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RESEARCH ARTICLE

Extracts of *Ficus exasperata* leaf inhibit topical and systemic inflammation in rodents and suppress LPS-induced expression of mediators of inflammation in macrophages

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Abstract

The leaves of *Ficus exasperata* are mashed and prepared as poultices that are placed on swellings, wounds, and arthritic joints to relieve swelling and pains by the Igede tribal community of Nigeria. The leaf and stalk are also squeezed and used to mitigate itching or inflammation. These claimed benefits inspired this study in which topical and systemic (acute, chronic) anti-inflammatory activities of a methanol/methylene chloride leaf extract of *F. exasperata* (MFE) were assessed in rodents. Effects of an aqueous leaf extract (AFE) on lipopolysaccharide-induced expression of interleukin-1β (IL-1β), tumor necrosis factor (TNF)-α, and inducible nitric oxide (iNO) were also investigated in murine bone marrow-derived macrophage (BMDM) cultures. Treatment of rats with MFE (200 and 400 mg/kg) led to significant inhibition of acute and chronic inflammation induced by, respectively, agar and formaldehyde in the paws. Topically, pre-application of mice with MFE (5 µg/ear) also significantly inhibited (by up to 21%) ear edema induced by xylene. *In vitro*, pre-treatment of BMDM with 5–100 µg AFE/ml significantly inhibited IL-1β, TNFα, and iNO production in a dose-related manner. BMDM viability was not significantly affected AFE at concentrations up to 200 µg/ml. Initial studies showed that flavonoids, alkaloids, and terpenoids were the predominant phytoconstituents in each extract. In conclusion, the results of the various investigations indicated that *F. exasperata* leaf extracts possess anti-inflammatory properties that could underlie the benefits associated with the folklore use of the plant. The results also show that the extracts may be acting through a suppression of mediators of inflammation, such as IL-1β, TNFα, and iNO.

Keywords: Arthritis, chronic inflammation, Ficus exasperata, inflammatory mediators, macrophages, paw edema

Introduction

Inflammation is a complex and dynamic biological response that is elicited in response to harmful stimuli, such as pathogens, mechanical injuries, burns, irritants, and other noxious stimuli that may threaten the well-being of the host. Inflammation is marked by local response to cellular injury that is associated with capillary dilatation, leucocytes infiltration, redness, heat, pain, swelling, and often loss of function that serves to initiate mechanisms of eliminating the noxious agents and damaged tissues. It involves a complex array of enzyme activation, inflammatory mediators, fluid extravasation, cell migration, tissue breakdown, and repair (Vane and Bolting, 1995; Perianayagam et al., 2006).

Many human and animal diseases, such as arthritic disorders, lupus erythematosus, asthma, bronchitis, inflammatory bowel disease, ulcerative colitis, pancreatitis, hepatitis, cancer, and infections possess an inflammatory component. Conventional drug treatments are limited in their effectiveness in managing the incidence/outcome of many inflammatory diseases. They also present a significant number of side-effects.

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Recently, it has been shown that non-steroidal anti-inflammatory agents may even slow down the healing process in many diseases (Ayoola et al., 2009). Despite progress made in use of orthodox medicines for treatment of inflammatory states, there are still needs for effective, affordable, improved remedies devoid of gastro-erosive side-effects or other unwanted effects associated with steroidal anti-inflammatory therapy. In this regards, medicinal plants and herbal remedies have been employed in Complementary and Alternative Medicine (CAM) for the treatment of inflammation and disorders having inflammatory components. The treatment of inflammation and rheumatic disorder is an area in which the practitioners of traditional medicine enjoy patronage and success (Akah and Nwambie, 1994). Taking into account that many important anti-inflammatory prototypes (e.g., salicylates) were originally derived from plants, the study of plant species traditionally used as anti-inflammatory agents is still seen as a fruitful strategy in the search of new anti-inflammatory drugs.

The plant *Ficus exasperata* (*Moracea*) is a medicinal plant used in folk medicine to treat inflammation/inflammatory disorders by the Igede people of Benue state, Nigeria (Igoli et al., 2005). Use of this plant has produced claims of effectiveness in resolving topical inflammatory events and systemic rheumatic disorders. The leaves of *F. exasperata* are mashed and prepared as poultices that are placed on swellings, wounds, and arthritic joints to relieve swelling; the leaf and stalk are used to mitigate itching/inflammation as well. The use of *F. exasperate* leaves and roots in the treatment of high blood pressure, asthma, dyspnea, rheumatism, arthritis, intestinal pain, colic, epilepsy, bleeding, and wounds in African traditional medicine was well documented over a 40-year period (see Irvine, 1961 up through Chhabra et al., 1990). Several studies have validated some of these traditional uses in model systems. For example, Akah et al. (1997, 1998) reported significant anti-ulcer and anti-motility effects of a methanol *F. exasperata* leaf extract in rats. Ayinde et al. (2007) demonstrated that the aqueous leaf extract of *F. exasperata* caused a dose-related reduction in mean arterial blood pressure; this effect was also blocked by anti-histamine and -muscarinic agents. Recently, it was reported that the hydroalcoholic leaf extract of *F. exasperata* displayed anti-nociceptive and weak anti-pyretic properties and inhibited carrageenan-induced footpad edema in chicks (Woode et al., 2009). In another related study, the ethanol extract of these leaves was shown to possess anti-oxidant and anti-arthritic properties (Abotsi et al., 2010).

