43,44) Consistent with earlier reports, quercetin, other quercetin type flavonoids and other polyphenolics were found to be present in large amounts in the extract. (45) The suppression of inflammatory mediators and cytokines by different plant flavonoids, especially the quercetine and quercetin types, and other polyphenolic compounds such as the caffeic acid derivatives found in ES, have been extensively reported. (1,47–49) These studies showed that these compounds inhibited the expression of various pro-inflammatory cytokines and inflammation-related proteins/enzymes, at least partly, by suppressing the activation of transcription factors such as NF-κB and AP-1. (46) These suppressions might be mediated via inhibition of several protein kinases involved in the signal transduction pathway.

Conclusion

This study showed that the extract of ES traditionally used to treat inflammation could be explored as a valuable source of new and potent anti-inflammatory compounds. The extract may also have therapeutic potential for the modulation and regulation of macrophage activation and may provide safe and effective treatment options for a variety of inflammatory disorders. The results of these studies suggest that treatment with ES alleviated inflammatory responses possibly through a suppression of pro-inflammatory mediators and cytokines such as IL-1β, TNF-α, and iNO.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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