To establish a scientific basis (if any) for these traditional practices and to understand possible mechanism(s) by which these extracts may act, a study was designed to investigate the topical and systemic (acute and chronic) anti-inflammatory properties of *F. exasperata* leaf extracts in rodent models. Effects of the extracts on inducible expression of mediators of inflammation by murine macrophages were also assessed.

Materials and methods

Collection and extraction of plant material

Fresh leaves of *Ficus exasperata* were collected in March 2009 from Nsukka, Enugu State, Nigeria and authenticated by Mr Alfred Ozioko of the Bioresources Development and Conservation Program (BDCP) Centre, Nsukka. The leaves were cleaned, shade-dried, and pulverized to coarse powder using a laboratory-scale slow speed electric blender. A portion of the powdered aerial parts (2 kg) was exhaustively extracted with a 1:1 mixture of methylene chloride and methanol by continuous cold maceration for 48 h with intermittent agitation. The extract solution was then filtered through Whatman No. 1 filter paper and concentrated by evaporation *in vacuo* to obtain a dried extract (MFE; 3.14% [w/w] recovery). The aqueous leaf extract of *Ficus exasperata* (AFE) used for *in vitro* cell culture experiments was obtained by macerating 250 g of the leaf powder for 2 h in 400 ml warm sterile distilled water, with intermittent agitation. The extract was then filtered and lyophilized to yield a dried extract (AFE; 8.27% [w/w] recovery). Both extracts were aliquoted and stored at –20°C until used for experiments. The endotoxin level in a 1 mg/ml solution of AFE was determined using a Limulus Amoebocyte Lysate kit (LAL; Endosafe®; Charles River, Sulzfeld, Germany). The results showed that the level of any endotoxin present in the extract was below detection (< 0.03 EU).

Animals

Adult Wistar rats (150–250 g) and Swiss mice (15–25 g) of both sexes were obtained from the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka (UNN). Balb/c mice (20–25 g), obtained from Centre d'Elevage (Janvier, France), were used for the *in vitro* studies. The animals were housed in institutional facilities under standard conditions (25 ± 2°C and a 12-h light/dark cycle) and provided access to standard pellets and drinking water *ad libitum*. Use and care of the animals in this 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. Prior to use in the different experiments, the animals were allowed at least 5 days to acclimatize.

Preliminary phytochemical tests

Preliminary phytochemical tests were carried out on the extracts using standard procedures previously described (Evans, 1998; Harborne and Harborne, 1998). Phytochemical tests were conducted on MFE and AFE for the presence of flavonoids, alkaloids, sterols, triterpenoids, saponins, glycosides, and tannin.

Acute toxicity (LD_{50}) test

The acute toxicity (LD_{50}) of the methanol/methylene chloride extract of *F. exasperata* (MFE) was estimated in

mice by oral and intra-peritoneal routes of administration using the method described by Lorke (1983). Briefly, the tests involved two phases. The first involved the determination of the toxic range. The mice were placed in three groups (n = 3/group) and MFE (10, 100, or 1000 mg/kg) was administered either intraperitoneally (IP) or *per os*. The injection and gavage volumes were always 200 µl. The treated mice were then observed for 24 h for mortality. Mortality in the first phase determined the four different doses of MFE administered (IP or *per os*) in the second phase. After treatment, the animals were observed for lethality/signs of acute intoxication for the next 24 h. The LD_{50} was calculated as the geometric mean of the highest non-lethal dose and the least toxic dose.

Effect of MFE on acute inflammation in mouse ear induced by xylene

The effect of MFE on acute topical inflammation was evaluated using earlier described methods (Tubaro et al., 1985; Gad et al., 1987; Atta and Alkohafi, 1998). For each experiment, Swiss mice of either sex were divided into three groups (n = 8/group). The treatment groups received MFE extract (5 mg/ear; applied in 50 µl 2% Tween) applied to the anterior surface of the right ear. Topical inflammation was instantly induced on the posterior surface of the same ear by application of xylene (30 µl/ear). Negative and positive control mice received 2% Tween and indomethacin (5 mg/ear; applied in 50 µl 2% Tween), respectively. Two hours after induction of inflammation, mice were euthanized by ether anesthesia and both ears were removed. Circular sections (4 mm diameter) of both the right (treated) and left (untreated) ears were isolated using a cork borer and weighed. Edema was quantified as the weight difference between each plug. Anti-inflammatory activity was evaluated as per cent edema reduction/inhibition in the treated animals relative to the edema in control hosts (Tubaro et al., 1985; Asuzu et al., 1999) using the formula: % inhibition/reduction = $100 \times (1 - \{[R_t - L_t]/[R_c - L_c]\})$ where; R_t = treated animal right ear plug mean weight; L_t = treated animal left ear plug mean weight; R_c = control animal right ear plug mean weight; and L_c = control animal left ear plug mean weight.

Effect of MFE on agar-induced acute paw edema in rat

The effect of MFE on rat paw edema was assessed by the method of Winter et al. (1962). Acute inflammation was measured in terms of change in volume of the rat hind paw (Backhouse et al., 1996) induced by injection of agar. Rats were randomized into four groups of five animals each. The treatment groups received MFE (200 or 400 mg/kg; orally) while the negative and positive control groups were given equivalent volume of the vehicle (distilled water in 2% Tween) or diclofenac (50 mg/kg), respectively. One hour after the treatments, edema was induced in the right hind paws of the rats by sub-plantar injection of an agar suspension (2% [w/v]; 100 µl/footpad) as the phlogistic agent. Edema in the treated paw was measured by the volume of distilled water displaced by each paw before and 1, 2, 3, 4, and 5 h after induction of edema. Measurement of rat paw edema followed the previously reported procedure of Fereidoni et al. (2000). Inflammation was assessed as the difference between the paw volume at zero time of the treated paw (V_o) and the paw volume at the various time intervals (V_t) after the administration of the phlogistic agent. Percentage inhibition of edema was calculated using the relation (Ahmed et al., 1993; Perez et al., 1996): % inhibition = $100 \times (1 - \{[a - x]/[b - y]\})$ where; a = mean paw volume of treated rats at various times after agar injection; x = mean paw volume of treated rats before agar injection; b = mean paw volume of control rats at various time after agar injection; and y = mean paw volume of control rats before agars injection.

Effect of MFE on formaldehyde-induced arthritis in rats

The effect of MFE on chronic inflammation was assessed using the model of rat arthritis induced by formaldehyde described by Selye (1949). Here, rats of either sex were randomized into four groups (n = 5) for the study. The first two groups were treated with MFE (200 or 400 mg/kg; *per os*); the negative controls received (*per os*) an equivalent volume of vehicle (2% Tween) or indomethacin (50 mg/kg). Arthritis was induced 1 h after the various treatments on the first and third day by sub-plantar injection of 100 µl of a 2.5% formaldehyde solution. Arthritis was assessed by measuring the volume of distilled water displaced by the paw before arthritis induction and once daily for 10 days, starting from the day after induction. Treatments with MFE and the controls continued once daily for 10 days. Global edematous responses were quantified as the area under the curve (AUC; calculated using trapezoidal rule) of the time-course of the arthritic event. The level of inhibition of arthritis was calculated using: % inhibition = $100 \times (1 - \{AUC_t]/[AUC_c]\})$, where AUC_c = AUC of control; AUC_t = AUC of treated hosts.

Isolation and culture of bone marrow-derived macrophages (BMDM)

Murine BMDM were generated from BM cells of the tibia, humerus, and femur of BALB/c donor mice using methods previously described (Lin et al., 2001; Weischenfeldt and Porse, 2008). BM cells were harvested and cultured in monocytic cell culture medium containing RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 5% heat-inactivated fetal calf serum (FCS), 50 µM 2-mercaptoethanol (Gibco), 1% L-glutamine, 1% non-essential amino acids, 1 mM pyruvate, 100 U penicillin/ml, 100 µg streptomycin/ml, and 10 ng/ml recombinant murine colony-stimulating factor (rmCSF-1; Immunotools, Friesoythe, Germany) in T-75 culture flasks. The cells were incubated at 37°C and 5% CO_2 for 24 h to adhere and allow for removal of stromal cells and mature BM resident macrophages. Non-adherent cells, which are mainly progenitor cells, were recovered after

24 h of incubation and further incubated in 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. Viability of the cells (macrophages) was assessed by trypan blue exclusion. The BMDM generated were plated and used for the *in vitro* studies of the effects of AFE on inducible NO and pro-inflammatory cytokine production.

Viability of AFE-treated BMDM assessed using an MTT assay

The viability of the BMDM after treatment with AFE extract was determined using cellular respiration as an indicator. Cell viability was determined on the basis of mitochondrial-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (Mosmann, 1983). BMDM were cultivated in 96-well plates (1×10^5 cells/well) for 24 h. The cells were then treated with various concentrations (0, 5, 25, or 100 μg/ml) of AFE in a 100 μl volume. After 24 h of incubation at 37°C, the medium in each well was discarded; the cells were then incubated with fresh medium containing 5 mg MTT/ml for 4 h. The formazan that formed in the cells was then dissolved by addition of 150 μl dimethyl sulfoxide to each well (for 10 min at 37°C) and the optical density of the solution in the well was measured at 550 nm in a microtiter plate reader (Tecan, Grödig, Austria).

Effect of AFE on the expression of LPS-induced nitric oxide (iNO) by BMDM

BMDM were cultivated in 48-well plates (5×10^5 cells/well) at 37°C in a 5% CO_2 incubator for 24 h. Thereafter, the cells were then pre-treated with graded concentrations of AFE (0, 5, 25, or 100 μg/ml) and incubated for 2 h. The culture medium was then removed and replaced with fresh medium and the cells then treated with 10 μg/ml of LPS (lipopolysaccharide; serotype 0128:B12; Sigma, Munich, Germany) or culture medium (as control). Conditioned supernatants were collected after 24 h of incubation and stored at –80°C. Nitrite levels in the supernatant were measured in 96-well microtiter plates by mixing 100 μl of cell-free culture supernatant with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydro-chloride and 1% sulphanilamide in 5% phosphoric acid; Applichem, Darmstadt, Germany) and then incubating at room temperature for 10 min. The NO concentration was then determined at 550 nm in the plate reader by extrapolation from a standard curve generated using $NaNO_2$ standards that had been included in each measurement plate.

Effect of AFE on expression of inducible pro-inflammatory cytokine secretion

BMDM (5×10^5 cells/well) was seeded in 48-well plates and cultured for 24 h. Thereafter the cells were pre-treated with graded concentrations of AFE (0, 5, 25, or 100 μg/ml) and incubated for 2 h. The culture medium was then removed and replaced with fresh medium and the cells then treated with 10 μg LPS/ml or culture medium (control). After 24 h of incubation at 37°C, the conditioned culture medium in each well was collected and stored at –80°C. Concentrations of interleukin-1β (IL-1β) and tumor necrosis factor (TNF)-α in the harvested supernatants were determined using commercial ELISA kits (PeproTech, Hamburg, Germany). The sensitivity of the IL-1β and TNFα kits were, respectively, 0.063 and 0.016 ng/ml.

Statistical analysis

Results are presented as mean and standard error of the mean (SEM) of at least triplicate determinations for *in vitro* experiments and a group size of five for *in vivo* experiments. To demonstrate statistical significance of data, a one-way Analysis of Variance (ANOVA) using Prism5 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

Extraction of plant material and phytochemistry studies

The extraction process yielded 62.80 g (3.14% [w/w]) of methylene chloride/methanol extract of *F. exasperata* (MFE). The aqueous extraction of *F. exasperata* yielded 20.68 g (8.27% [w/w]) of extract (AFE). Preliminary phytochemical analysis showed the presence of alkaloids, flavonoids, resins, carbohydrate, proteins, oil, and terpenoids in the MFE (Table 1). The AFE tested positive for glycoside, saponins, alkaloids, flavonoids, resins, carbohydrate, proteins, acidic compounds, and terpenoids (Table 1).

Acute toxicity test

An acute toxicity test carried out on the MFE (using oral and intraperitoneal routes) showed there were no deaths and no observable signs of acute intoxication in the mice after a 24 h observation in the two stages of the study. Thus, the LD_{50} of the test extract was set at > 5 g/kg body weight (Lorke, 1983) in mice for either route.

Effect of MFE on topical (acute) inflammation

The MFE significantly inhibited xylene-induced ear edema in mice (Table 2). Mice treated with MFE had a mean ear edema of 7.88 ± 1.46 mg; this represented a 21.2% inhibition of inflammation from a 10.00 ± 1.65 mg level for the untreated group. Indomethacin (used as standard anti-inflammatory agent) produced mean ear edema of 4.92 ± 1.03 mg, representing a 50.8% inhibition) (Table 2).

Effect of MFE on acute inflammation in rats

Pre-treatment of rats with the MFE caused significant inhibition of agar-induced inflammation and a more

rapid resolution of paw edema over a 5-h period of measure (Figure 1). Edema was always greater in control rats at every timepoint compared to in rats treated with extract. Rats that had received 200 or 400 mg MFE/kg had only 0.28 ± 0.02 and 0.32 ± 0.04 cm^3 mean paw edema values, respectively, after 1 h, while negative controls had a value of 0.42 ± 0.04 cm^3 at that time. In general, both MFE treatments resulted in inhibited edema formation at each of the measured timepoints (Figure 1).

Effect of MFE on formaldehyde-induced arthritis

Anti-arthritic activity was evaluated in a formaldehyde-induced arthritis model in Wistar rats. Treatment with 200 and 400 mg MFE/kg produced significant (apparent dose-trend) inhibition of the induced arthritis (Table 3). The mean cumulative arthritic edema measured as the area under the edema-time curves (AUC) were 1.84 ± 0.57 (15.3% inhibition) and 1.69 ± 0.30 (22.0% inhibition) for the 200 and 400 mg MFE/kg groups, respectively, as compared to an AUC of 2.17 ± 0.31 for the negative control group. The maximum inhibition of arthritic edema (22.0% with 400 mg MFE/kg) was comparable to a 26.9% inhibition due to 50 mg indomethacin/kg (used as standard anti-arthritic agent) (Table 3).

Viability of AFE-treated BMDM

The toxicity of AFE against the BMDM was determined using a modification of the MTT cytotoxicity assay. The results showed that treatment of BMDM with 10–200 μg AFE/ml did not significantly affect viability.

Table 1. Phytochemical constituents of aqueous and methylene chloride/methanol extracts of F. exasparata.

Phytoconstituent	MFE	AFE
Carbohydrate	++	+
Alkaloids	++	+
Glycoside	–	++
Saponins	–	++
Tannins	–	–
Flavonoids	+++	++
Resins	++	+
Proteins	+	+
Oil	++	–
Steroids	–	–
Terpenoids	++	++
Acidic compounds	–	+

–, Absent; +, present in low concentration; ++, present in moderate concentration; +++, abundantly present.
MFE, methylene chloride/methanol extract of *F. exasparata*; AFE, Aqueous extract of *F. exasparata*.

Table 2. Effect of MFE on xylene-induced ear edema in mice.

Treatment	Dose (mg/ear)	Edema (mg)	Inhibition (%)
Vehicle	0	10.00 ± 1.66[a]	—
MFE	5.0	7.88 ± 1.46*	21.20
Indomethacin	5.0	4.92 ± 1.03*	50.80

* Value significantly different from vehicle control at $p < 0.05$.
[a] Value is mean ± SE.

Inhibition of LPS-induced IL-1β and TNFα production in BMDM treated with AFE

High levels of IL-1β and TNFα were measured in culture supernatants following treatment of the BMDM with 5 μg LPS/ml; however, pre-treatment of the cells with AFE (5, 25, or 100 μg/ml) significantly inhibited IL-1β and TNFα formation/release in a concentration-related manner (Figures 2 and 3) when compared to the cells treated with LPS alone. Pre-treatment with 5, 25, and 100 μg AFE/ml resulted in mitigation of LPS-induced TNFα release from a mean level of 3.52 ± 0.19 ng/ml secreted by cells treated with LPS alone to 2.00 ± 0.12, 1.32 ± 0.04, and 0.93 ± 0.14 ng/ml, respectively (Figure 2). These reflect an inhibition of 43.1 ± 3.3, 62.6 ± 2.2, and 73.5 ± 10.4%, respectively. Pre-treatment AFE also resulted in the mitigation of LPS-induced IL-1β release from a mean of 0.50 ± 0.03 ng/ml to 0.31 ± 0.02, 0.21 ± 0.02, and 0.14 ± 0.01 ng/ml, respectively (Figure 3). These reflect an inhibition of 38.0 ± 4.2, 57.7 ± 3.9, and 73.0 ± 1.70%, respectively.

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

Expression of inducible nitric oxide (iNO) by macrophages was determined indirectly using Griess reagent. As shown in Figure 4, the NO concentration in the medium of LPS-stimulated control cell cultures was

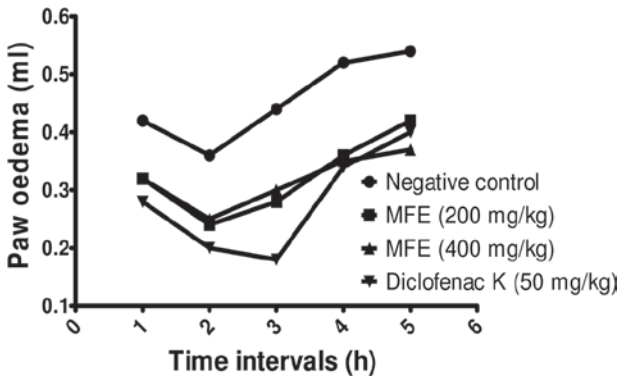

Figure 1. Anti-inflammatory activity of MFE on carrageenan-induced rat paw edema. Rats were randomized in groups ($n = 5$) and then treated with MFE (200 or 400 mg/kg, *per os*), diclofenac potassium (50 mg/kg, *per os*), or vehicle. One hour after the drug administration, agar suspension (2% w/v) was injected into the sub-plantar surface of each rat hind paw (100 μl/footpad). The edema produced in the treated paw was measured by the volume of distilled water displaced by each paw before and 1, 2, 3, 4, and 5 h after induction of inflammation.

Table 3. Effect of MFE on formaldehyde-induced chronic inflammation in mice.

Treatment	AUC	Inhibition (%)
Control	2.17 ± 0.31[a]	—
MFE (200 mg/kg)	1.84 ± 0.57	15.26
MFE (400 mg/kg)	1.69 ± 0.30	22.00*
Indomethacin (50 mg/kg)	1.59 ± 0.13	26.88*

* Value significantly different from vehicle control at $p < 0.05$.
[a] Value is mean ± SE.

significantly higher than those in unstimulated control wells (31.00 ± 2.08 μM vs 1.900 ± 0.21 μM). This LPS-evoked release of iNO was significantly inhibited in a concentration-dependent manner in BMDM pre-treated with 5, 25, and 100 μg AFE/ml. AFE pre-treatments resulted in reductions of NO levels to 24.50 ± 1.32, 19.33 ± 1.16, and 15.67 ± 2.52 μM, respectively. These represents reductions of 20.96, 37.65, and 49.45% compared to that by cells treated with LPS alone (Figure 4).

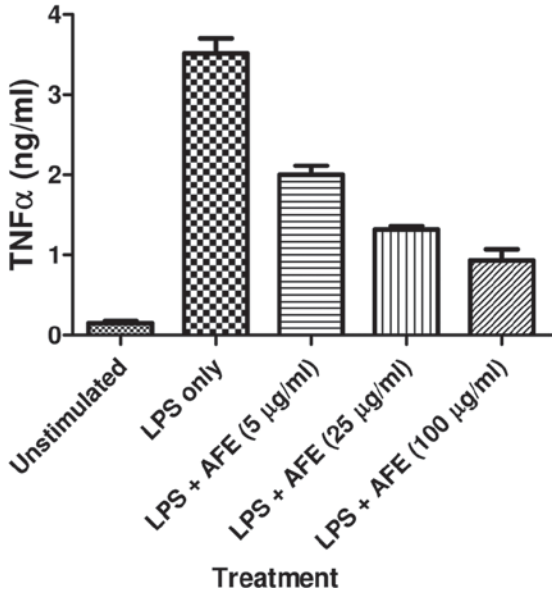

Figure 2. Effect of AFE on LPS-induced TNFα production *in vitro*. BMDM were pre-treated with AFE (0, 5, 25, or 100 μg/ml) for 2 h. The medium was then removed and replaced with fresh medium and the cells then treated with 10 μg LPS/ml (or medium) for 24 h. The supernatant in each culture well was then collected and assayed for TNFα via ELISA. Values shown are means (± SE) from n = 3/treatment group. * Value significantly different ($p < 0.05$) compared with that of 'LPS alone' control.

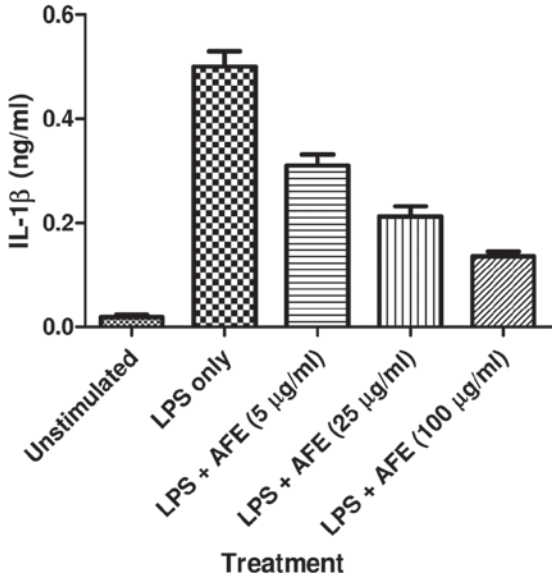

Figure 3. Effect of AFE on LPS-induced IL-1β production *in vitro*. BMDM were pre-treated with AFE (0, 5, 25, or 100 μg/ml) for 2 h. The medium was then removed and replaced with fresh medium and the cells then treated with 10 μg LPS/ml (or medium) for 24 h. The supernatant in each culture well was then collected and assayed for IL-1β via ELISA. Values shown are means (± SE) from n = 3/treatment group. * Value significantly different ($p < 0.05$) compared with that of 'LPS alone' control.

Discussion

Inflammation is fundamentally a protective response and is ultimately aimed at ridding the organism of both the initial cause of cell injury (e.g., microbes, toxins) and the sequelae of such injury (e.g., necrotic cells and tissues). Inflammation is terminated when the offending agent is eliminated and the secreted mediators are broken down or dissipated. In addition, there are innate anti-inflammatory mechanisms that serve to control the response and prevent it from causing excessive damage to the host. When these counter-regulatory mechanisms fail or are overwhelmed by the offending agents, an inflammatory disease supervenes (Cotran et al., 1998; Abbas and Lichtman, 2011). Inflammatory diseases, such as rheumatism and arthritis, have continued to be a significant cause of debilitation, morbidity, and mortality globally. Since the discovery of acetylsalicylic acid (aspirin) from the bark of the Willow plant (*Salixalba*) more than 100 years ago, many other non-steroidal as well as steroidal anti-inflammatory drugs have been introduced for clinical management of inflammatory disorders. However, the prolonged use of most of these medications is associated with some unwanted and often serious side-effects, mainly renal problems, gastrointestinal

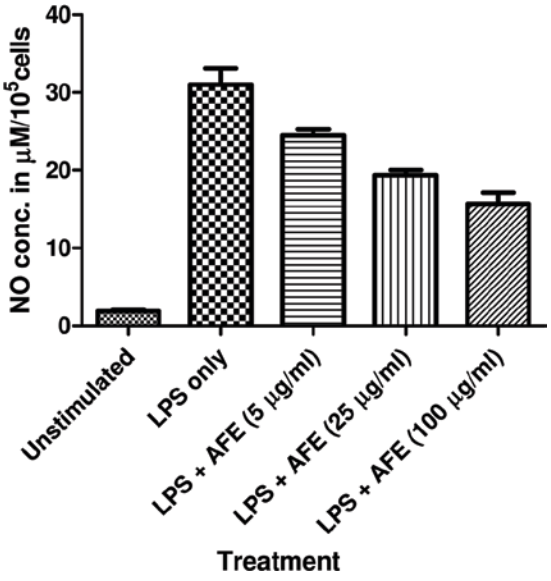

Figure 4. Effect of AFE on inducible (iNO) production. Effect of AFE on LPS-induced NO production. BMDM were pre-treated with AFE (0, 5, 25, 100 μg/ml) for 2 h. The medium was then removed and replaced with fresh medium and the cells then treated with 10 μg LPS/ml (or medium) for 24 h. The supernatant in each culture well was then collected for and assayed for NO using a Griess reagent protocol. Values shown are means (± SE) from n = 3/treatment group. * Value significantly different ($p < 0.05$) compared with that of 'LPS alone' control.

irritation, and ulcerations (Bertolini et al., 2001; Green, 2001). Therefore, there is still an unmet need of discovering potent anti-inflammatory molecules that are devoid of the limitations of the present therapeutic options. This has encouraged more research on medicinal plants that are used in complementary and traditionally medicine for the treatment pains, fever, and rheumatic pains (Basu and Hazra, 2006).

The study reported here investigated the anti-inflammatory activities and mechanisms of *Ficus exasperata* leaf extracts. The leaf and stalk of *F. exasperata* are used by the Igede people of Nigeria to mitigate itching or inflammation, and are prepared as poultices that are applied to swellings/arthritic joints. The beneficial claims in these traditional practices inspired this study in which topical and systemic (acute and chronic) anti-inflammatory activities of the extracts were studied in rodents. Because increased expression and release of pro-inflammatory cytokines and mediators are involved in inflammatory processes, the study included experiments to assess effects of the extracts on inducible IL-1β, TNFα, and iNO formation/release by cultured macrophages.

The results show that MFE significantly inhibited acute inflammation *in vivo*. Rats pre-treated with MFE showed significant increases in remission of edema induced by agar. This data supports the results of a recent study in which a hydroalcoholic leaf extract of *F. exasperata* was evaluated for anti-nociceptive, anti-inflammatory, and anti-pyretic properties in chicks (Woode et al., 2009). That study reported that the leaf extract (given at 10–300 mg/kg *per os*) yielded a dose-dependent anti-inflammatory activity against carrageenan-induced footpad edema, with an IC_{50} of ≈ 46 mg/kg. Acute inflammation induced by xylene was also inhibited in mice by topical application of MFE. The method of xylene-inducible ear edema has some advantages in natural product testing, including a good predictive value for the screening of anti-inflammatory agents. Xylene causes an instant irritation of the ear that leads to fluid accumulation/edema characteristic of an acute inflammatory response; suppression of this response is indicative of an anti-phlogistic effect. The eventual increase in ear weight and inflammation seen in this model is due to neutrophil accumulation. This cellular influx plays a critical role in cutaneous inflammatory diseases like dermatitis, and is related to the pathological mechanism of the disease (Bradley et al., 1982).

Inhibition of formaldehyde-induced pedal edema in rats has been reported, and is often used, as a suitable model to assess potential anti-arthritic/-inflammatory agents. The model is believed to closely resemble human arthritis (Greenward, 1991). It is also remarkable that daily administration of MFE (200 and 400 mg/kg) for 10 days decreased formaldehyde-induced arthritis (a manifestation of chronic inflammatory processes) in the test rats. This outcome is also akin to that in a recent report that showed that the administration of an ethanolic *F. exasperata* leaf extract (given at 30–300 mg/kg, *per os*) significantly reduced Freund's adjuvant-induced arthritic edema in the ipsilateral paw of rats (with a maximal inhibition of ≈ 34%) and also prevented the spread of edema from the ipsilateral to contralateral paw, indicating inhibition of systemic spread (Abotsi et al., 2010).

The results of the *in vitro* studies showed that pre-treatment of bone marrow derived macrophages with AFE led to a concentration-dependent suppression of the formation/release of IL-1β, TNFα, and iNO. Unregulated levels of pro-inflammatory cytokines have been implicated as a potential etiological factor in the development of several acute and chronic inflammatory diseases (Esposito and Giugliano, 1994; Ohshima and Bartsch, 1994; Krakauer, 2004). LPS is a potent inducer of inflammatory agents in macrophages; as such, LPS stimulation is frequently used to evaluate the efficacy of potential drugs/products against inflammatory response (Hong et al., 2009; Pearson et al., 2010). Based on the findings here, the inhibition of inducible IL-1β, TNFα, and iNO production by the extracts of *F. exasperata* could explain, at least in part, the anti-inflammatory activities of the plant reported in the two edema models here and, more importantly, in the outcomes ascribed to their ethno-medicinal use.

While NO is critical in host defense against microorganisms and tumor cells, excess NO production is associated with several inflammatory diseases, e.g., arthritis, autoimmune diseases, septic shock. In these disorders, NO contributes to the inflammatory cascade by increasing vascular permeability and extravasation of fluid/proteins at sites of inflammation (Moncada et al., 1991; Snyder and Bredt, 1992; Guzik et al., 2003). NO is produced by NO synthase (NOS) (Korhonen et al., 2005); after exposure to LPS, iNOS is quantitatively induced in macrophages (Duval et al., 1996). As such, inhibition of NO production has been a therapeutic strategy increasingly used in the treatment of various inflammatory diseases. This may also be a contributing factor underlying the observed anti-inflammatory activities of MFE.

The inhibitory effects of AFE on IL-1β, TNFα, and iNO formation/release by the BMDM were not due to cytotoxicity. Viability of these macrophages was not affected by AFE at the concentrations used in the *in vitro* studies. Similarly, the acute toxicity studies in mice did not suggest severe untoward effects after oral and intraperitoneal administrations at doses up to 5000 mg MFE/kg (Lorke, 1983).

Preliminary phytochemical studies showed that the extracts are rich in flavonoids, terpenoids, and alkaloids among other constituents. Although the study has not associated any of these constituents with the activities recorded, some of these bioactive constituents have been reported in previous studies to inhibit inflammation and suppress the production and release of pro-inflammatory mediators by monocytic cells. For examples, plant terpenoids have been reported to possess anti-inflammatory activities (Calou et al., 2008; Salinas-Sánchez et al., 2012; Zhang et al., 2012). Plant alkaloids have also been reported, in previous studies, to suppress inflammatory responses in rodents (Chakraborty and Brantner, 2001).

Similarly, many studies have shown that plant flavonoids possess anti-inflammatory activities *in vitro* and in cellular models which involve the inhibition of the expression and actions of different pro-inflammatory mediators such as eicosanoids, cytokines, adhesion molecules, and C-reactive protein (Hämäläinen et al., 2007; Serafini et al., 2010). Prostaglandins and nitric oxide biosynthesis are involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of inflammatory mediators. It has also been demonstrated that flavonoids are able to inhibit both enzymes (Marcinkiewicz, 1997; Chen et al., 2001; Shen et al., 2002). Flavonoids modulate the cascade of molecular events leading to the over-expression of these mediators which include inhibition of the transcription factors such as nuclear factor-κB (NFκB) and activating protein-1 (AP-1), through the inhibition of protein kinases involved in signal transduction (González-Gallego et al., 2007; Tuñón et al., 2009).

Conclusion

In summary, the results of the various investigations show that *F. exasperata* leaf extracts possess anti-inflammatory properties that could underlie the benefits associated with the folklore use of the plant. The results also show that the extracts may be acting through the suppression of mediators of inflammation such as IL-1β, TNFα, and iNO. This means that *F. exasperata* should be explored further as a potential source of anti-inflammatory compounds.

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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